THE EFFECT OF HYDRAULIC RETENTION TIME ON RECOVERABLE AMMONIA, VIRUS-PARTICLE ASSOCIATION, AND BACTERIAL, FUNGAL, AND VIRAL POPULATIONS IN A BENCH SCALE ACTIVATED SLUDGE MUNICIPAL WASTEWATER TREATMENT SYSTEM

By

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ABSTRACT

The objective of the study was to characterize the effect of shortening hydraulic retention time in a bench-scale activated sludge wastewater treatment system on ammonia oxidation, viral-particle attachment, and the bacterial, viral, and fungal communities. The scope of wastewater treatment now includes the desire to create a circular economy. By harvesting nutrients like ammonia, local governments and utilities can sell recovered products as raw materials to manufacturers and recuperate operating costs. This requires changes to the activated sludge process, specifically to the hydraulic retention time (HRT). Process changes like these could have unforeseen impacts on the quality of treated effluent. The specific impact studied here is the association of bacteriophages, a viral indicator, specifically phage that infects *E. coli*, with particles in these systems at different HRTs. Particle association affects virus removal by treatment. In this study, a bench-scale activated sludge reactor was set up and run in triplicate. The data show that recoverable ammonia in reactor effluent rose from 0.05 to 7 to 8.9 mg/L in the 24-, 16-, and 8-hour HRTs, respectively. In the influent sewage, 62.1% of somatic coliphage and 83.3% of f-specific coliphage were associated to particles of 0.45 μm or larger. The main finding of this work was that as HRT decreased, particle attachment decreased for both somatic and f-specific coliphage. The percent coliphage associated with particles of 0.45 μm or larger was 66.6, 51.4, and 47.2% for somatic and 88.2, 66.2, and 74.2% for f-specific at the 24, 16, and 8-hour HRTs, respectively. The functional gene count of ammonia oxidation related genes fell as HRT decreased. *Nitrosomonas* was the dominant ammonia oxidizing bacteria in this system, while *Nitrospira* was the main nitrate oxidizing bacteria. The dominant genera of fungi and viral families, with two exceptions, did not change across HRT. Only one fungal nitrifier was found in sludge samples, *Aspergillus flavus*. Its relative abundance did not change with HRT.

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AOA	ammonia oxidizing archaea
AOB	ammonia oxidizing bacteria
BGM	buffalo green monkey
CAS	conventional activated sludge system
COD	chemical oxygen demand
CV	coefficient of variance
DO	dissolved oxygen
ELWWTP	East Lansing Wastewater Treatment Plant
FISH	fluorescent in situ hybridization
GSBR	granular sequencing batch reactors
HRT	hydraulic retention time
ID	inner diameter
MAS	microaerophilic activated sludge system
MBR	mixed batch reactors
MGD	million gallons per day
MLSS	mixed liquor suspended solids
nm	nanometer
NOB	nitrite oxidizing bacteria
OD	outer diameter
PEG	polyethylene glycol
pfu	plaque forming units
pI	isoelectric point

PPMoV	pepper mild mottle virus
rpm	rotations per minute
SBR	sequence batch reactor
SRT	solids retention time
TN	total nitrogen
TSS	total suspended solids
UV	ultraviolet
WSP	water stabilization pond
WWRF	wastewater recovery facility
μm	micrometer

CHAPTER 1: INTRODUCTION

1.1 Significance of the Study

Water is integral to life on earth. As the global population rises and climate changes, so will the need for two critical resources: safe drinking water and agricultural fertilizers. The supply of both may be intrinsically related to resource recovery from wastewater. Spent water is a constant byproduct of human life. Its treatment for recycled use is one way to ensure its continuous availability. However, wastewater treatment can be costly, with start-up and maintenance costs being the barriers of entry to the establishment of treatment plants. Resource recovery can offset these costs and, in some cases, offer an attractive investment for local governments and entrepreneurs. Wastewater management has evolved from the need to remove polluted water from its source to its treatment and disinfection. Recently, the industry has faced a new task, developing and integrating technology and biological processes with which to harvest usable resources from waste streams.

Access to safe drinking water and adequate wastewater sanitation is limited in developing countries and some cities in developed nations struggle to fund updates to their existing sanitation framework (Holmes et al. 2019, Levenson 2023). The UN World Water Development Report for 2023 details that about 26% of the world population does not have access to safe drinking water. Additionally, 46% of the population does not have adequate wastewater sanitation. Building and maintaining a treatment facility is estimated to cost about $12 million for each million gallons per day (MGD) expected load (Liu et al. 2017). As a reference, the East Lansing Wastewater Treatment Plant (ELWWTP) processes 12.6 MGD from Michigan State University and the surrounding area (East

Lansing, accessed 2024). As populations rise, these limitations will only be exacerbated, therefore it is necessary to find sources of funding for wastewater sanitation and infrastructure.

According to the 2024 Global Report on Food Crises, carried out by the Food Security Information Network, almost 282 million people experienced acute hunger in 2023. Along with an increased need for clean water and sanitation, the demand for agricultural products will also rise with the growing global population. It has been estimated that fertilizer production must increase at an annual rate of 1.8% to guarantee food security (Ledezma et al. 2015). The ammonia derived from biological nitrogen fixation is not enough to satiate this demand (Ye et al. 2018). Existing processes, like the Haber-Bosch method of ammonia production from molecular nitrogen, are energy intensive and produce harmful greenhouse gases (Ye et al. 2018). This highlights the need for an environmentally safe and sustainable ammonia supply.

A circular economy created around wastewater treatment could help fund updates for existing infrastructure and attract investment in new facilities by recovering resources and selling them as raw materials to other industries. Nitrogen is a valuable resource that exists in municipal wastewater streams in the form of ammonia at a concentration of 10-200 mg/L (Adam et al. 2019). Ammonia has a wide range of applications, including use as an agricultural fertilizer, and is increasingly regarded as a future alternative to fossil fuels (Holmes et al. 2018, MacFarlane et al. 2020). Currently, the wastewater treatment process aims at removing nitrogen before discharging water to the environment. This is typically done through nitrification-denitrification cycling, aerobic-anoxic processes respectively (Holmes et al. 2018). During aerobic nitrification, ammonia is converted to

nitrate with a nitrite intermediate (Holmes et al. 2018). Afterward, an anoxic environment is created, and denitrification occurs, converting nitrate into dinitrogen gas (Holmes et al. 2018). With nitrogen removed from treated water, treatment plants then discharge effluent into the environment, limiting the effects of eutrophication (Adam et al. 2019). The aerobic activated sludge treatment method is the most common form of biological wastewater treatment but modifications to this process are needed to address resource recovery and to innovate future facilities around the world (Tran et al. 2022). Modifications to this process, such as gas permeable membrane technology, could help shift the focus from removal to recovery (Beckinghausen et al. 2020).

While resource recovery is important, it is also necessary to maintain plant effluent standards, as discharge should still be of sufficient water quality to avoid waterborne diseases, before re-entering the environment or for potential reuse. In the face of climate change, cities are considering and investing in water reuse projects as a partial solution to water scarcity in drought-stricken areas of the US (Harris-Lovett 2024). As such, it is important to consider how methods for ammonia recovery would affect plant effluent quality. One parameter of interest is the removal and inactivation of human enteric viruses. Human enteric viruses, viruses primarily transmitted through the fecal-oral route, are shed into wastewater by infected individuals (Tarris et al. 2021). Consumption of contaminated water can cause severe illnesses, like gastroenteritis and conjunctivitis (Ahmad et al. 2021). These viruses can attach to particles, which leads to their protection against and survival of wastewater treatment methods (Gerba et al. 1978). This aspect of wastewater treatment and virus control has not been well studied while examining approaches to enhance resource recovery.

Water and food are critical to all communities. The creation of wastewater treatment infrastructure in developing countries and maintenance of existing infrastructure in developed nations is an ongoing issue that will only be magnified as the global population rises. Coupled with this, the demand for fertilizers needed to maintain and increase crop yields to feed these populations will also rise. Resource recovery from wastewater could be one piece of the puzzle to meeting these growing needs.

1.2 Literature Review: Increasing Ammonia Content in Effluent by Varying Hydraulic Retention Time

Several studies have focused on varying parameters of activated sludge systems, such as dissolved oxygen (DO) and hydraulic retention time (HRT), to examine their effects on nitrification and nitrifying bacterial populations. However, none have characterized the effect of HRT on fungal and viral communities.

Nitrification is the oxidation of ammonia (NH_3) to nitrate (NO_3^-) through a nitrite (NO_2^-) intermediate (Colliver and Stevenson, 2000). Dissolved oxygen is a crucial component of nitrogen oxidation. When there is insufficient oxygen supply in an aerobic system this can lead to incomplete nitrification, causing nitrous oxide production (Colliver and Stevenson, 2000). Several studies on different aerobic systems characterized nitrous oxide emissions in relation to DO levels, these are reviewed below.

Nitrous oxide (N_2O) is produced when there is an insufficient oxygen supply in the activated sludge treatment process, causing partial nitrification (Colliver and Stevenson, 2000). Denitrification is the conversion of nitrate to nitrogen gas (N_2) and nitrous oxide is produced when there is oxygen present in the anoxic zone, causing partial denitrification (Liu and Wang, 2013). A study done on a full-scale plug flow activated sludge wastewater treatment plant in the UK found that increasing DO in the aeration lanes of

the plant decreased the N_2O side reaction (Aboobakar et al. 2013). Another article studying a full-scale sequence batch reactor (SBR) found the same, DO was negatively correlated with nitrous oxide emissions. Additionally, this study found that higher treatment temperature led to more complete nitrification (Sun et al. 2013).

Other studies varying DO in activated sludge systems focused on the shifts in ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) populations. In a study by Park and Noguera (2004) two lab-scale chemostats and two full-scale reactors were analyzed. One bench-scale chemostat was run at low DO (0.12-0.24 mg/L) and the other at high DO (8.5 mg/L). They found that the AOB populations mainly consisted of *Nitrosomonas europaea* in both low and high DO environments. However, the low DO environment had two distinct lineages of *N. europaea*. They found they were able to enrich the AOB population, promoting nitrification at low DO, by extending the operation time of the chemostat. This allowed *N. europaea* communities to establish themselves. The study then focused on a full-scale treatment plant where one train was kept between 0.6 and 7.4 mg/L DO and the other had a low DO of 0.2 to 0.8 mg/L. In this experiment, *N. oligotropha* was the dominant AOB in the high DO train, while *N. europaea* dominated the low DO train.

Similarly, Liu and Wang 2013 studied bench-scale complete mix reactors at long solid retention time (SRT) (10-40 days) and low DOs (0.16-0.37). SRT refers to the average time that sludge solids remain in the aeration chamber before they are exhausted. Traditionally, low DO levels lead to incomplete nitrification, producing nitrous oxide. However, they found that at long SRTs complete nitrification was achieved even at low DO. With the longer SRT, the AOB and NOB populations increased in size, therefore

increasing the sludge nitrification capacity. At low DO, NOBs shifted to bacteria that were better oxygen competitors, ensuring complete ammonia conversion to nitrate and mitigating nitrous oxide production. *N. europaea* remained the dominant AOB, as in the study by Noguera and Park mentioned above, but NOB dominance shifted from *Nitrobacter*-like NOB to *Nitrospira*-like NOB.

Another important operational parameter in activated sludge treatment systems is hydraulic retention time (HRT). This is the amount of time that influent sewage spends in contact with activated sludge in an aeration tank. Several studies have varied HRT in activated sludge systems and analyzed the effect on nitrifier populations and nitrification. One study looked at the effect of lowering HRT on nitrification and the nitrifier populations in a conventional activated sludge system (CAS) operated for 260 days using synthetic wastewater (Li et al. 2013). They found that decreasing the HRT from 30 hours to 10 hours initially decreased the specific rate of ammonia oxidation from 0.45 to 0.32 kg NH^{+}_{4}–N/ kg mixed liquor suspended solids (MLSS) per day. However, the rate increased over time until it reached 0.45 kg NH^{+}_{4}–N/ kg MLSS per day again at the end of the experiment. Additionally, specific nitrate forming rates increased from 0.11 to 0.50 kg NO^{-}_{3}-N/kg MLSS per day. These results indicate that at lower HRT the first step of nitrification (NH_4^+ to NO_2^-) was able to occur at the same rate as higher HRT and the second step (NO^{-}_{2} to NO_3^-) occurred more rapidly at the lower HRT. Upon performing fluorescent in situ hybridization (FISH) to identify bacterial populations in the reactor sludge, they found that *Nitrosomonas* was the dominant AOB across both HRTs. However, the AOB relative percentage fell from 33 to 15% with the drop in HRT while

NOB percentage rose from 4 to 15%. With this rise in NOBs, *Nitrobacter* replaced *Nitrospira* as the dominant NOB (Li et al. 2013).

Another study varied HRT in an aerobic granular sequencing batch reactors (GSBR) fed high-nitrogen wastewater. The reactors were operated at 10-, 13-, and 19-hours HRT and were run once with continuous aeration and again with an anoxic phase before aeration. At all HRTs the ammonia nitrification efficiency was above 90%, however denitrification decreased with longer HRTs. AOBs dominated the continuously aerated population of nitrifiers, while annamox bacteria dominated during the anoxic/aerated treatment (Cydzik-Kwiatkowska and Wojnowska-Baryła, 2015).

At a difference to studies varying one operational factor and characterizing the shift in nitrifier populations, others have characterized nitrifying bacterial populations at multiple operating parameters in full scale activated sludge treatment facilities and bench scale operations. A study published in 2007 analyzed the effects of DO, HRT, influent COD and NH_4^+ concentration and influent toxic shock on nitrifier populations. They looked specifically at *Nitrosomonas* and *Nitrosospira* AOB's and *Nitrospira* and *Nitrobacter* NOB's as the previously identified main nitrifying populations. They found that *Nitrosomonas* and *Nitrospira* were dominant in conditions favorable to nitrification: high NH_4, high DO, long HRT, and low influent toxic shock. However, *Nitrosospira* outcompeted *Nitrosomonas* in opposite conditions while *Nitrobacter* outcompeted *Nitrospira* at high influent COD and NH_4^+. AOB dominated over NOB and grew even more at low DO and short HRT (Li et al. 2007).

In one study, four treatment plants in Thailand, South Korea, and the US were divided into two categories: preanoxic without anaerobic processes and preanoxic with anaerobic

processes. In the treatment plants without anaerobic processes: at low COD/total nitrogen (TN) feed ratios and DO levels of 4.0 mg O_2/L or lower, longer HRT and SRT improved nitrogen removal while short HRTs and SRTs were needed at higher DO levels. High COD/TN ratios required an HRT between 9-15 hours, an SRT between 12-19 days, and a DO level of 1.3-2/6 mg-O_2/L. *Nitrosomonas* was found at all wastewater recovery facilities (WWRFs). Facilities with low DO levels showed an abundance of ammonia oxidizing archaea (AOA). *Nitrospira* was only found at low COD/TN ratios with long SRTs. *Nitrobacter* populations were found to be proportional to low DO levels. In addition, there was an abundance of nos-Z type nitrifiers (Phanwilai et al. 2022).

The study above characterized full scale facilities and focused on nitrogen removal (nitrification), in contrast, a lab-scale microaerophilic activated sludge system (MAS) was run for 300 days using synthetic wastewater to analyze ammonia retention and microbial communities in its sludge. At DO levels less than 0.2 mg/L, SRTs less than 5 days, and with an HRT of 11.2 hours, researchers found that all nitrifying bacteria were eliminated, leaving ammonia concentrations high in the treated effluent, and reduced COD by 85.5 ± 8.9% (Tsukamoto et al. 2023). Researchers from the same group used the data from the MAS system and biokinetics to develop a mathematical model that would predict the ideal SRT and HRT for COD reduction and ammonia retention in the MAS system. They found the ideal operating parameters to be an SRT between 3-50 days and an HRT of 0.1-1 day (Duan et al. 2023).

Whang et al (2009) examined MLSS, DO, and HRT and their effect on nitrogen removal. Three mixed batch reactors (MBRs) were run at 4,000, 6,000, and 8,000 mg/L MLSS, respectively. These reactors' HRTs were then varied from 4 to 6 to 8 hours. At each HRT,

the DO varied from 0.51 to 1.52 mg/L. They found that at high DO, increases in HRT and MLSS produced a slight increase in nitrification; while at low DO, increases in HRT and MLSS produced a large increase in nitrification.

Numerous studies have been conducted on activated sludge systems, both bench-scale and full scale, varying single parameters like DO, HRT, and SRT as well as a panel of parameters, and characterizing the effects on nitrification and nitrifier bacterial populations in reactor sludge. However, the shift of fungal and viral populations within these systems has not been a focus in these studies.

1.3 Literature Review: Viral Attachment to Particle Profiles with Varying HRTs

The demand for clean, potable water is set to increase around the world as populations rise and weather patterns are altered by climate change (Chahal et al. 2016). With freshwater sources already stretched thin in many countries, some are turning to water reuse to satiate need (Olivieri et al. 2020). One concern with current wastewater treatment methods is the survival of pathogenic human enteric viruses. Human enteric viruses come specifically from the human gut and are excreted in feces (Gerardi and Zimmerman, 2004). Examples of these include adenovirus, norovirus, hepatitis A virus, and enterovirus. These can cause many illnesses, including meningitis, gastroenteritis, and hepatitis (Ashbolt et al. 2004). A viral particle exposed to the environment will eventually lose its infectivity, even more so when subjected to inactivation processes, such as biological treatment, UV light, etc. (Templeton et al. 2008). However, when viruses are associated with particles, such as fecal matter, colloidal clays, and soils, the effectiveness of inactivation techniques is reduced, and active virions can contaminate treatment effluent even after upstream disinfection processes (Templeton et al. 2008).

Certain associated viruses have even been found to survive more than two times longer than their unassociated counter parts in environmental samples (Rao et al. 1984). Due to low pathogenic dose, presence in the environment, and survival of disinfection processes, it is imperative to understand their particle attachment characteristics to improve current wastewater treatment and fit the existing model to future wastewater reuse needs. Colloid particles are one of the main contributors to turbidity and do not settle during sedimentation, the first stage of wastewater treatment. Aquatic colloids are microscopic particles, between 1 nm and 1 μm (micron) in size. (Lead and Wilkinson 2006). They provide large amounts of attachable surface area and are strong adsorbents (Templeton et al. 2008). Examples of particle associated bacteriophage (bacteria infecting virus) can be found in figure 1.1 below.

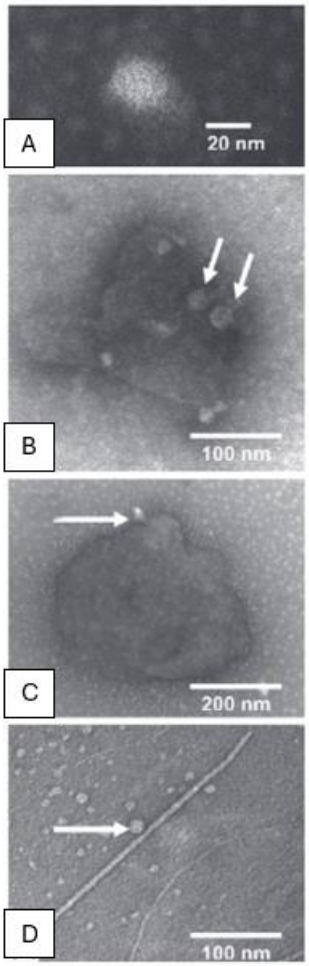

Figure 1.1: Particle associated MS2 *E. coli* phage. Image A is a free floating MS2 coliphage. Image B shows MS2 associated with colloid clay particles. C is an MS2 phage associated with humic acid flocs. Image D shows MS2 coliphage associated with a bacterial flagellum. Adapted from *Templeton et al. 2005.*

Some viral particle association pathways have been characterized and behavior depends greatly on the specific charge on the surface of the viral protein capsid, as well as the charge and shape of the suspended particles (Gerba et al. 1984). Each type of virion and particle has a specific isoelectric point, a pH at which it has a net charge of 0 (Gerba et al. 1984). Above their isoelectric point (pI), viruses will have a negative charge, and vice versa. Their interactions with particles depend on both the virion and the particle's

surface pI (Gerba et al. 1984). Table 1.1 lists three human enteric viruses of interest with their proposed shape, isoelectric point, and associated illnesses.

Table 1.1: Various human enteric viruses and their characteristics.

Virus	**Associated Illnesses**	**Size (nm)**	**Structure**	**Isoelectric point (pI)**	**Reference**
Adenoviruses (dsDNA)	Acute respiratory disease, pneumonia, acute follicular conjunctivitis, cystis, and gastroenteritis	70-100	icosahedral	2.6	(Doefler 1996) (Heffron and Mayer 2021)
Hep A (ssRNA)	Infectious hepatitis	27-32	icosahedral	2.8-5.5	(Gholizadeh et al. 2023) (Kusov et al. 2007) (Chahal et al. 2016)
Norovirus (GI, GII, and GIV) (ssRNA)	Gastroenteritis	38-40	icosahedral	5.5-6.9*	(Chan et al. 2017) (Venkataram et al 2016) (Goodridge et al. 2004)

*determined using virus-like particles (VLPs)

It should also be noted that particles can have multiple charges, depending on their composition, and while one portion of the particle may be neutral, others may have a charge promoting attachment (Templeton et al. 2008). Because of issues like low virion numbers in the environment and difficult culturability, somatic and f-specific phage infecting *E. coli* bacteria have been identified as viable surrogates for the study of human enteric viruses (Monis 2015). These phages display similar sizes, shapes, isoelectric points, and survival characteristics to human enteric viruses of interest (Skraber et al. 2004). Table 1.2 lists coliphages used as surrogates for the study of human enteric viruses and some of their characteristics. Coliphages are viruses that infect an *E. coli* host.

Table 1.2: Bacteriophages (specifically *E. coli* phages) used as surrogates for human enteric viruses.

Coliphage	**Size (nm)**	**Shape**	**Isoelectric point (pI)**	**Reference**
F-specific (ssRNA)	27	icosahedral	2-6.37	(Heffron and Mayer 2021), (Kuzmanovic et al. 2003), (Singh et al. 2022)
F-specific (ssDNA)	6	filamentous	4-7	(Singh et al. 2022)
Somatic (dsDNA)	25-90	elongated icosahedral or icosahedral	3-7.8	(Heffron and Mayer 2021), (Singh et al. 2022)

The concept of viral-particle association is not new. Wellings et al. (1976) concentrated animal viruses found in influent, effluent, and chlorinated effluent from a small municipal wastewater treatment plant via a PEG precipitation method. They demonstrated that 23.4-80.8% of virus in the plant influent was associated to particles 0.45 μm in size or greater, while 90-100% of virus in the plant effluent were particle associated. They postulate that

the agitation of wastewater and sludge could be producing smaller particles, creating more surface area for the surviving viruses to adsorb to. This paper also makes the important distinction between adsorbed and embedded viruses. While adsorbed viruses could be eluted using certain protocols, embedded viruses could not be recovered and may benefit from a greater shielding effect.

Not much later, Gerba et al. (1978) sampled effluent from the outlets of two final settling tanks (clarifiers) after activated sludge treatment and found that 1-24% of f-specific coliphage was associated to particles. Furthermore, 3-100% of animal viruses sustained by Buffalo Green Monkey (BGM) host were particle associated. Gerba et al. (1978) also found that 78% of total solids by weight were between 1-100 μm in size, compared to 35% before treatment. This could be due to fragmentation of larger particles during the activated sludge process, or the settling of larger particles during clarification, the step after activated sludge treatment, excluding them from the final effluent.

Supporting the above two studies, Hejkal et al. (1978) found a three-fold increase in virus titer after a fecal particle homogenate was passed through a 0.22 μm filter and sonicated. This suggests that most viruses are associated with particles of 0.22 μm or greater. When comparing influent versus unchlorinated effluent, they found that total virus was reduced by 92%. In addition, the percentage virus associated to particles 0.3 μm or greater dropped from 28% to 3.4%. They concluded that the originally associated virus in the influent settled out during clarification. However, after effluent chlorination, the percentage phage associated with particles 0.3 μm or greater increased to 7.7%.

A study by Guskey (1983) focused on enterovirus in the influent and effluent from a wastewater treatment plant on Jones Island, Wisconsin, on Lake Michigan. Samples were

collected and centrifuged to concentrate settlable particles. Viruses were then eluted from the sediment using fetal bovine serum. At least 35% of viable enterovirus was associated with these larger particles in sewage. Additionally, polio virus was found associated to pelleted particles in chlorinated plant effluent.

More recent papers support the theory that virions associate heavily with colloidal particles rather than larger particles that settle during primary sedimentation. Greaves et al. (2022) found that pepper mild mottle virus (PPMoV), crAssphage, Adenovirus, Human Polyomavirus, HF183/MacR287 and Norovirus GII in wastewater treatment plant influent mostly associated with particles between 20 and 0.45 µm. Another paper, Silva et al (2008), focused on Norovirus GI and GII in a waste stabilization pond (WSP) and found that they primarily associated with particles between 180 µm and 0.45 µm in size. Symonds et al. (2014) found similar association profiles in a wastewater treatment pond when examining enterovirus, norovirus, rotavirus and PPMoV. These viral targets associated with particles between 0.45 to 180 µm.

The association of viruses with particles is not a new topic. It has been found that these associated particles are able to survive longer than their free-floating counterparts in environmental samples (Rao et al. 1984). Additionally, viruses can survive the most common biological wastewater treatment method, activated sludge, and subsequent disinfection by associating with particles that occlude them from treatment (Chahal et al. 2016). This indicates that viruses are being released into the environment and current wastewater treatment methods are not sufficient for complete virus removal. This problem is magnified when considering water reuse as populations rise and access to clean water becomes even more limited. Retrofitting current wastewater treatment

methods may be an attractive option for communities looking to utilize current infrastructure to address this new issue of water reuse or mitigate the discharge of contaminants into the environment. While some viral attachment profiles have been characterized, no studies have investigated the effect of activated sludge process parameters on viral-particle association.

1.4 Literature Review: Bacteria, Fungi, and Viruses Involved in Wastewater Nitrification

The first step in the nitrification process is the conversion of ammonia (NH_3) to nitrite (NO_2^-) (Lehtovirta-Morley 2018). This is carried out by ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) (Lehtovirta-Morley 2018). The second step in the nitrification process, nitrite (NO_2^-) to nitrate (NO_3^-), is facilitated by nitrite oxidizing bacteria (NOB) (Lehtovirta-Morley 2018). There are some bacteria that can catalyze both nitrification steps mentioned above on their own. These are known as comammox bacteria (Daims et al. 2016). The functional genes associated with these conversions are illustrated in figure 1.2 (Lehtovirta-Morley 2018, Levy-Booth et al. 2014). Ammonia monooxygenase (*amo*) carries out the first step in ammonia oxidation. The amo enzyme uses O_2 to oxidize NH_3. The product of this step is hydroxylamine (NH_2OH). There are several enzymatic subunits which can differ, but the *amo* enzyme is shared by all three of the ammonia oxidizers mentioned in the following paragraphs (Lehtovirta-Morley 2018, Martikainen, 2022). However, not all AOBs have the *amo* enzyme (Martikainen, 2022). Hydroxylamine is further oxidized to NO_2^- by hydroxylamine oxidoreductase (*hao*) (Soler-Jofra et al. 2021). However, it has been postulated that *hao* may only reduce hydroxylamine to NO and another, not yet identified enzyme further oxidizes to NO_2^- (Soler-Jofra et al. 2021). Once *hao* reduces

hydroxylamine to NO_2^-, nitrite oxidoreductase (*nxr*) found in NOBs further reduces to NO_3^- (Daims et al. 2016). Commamox bacteria use all three enzymes mentioned above: *amo*, *hao*, and *nxr* to completely nitrify ammonia to nitrate.

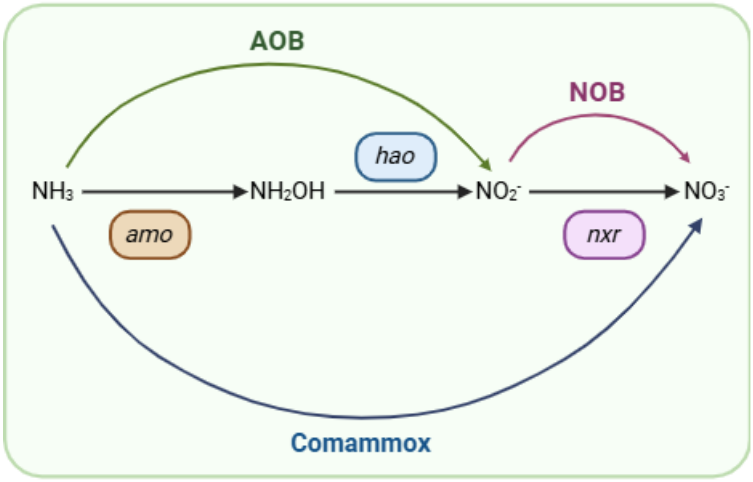

Figure 1.2: Bacterial ammonia oxidation pathway and related functional genes.

AOBs have two main phylogenic groups: β-proteobacteria, found in soil and wastewater treatment plants, and γ -proteobacteria, found in marine habitats or acidic wastewater treatment plants (Daims et al 2016). *Nitrosomonas* and *Nitrosospira* are β-proteobacteria. *Nitrosococcus* is a γ -proteobacteria. Because of their high half saturation constant (K_m), AOBs they are less efficient at utilizing ammonia at low concentrations than AOA's and some comammox bacteria. They are found to dominate in high ammonia environments, like wastewater treatment plants (Daims et al. 2016).

Ammonia oxidizing archaea traditionally thrive in environments unsuited to AOBs, such as those with low ammonia concentrations, acidic pH, and/or extreme temperature (Lehtovirta-Morley, 2018). One example is *Nitrosopumilus maritimus*, isolated from sea water, a low ammonia environment (Martikainen, 2022). *Nitrososmicus exaquare* was isolated from a wastewater treatment plant, one of the exceptions to low ammonia environment affinity (Sauder et al. 2017). AOA are more abundant in the environment

than AOB, but AOB dominate in wastewater treatment plants (Mussmann et al. 2011). Additionally, AOB are more resistant to environmental changes than AOA (Placella and Firestone, 2013). While AOAs have the *amo* enzyme, they do not display an analog *hao* enzyme. It has instead been postulated that they use quinone reductase (QRED) coupled with a hydroxylamine ubiquinone redox module (HURM) to achieve the reduction of hydroxylamine to nitrite (Wright and Lehtovirta-Morley, 2023).

Nitrite oxidizing bacteria catalyze the second step of nitrification, nitrite (NO_2-) to nitrate (NO_3-). This step is integral to ensuring that nitrite does not leach into the environment (Daims et al. 2016). NOBs are a diverse and complex functional group (Lehtovirta-Morley, 2018). The main groups found in engineered environments are: *Nitrobacter, Nitrotoga, Nitrococcus, Nitrolancea, Nitrospina,* and *Nitrospira* (Daims et al. 2016). *Nitrospira* lineages I and II are the main NOBs found in wastewater treatment plants (WWTPs) (Daims et al. 2016, Juretschko et al. 1998). In 2015, Lücker et al. found that *Nitrotoga* was also present and coexisting with *Nitrospira* in a full-scale wastewater treatment plant. However, it was found by Alawi et al. that *Nitrotoga* thrives in WWTPs operated at low temperatures, otherwise, *Nitrospira* dominated. Similarly, a study by Hüpeden et al. in 2016 found that *Nitrotoga* preferred growing at 22 ºC and pH 6.8, while *Nitrospira* preferred 32 ºC and pH 7.3.

Nitrospira is the major genus in the comammox category (Daims et al. 2016). It is a relatively new discovery that some, not all, *Nitrospira* lineages are able to directly convert ammonia to nitrate (van Kessel et al. 2015). Not much is known about the contribution of comammox bacteria to the nitrogen cycle, however they are known to be higher in abundance than other ammonia oxidizers in engineered environments like

wastewater treatment plants (Lehtovirta-Morley, 2018). The key enzyme found in NOBs is the nitrite oxidoreductase (*nxr*) enzyme (Daims et al. 2016). Sequencing of *nxrB* found at least 120 *Nitrospira* species in the activated sludge of a wastewater treatment plant (Gruber-Dorninger et al. 2015).

Because viruses can infect bacteria, they have an influence on the shape of microbial communities. In WWTPs, more than 96% of DNA viruses found are bacteriophages (Wang et al. 2018). Wang et al. (2018) studied a WWTP in Hong Kong over a period of nine years and found that the abundance of viruses was cyclical, often with blooms occurring after sludge bulking caused by certain bacteria. Additionally, when comparing viral clusters in the activated sludge of ten other WWTP around the world, they found a high number of shared clusters. They posed the hypothesis that there may be a generalized activated sludge viral community shared globally. Garcia-Fontana et al. (2020) sequenced samples using Illumina HiSeq from a WWTP using a conventional activated sludge treatment system in Andalusia, Spain for DNA viruses over a period of four months. They also found bacteriophages to be the dominant DNA viruses in the WWTP, with specific phage found to be dominant throughout the study. They found specific *E. coli, Edwardsiella, Pseudomonas, Enterobacteria,* and *Aeromonas* phages to be dominant in the treatment plant mixed liquor. The use of phage in controlling host growth has emerged as an area of interest in the medical field, however, research into phage-bacteria pairs specifically targeting nitrifying bacteria is limited. Only one pair can be found in literature, a *Nitrosomonas* infecting phage named PhiNF-1 identified by Quirós et al. (2023). Several studies have investigated the importance of phage in controlling host populations (Braga et al. 2020). These studies highlight their use as

microbiome tailors, able to enhance substrate quality by inhibiting host growth (Braga et al. 2020). Some studies have focused on the virome of activated sludge in WWTPs, with one of the main focuses of viral studies being the characterization and monitoring of pathogenic viruses in sewage influent and treated effluent (Fernandez-Cassi et al. 2018). None of the studies looked directly at the change in viral communities of activated sludge at varying HRTs.

Another understudied area in engineered aquatic systems like WWTPs is the presence of nitrifying fungi. Nitrification pathways in bacteria and fungi are different (Martikainen, 2022). While bacterial pathways are mostly understood, fungal nitrification pathways have not been an area of focus (Martikainen, 2022). The *amo* enzyme used by AOBs, AOAs, and comammox bacteria has not been found in nitrifying fungi (Martikainen 2022). One of the roadblocks in studying fungal nitrification is that some isolated fungi lose the ability to nitrify once cultured (Martikainen, 2022).

Most of the studies on nitrifying fungi are on soil, not aquatic environments. Early on, Eylar and Schmidt (1959) identified *A. flavus* when isolating nitrifiers in nitrifying soils. They also isolated and identified *Penicillium sp.* and *Cephalosporium sp. Aspergillus flavus* was found to prefer neutral pH, common in conventional activated sludge wastewater treatment plants (Hirsch et al. 1961). Another study in 1978 found that *Mortierella* spp. was a soil nitrifier under acidic conditions (Johnsrud, 1978). Land and Jagnow (1986) found *Verticillium lecanii* to be the main ammonia nitrifier in podzolic brown earth. In the same study, the optimal performance of this fungi was determined to be pH 3.55, not a pH traditionally obtained in a wastewater treatment plant. However, it did exhibit nitrifying properties at higher pHs. *Penicillium nigricans* was isolated from

forest soil by Stams et al. (1990) and was able to nitrify NH_4^+ within a 3-7 pH range. There are recent studies analyzing the importance of fungi in activated sludge wastewater treatment plants when trying to enhance chemical oxygen demand (COD) removal (Li et al. 2022). However, there are no direct studies involving activated sludge and nitrifying fungi.

1.5 Objectives of the Study

Activated sludge wastewater treatment is the most common method of biological treatment. Wastewater sanitation could be a renewable source of clean water and recoverable raw materials. Raw materials, such as ammonia, could be recovered and sold to the agriculture industry. This revenue model could serve to attract investment in new sanitation infrastructure for underserved communities and fund updates to existing infrastructure as well as support water security. Shortening hydraulic retention time, the amount of treatment time sewage spends with activated sludge, increases recoverable ammonia levels in reactor effluent. However, it is important to balance resource recovery with treated effluent quality. Shortening the time sewage spends in treatment could affect the way viruses attach to particles. Additionally, it is important to understand how hydraulic retention time affects the bacterial, viral, and fungal populations in the activated sludge to use this information to better tailor engineered environments toward resource recovery. The objectives of the study were to characterize the effect of shortening hydraulic retention time in a bench-scale activated sludge wastewater treatment system on 1) ammonia oxidation, 2) viral-particle attachment, and 3) the bacterial, viral, and fungal communities. The hypothesis is that decreasing hydraulic retention time in a continuous flow activated sludge bench-scale system will 1) increase

ammonia in treated effluent, 2) increase viral-particle attachment in effluent due to shorter treatment time, and 3) decrease nitrifier populations in sludge and change the overall bacterial, fungal, and viral populations. Potentially, the decrease in nitrification and known nitrifiers could be correlated to fluctuations in populations that have not been previously known to be associated with nitrification.

CHAPTER 2: MATERIALS AND METHODS

2.1 Reactor Setup and Materials

Three reactors were constructed to be run in parallel, meaning that all three were constructed and run identically to obtain an n=3 for each hydraulic retention time (HRT). Figure 2.1 shows the set up for the bench scale system used.

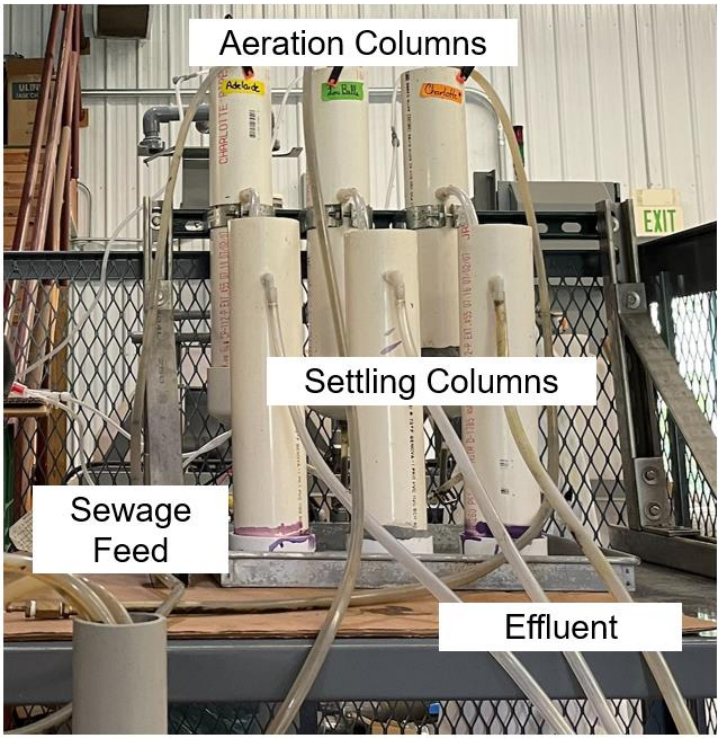

Figure 2.1: The configuration of the aeration column, settling column, and effluent cylinders of the bench-scale activated sludge wastewater treatment system.

The set up was split into three segments: aeration, settling, and effluent collection. The aeration and settling columns were made from PVC pipes with an outer diameter (OD) of 3.5 inches and inner diameter (ID) of 3 inches. The height of the aeration columns was 20 inches, with the exhaust spigot located 7.5 inches from the top of the column. Each aeration column contained an air stone that delivered a constant supply of air to the activated sludge for the duration of the experiment. The air pumps used, one for each reactor, were adjustable aquarium air pumps manufactured by Zhongshan Ibay Electric

Appliance Co., LTD, model number AC100-120V 50/60Hz. The three aeration columns were attached to a bar suspended above the settling columns so that treated water was positively displaced by the sewage feed loading from the top of the column. The treated water ran downward by gravity from the aeration spigot into the settling column through a clear vinyl tube with a 0.5 inch OD and a 0.375 inch ID.

The settling column was constructed using the same size PVC pipe as the aeration column, but had a height of 16 inches, with the exhaust spigot located 3 inches below the top of the column. The effluent from this settling column was positively displaced and ran down by gravity using the same clear vinyl tubing material as mentioned above, into a graduated cylinder located below the aeration and settling columns. This graduated cylinder was used to verify that flow, in mL, was identical across the three reactors during the experiment.

Sewage feed came from a 5-gallon bucket located behind the reactor system. The sewage was pumped to the aeration tank through the same type of clear vinyl tubing mentioned above, into the top of the aeration column. The pump used to funnel sewage to the aeration columns was a Thermo Scientific cartridge peristaltic pump with 4 rollers, model number 72-320-126 0.8 rpm-80rpm, 0.1 Hp. The tubing used for the pump was Masterflex brand with part number 06409-16. The length of the tube was 16.25 inches, with plastic stoppers located 4.5 inches apart, placed across the Thermo Fischer pump and secured using Thermo Fischer Scientific peristaltic pump cartridges, part number 72-560-100, 54321. The pump was operated in “Continuous” mode. The rpm setting was increased depending on the HRT.

2.2 Reactor Feeding

Primary sewage was collected from the East Lansing wastewater treatment plant (ELWWTP) daily using a five-gallon bucket. The sewage feed was collected between the hours of 9:00 am and 10:00 am and transported to the Anaerobic Digestion Research and Education Center (ADREC) bioreactor site at Michigan State University. The previous day's sewage was replaced with fresh sewage within thirty minutes of collection from the plant. The reactor feed lines were inserted into the new sewage bucket and capped with a five-gallon bucket plastic lid with a seal to prevent vapor escape. The sewage temperature was taken upon arrival at the bioreactor site using a Thermochemical liquid in glass thermometer. The thermometer was inserted into the wastewater collection bucket and allowed 5 minutes to equilibrate before the temperature was recorded.

2.3 Sludge Return

As the reactors ran, fluid from the aeration chamber moved to the settling column where supernatant and sludge were separated by gravity. The supernatant was either collected for testing or poured off, while the sludge was recycled to the appropriate reactor's aeration column manually. This was set up to mimic the return mixed liquor process at the wastewater treatment plant. However, sludge return on the reactor system was only done between once a day, versus a continuous return at the wastewater treatment plant. Additionally, there was no wasted sludge in this system. The only sludge that left the system, exited in the form of 200 mL per week of aeration chamber liquid used for testing. To return the sludge after the supernatant was poured off, the air stones in the aeration columns were shut off for 5 minutes after sludge was poured back in. Column nozzles were turned upward to avoid spillage. Sludge was allowed to settle for 5 minutes

after which nozzles were turned downward. Resulting overflow liquid was disposed of. Air stones were turned back on and allowed to operate for 30 minutes before dissolved oxygen (DO) was remeasured.

2.4 Flowrate

Flowrate was set at each HRT iteration by adjusting the continuous flow rotations per minute (rpm) and the pump cartridge tightness around the feed tubes. At the start of each iteration, a graduated cylinder was placed at the end of each feed tube and the mL per minute feed was measured and adjusted. For the 24-hour HRT, 1.5 L (the volume of each aeration column) was fed to the system in 24-hours. The formula used and the mL/min at each HRT are shown below.

Flowrate calculation: $\frac{Column\ volume\ (mL)}{HRT\ (minutes)}$

Table 2.1: The flowrate in mL/min at each HRT iteration.

HRT iteration	Flowrate (mL/min)
24-hours	1.0
16-hours	1.6
8-hours	3.1

Flowrate was measured once per day throughout the experiment for each bench scale reactor. A graduated cylinder was placed underneath the aeration column which doubled as the settling chamber. Before recycling sludge, the volume in the graduated cylinder was recorded and correct flowrate was verified. Adjustments to the pump lines were made accordingly.

2.5 Pre and Post Stability System Sampling and Testing

There were two sampling phases, pre and post process stability. The activated sludge stability was determined using chemical oxygen demand (COD) and total solids (TS). Once COD and TS did not vary more than 25% between sampling events, the reactors were considered stable. At this point, the post-stability sampling began.

Pre-stabilization sampling and testing consisted of sampling from each reactor's aeration column twice a week for COD and TS. The methods used for measuring these are outlined in section 2.6 below.

Once the reactors were considered stable, 5 testing events were carried out, each separated by at least 24 hours. Post-stabilization testing included ELWWTP sewage after primary settling, all three reactor effluents, and sludge from all the aeration chambers. These samples were subjected to a panel of physiochemical analysis outlined in table 2.2 and described in section 2.6. Although total phosphorus (TP) was not the focus of this study, activated sludge is known to accumulate phosphorus in the form of orthophosphate (Jupp et al. 2021) Since phosphorus is also an important recoverable resource from wastewater, this parameter was measured as a characterization of the system. All three reactor effluents were assayed for somatic and f-specific coliphage using the methods outlined in section 2.7.1. Once it was determined that reactor effluents were performing similarly, a single reactor's effluent was used to evaluate viral-particle association using the separation method described in section 2.7.2. Primary sewage from ELWWTP was also assayed using the viral particle separation method.

Table 2.2: List describing all parameters analyzed (physiochemical and biological) post reactor stabilization.

Sample Type	pH	Turbidity	COD	TS	TP	NH_4^+-N	Coliphage Plating	Particle Separation
Reactor Effluent	✓	✓	✓			✓	✓	✓*
Reactor Sludge	✓		✓	✓	✓			
Sewage Feed	✓	✓	✓		✓	✓	✓	✓
ELWWTP Effluent	✓	✓					✓	✓

* Only one reactor's effluent was used for the particle separation experiment since reactors were determined to perform similarly.

2.5.1 Sampling Methods

Samples were taken using a 50 mL serological pipette and pipette bulb by inserting pipette into the sewage bucket, aeration and settling PVC columns. These were sampled from approximately the center of the column and bucket. Two 40 mL samples were taken from the sewage bucket containing the previous day's sewage feed, the bucket containing the current day's sewage feed, and settling column (effluent) and placed into 50 mL centrifuge tubes. One set was immediately submitted for physiochemical testing, while the other was refrigerated at 4℃ until it was transported to the coliphage testing lab. One set of samples was taken from the aeration column and submitted for COD and TS analysis. No coliphage testing was done on this sample type. All physiochemical analyses were started within 15 minutes of sample collection.

Post and pre-stabilization sampling varied only in that an extra 40 mL of aeration sludge was harvested from each reactor three separate times during the stability phase and frozen immediately at -80℃ until DNA extraction could be performed for shotgun sequencing, described in section 2.8.

2.6 Physiochemical Parameters

2.6.1 Dissolved Oxygen

The HACH IntelliCAL LDO101 probe was used to measure the dissolved oxygen DO in the aeration chambers every day after sludge recycling. The HACH DO probe was inserted into aeration tube until the probe was half way submerged, ensuring that the probe tip was not in contact with the air stone at the bottom of the column. The probe was allowed 5 minutes to acclimate, after which the measurement was taken. The target DO was between 6 and 8 mg/L. If DO measurement was out of range, the air pump dial was adjusted, and the DO measurement was taken 30 minutes afterward until target DO was achieved.

2.6.2 Total Solids

The HACH Total and Volatile Nonfilterable Solids protocol methods 8158 and 8164 were used as a reference for the TS method used in this experiment. The weight of an empty 30 mL ceramic crucible was taken, and the scale was then tarred. Twenty mL of homogenized sample was added to the crucible and the weight was recorded, corresponding to the sample weight. The crucible with sample was then dried in an oven at 105 ℃ for 24-hours. After the 24-hour drying period, the crucible was placed in a desiccator until it reached room temperature and then was weighed. The weight of the dry sample was determined by subtracting the weight of the empty crucible from the weight of the crucible after drying. The weight of the dry sample was divided by the sample weight and percent TS was determined.

2.6.3 HACH Physiochemical Kits

Chemical Oxygen Demand (COD), Total Phosphorus (TP), and Nitrogen in the form of Ammonia (NH_4^+-N) were determined using HACH kits. The protocols for each kit can be found on the HACH website: https://www.hach.com/resources/water-analysis-handbook. The range for each kit was determined after preliminary testing and either ultra-low range (ULR), low range (LR), high range (HR), or ultra-high range (UHR) kits were selected for each parameter. The HACH kits used for the quantification of phosphate, nitrogen, nitrate, and ammonia are listed in table 2.3 along with their corresponding sample types.

Table 2.3: Kit numbers for HACH kits used to measure physiochemical parameters.

Test	Sample Type	Range	HACH Part Number
Total Phosphorous	Sewage and Effluent	UHR	TNT845
Ammonia	Sewage	HR	TNT832
Ammonia	Effluent	ULR or HR	TNT830
COD	Aeration	UHR	TNT824
COD	Sewage, Effluent	HR	TNT822

Note. COD (chemical oxygen demand), ULR (ultra-low range), LR (low range), HR (high range), UHR (ultra-high range). HR ammonia was used on effluents during the 16- and 8-hour HRT iterations due to a higher concentration.

2.7 Biological Analysis

2.7.1 Coliphage Quantification:

A modified double agar overlay method was used to quantify somatic and f-specific coliphage in sewage collected from the primary settling tank from the East Lansing Wastewater Treatment Plant (ELWWTP) and effluent coming from each of the bench scale reactors. The double agar overlay method was first outlined in Adams, 1959,

and an overview of the method can be found in Ács et al.(2020). The modified method was adapted from the EPA 2012 method 1602: Male-Specific (F+) and Somatic Coliphage in Water by Single Agar Layer (SAL) Procedure, April 2001). Briefly, while samples were analyzed for physiochemical parameters, another set of samples were transported on ice to the coliphage testing laboratory. Here, the samples were analyzed within 8 hours of collection. This was done to determine how effective the lab-scale reactors were in reducing fecal indicators, providing a parameter by which to measure the quality of the reactor effluents from each HRT (8, 16, 24). Samples were assayed for somatic and f-specific coliphage. An *E.coli* culture of CN-13 (ATCC#700609) was used as a host to assay for somatic coliphage, and an *E.coli* culture of F-amp (ATCC#700891) was used as a host for f-Specific phage. Cultures of each host were grown using trypticase soy broth (TSB), with antibiotics, incubated at 37 ℃ for 24 hours. A Naladixic acid solution was used for the CN-13 host, while an Ampicillin/Streptomycin solution was used as an antibiotic for the F-amp host. Antibiotics were added to achieve a 1% (v/v) concentration in TSB for host growth. The overnight cultures were used for the agar overlay by combining 1 mL of the overnight *E. coli* culture, 38.6 mL TSB, and 0.4 mL antibiotic. The host was incubated for 4 hours at 37 ℃. After incubation, another 0.4 mL of the antibiotic were added to the cultures to bolster the concentration back to 1% (v/v), since some antibiotic may have been inactivated during incubation.

The overlay tubes were melted prior to use, containing 2.5 mL of 1.5% of Trypticase Soy Agar (TSA). This was kept in a liquid state incubated in a water bath at 49.5 ℃ until used. The tubes were then removed and quickly 0.5 mL of *E. coli* host was

added. Afterward, 2 mL of the sample was added to the 2.5 mL of 1.5% of liquid Trypticase Soy Agar (TSA) and 0.5 mL host. This mixture was poured over a 1.5% TSA plate. Five replicate plates were prepared for each host type. A total of 10 plates per sample were made. Plates were allowed to solidify and were then incubated at 37 ℃ for 18-24 hours. Plates were refrigerated until plaques could be counted. Plaques were counted within 24 hours after plates were removed from the incubator.

This coliphage assay was run over several days (x3) while reactors stabilized during the 24-hour HRT and five samples were collected over several days after stabilization of the reactors. The assay was run only after all three reactors stabilized as measured by COD and TS for the 24-,16- and 8-hour HRTs. Stabilization was defined by no more than a 25% change in COD and TS in reactor sludge.

2.7.2 Particle Separation Assay

A particle separation assay was used to determine the distribution of viruses associated with specific particle sizes in ELWWTP sewage and reactor effluents. Once reactors stabilized (parameters for reactor stability are described above) and were determined to perform in a statistically similar manner, one reactor's effluent was used to perform the particle separation experiment at each HRT iteration (24-,16-, 8-hours).

Forty-seven mm filters with 100-, 20-, 3-, and 0.45 μm pore sizes were used along with Whatman 420400 Swin-Lok plastic filter holders and luer lock syringes to filter 30 mL of sewage through each filter size. This was done in parallel. Nylon filters were used for the 100 and 20 μm pore sizes, while mixed cellulose ester filters were used for pore sizes 3 and 0.45. Each filter holder was bleached with a 10% bleach

solution for 10 minutes and then submerged in a 1% sodium thiosulfate sulfate solution (w/v) before use.

The filtrate from each filter was plated using the plaque assay described in section 2.7.1, using CN-13 as the somatic coliphage host and F-amp as the f-specific host. The viral concentration filtered out of solution at each filter size was considered to be associated with particles of that size or greater. To verify that the loss in phage was not due to the filtration method, a control experiment was performed using pure suspensions of PhiX-174 and MS2, described below.

2.7.3 Viral Particle Filtration Control Experiment

Pure suspensions of PhiX-174 (ATCC#13706-B1), somatic phage, and MS2 (ATCC#15597-B1), F-specific phage, were made in TSB to an approximate concentration of 80 plaque forming units (pfu)/mL. Lyophilized phage was rehydrated using TSB. One mL of log phase host (either CN-13 or F-amp) was added to a melted 1.5% TSA overlay along with 1 mL of phage suspension. This was poured onto a TSA plate. Several dilutions of the phage suspension were made and plated. After incubation, plates with high plaque counts were selected and TSB broth was added. These plates were incubated with shaking for one hour. Supernatant was pipetted off the plates and filtered through a 0.4 μm filter to remove bacterial particles. These suspensions were assayed to determine concentration. Once concentration was known, suspension was diluted to reach a concentration of 80 pfu/mL for the particle separation control experiment. Because these were pure suspensions, the phage was presumed unassociated, in contrast to the environmental

samples from the treatment plant and the bench scale reactors. Therefore, any loss could be attributed to the method and not particle association.

Thirty mL of suspension containing one phage type were filtered through each filter pore size outlined in the particle separation assay section above. The filtrate was plated using the modified plaque assay.

2.8 DNA Extraction and Shotgun Sequencing

Forty mL of sludge samples from each reactor's aeration chamber were taken on three of the five sampling days after reactor stabilization, for a total of 9 sludge samples. An additional 40 mL of sludge used to seed the reactors at the beginning of each experiment were frozen at -80℃. A total of 10 sludge samples were collected and frozen for DNA extraction at each HRT.

Once all samples were collected and frozen at -80℃, a total of 12 samples, they were thawed for DNA extraction. Once thawed, 250 uL of each was extracted using the Qiagen DNeasy PowerSoil Pro Kit according to manufacturer's instructions for a final volume of 100 uL. Nanodrop testing was done to determine the DNA purity and approximate concentration of the resulting extractions. According to Thermofisher *Nanodrop* "Interpretation of Nucleic Acid 260/280 Ratios", an A260/A280 ratio of 1.8 was accepted as indicative of "pure" DNA. Nucleic acids have a maximum absorption around 260 nm, and the ratio of absorption at 260 nm compared to 280 nm has been used as a parameter of purity. (Thermofisher, 2012) If samples were pure and above 5 ng/µl in concentration, thirty microliters of extractions were then sent for shotgun sequencing at the Michigan State University Research Technology Support Facility Genomics Core.

The genomic DNA was submitted for Illumina compatible library preparation and sequencing. The methods for Illumina shotgun sequencing used by the MSU Genomics Core and the corresponding technical documents can be found at the RTSF Genomics Core website: https://rtsf.natsci.msu.edu/genomics/.

This project was part of a shared lane on a NovaSeq S4 flow cell, targeting ~1,400 million read pairs. The resulting pools were combined with MSU Genomics core prepared Kapa HyperPrep libraries (Roche, Basel, Switzerland). The pools were then quantified using the Invitrogen Collibri Quantification qPCR kit (Invitrogen, Waltham, Massachusetts). One lane of a NovaSeq S4 flow cell was used for sequencing in a 2x150bp paired end format using the NovaSeq 6000 v1.5 500 cycle reagent kit. (Illumina Inc., San Diego, California) Base calling was prepared by Illumina Real Time Analysis (RTA) v3.4.4. QC was done using the FastQC format.

Shotgun sequence reads were analyzed using the Department of Energy Systems Biology Knowledgebase (KBASE) found at https://www.kbase.us/. The shotgun sequencing reads were uploaded to the database and the files were decompressed and imported into the staging area. Afterward, a series of applications available on the platform were used to trim and pair the shotgun reads, following the "*Metagenome-Assembled Genome Extraction from a Compost Microbiome Enrichment*" tutorial found on Kbase. (Chivian et al. 2023) "Run Fama Read Profiling" and "View Fama Functional Profile" apps were used to analyze the functional genes used in known nitrification pathways. This generated an EFPKG value. This is a normalization method used by Kbase to compare genetic potential across different samples. This method normalizes across library size, target gene size, and predicted average genome size. (Kazakov, 2019) However, this app does not

determine which functional genes were found in which bacterial genomes. The paired reads were also run through the "Kaiju" app configured to identify viral, fungal, or bacterial populations. For bacterial reads, this application also assigns a taxon found in the NCBI taxonomy to each sequencing read. The taxon is assigned by comparing it to a reference database containing microbial and viral protein sequences. The database "*Nitrifying Bacteria"* was used in the Kaiju application. (Chivian et al., 2023)

2.9 Statistical Comparisons

Statistical comparisons were made to determine if all three reactors performed similarly, and to determine if there were differences in performance (COD, coliphage removal, coliphage attachment to particles) across HRTs. An analysis of variance (ANOVA) was used to determine whether two means were statistically different from each other. The criteria for choosing which type of ANOVA or t-test depended on the distribution of values (Gaussian) and the assumption of equal standard deviation across groups of data. Tables 2.4 and 2.5 summarize the type of test used based on these two criteria.

Table 2.4: Type of t-test used when comparing two sets of values.

Type of t-test	Normal Distribution of data values (Gaussian)?	Assuming Equal Standard Deviation?
Mann-Whitney	No	-
Welch's	Yes	No
Standard	Yes	Yes

Table 2.5: Type of ANOVA test used when comparing three sets of values

Type of ANOVA	**Normal Distribution of data values (Gaussian)?**	**Assuming Equal Standard Deviation?**
Kruskal-Wallis	No	-
Brown-Forsythe and Welch	Yes	No
Ordinary	Yes	Yes

CHAPTER 3: RESULTS

3.1 Stabilization and Treatment Performance of the Bench Scale Activated Sludge System

3.1.1 Activated Sludge Chemical Oxygen Demand and Percent Total Solids at Stability for Each Hydraulic Retention Time

The stability for each HRT was defined by a less than or equal to 25% change in COD and TS of each reactor's activated sludge between sampling events. After stability, five samples were taken from each triplicate reactor and as seen in figure 3.1, the average COD of the activated sludge decreased at each HRT. At the 24-, 16-, and 8-hour HRTs, the average COD was 2519 (±334), 1961 (±448), and 1299 (±283), respectively. The COD was found to be statistically different across HRTs, with a P-value of <0.0001. When comparing the COD of each reactor's activated sludge across the five testing events, the results did not fluctuate more than 25%.

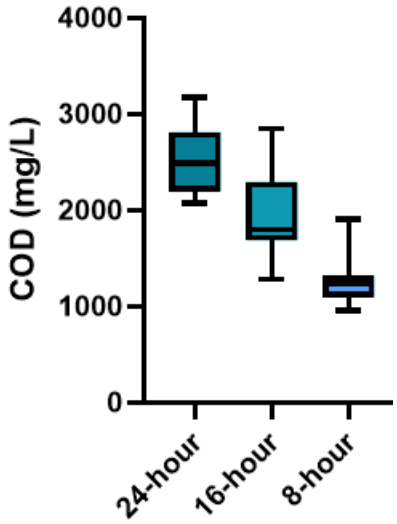

Figure 3.1: Average COD of reactors at each HRT. N=15 for each HRT.

The average percent TS decreased with HRT, as seen in figure 3.2, with average percent of 0.37% (±0.04), 0.30% (±0.06), and 0.22% (±0.04) at the 24-, 16-, and 8-hour HRTs, respectively. These were also found to be statistically different, with a P-

value of <0.0001. When comparing the TS of each reactor's activated sludge across the five testing events, the results did not fluctuate more than 25%.

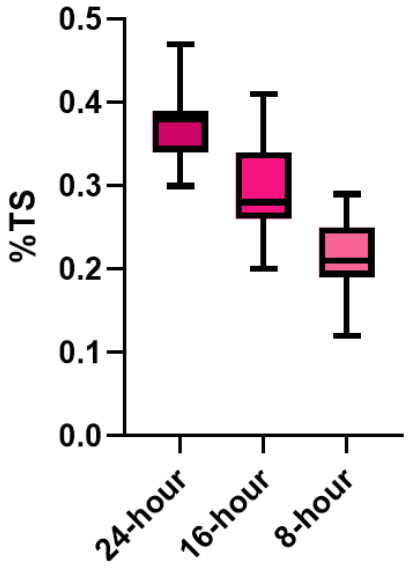

Figure 3.2: Average percent TS of reactors at each HRT. N=15 for each HRT.

3.1.2 Comparison of Chemical Oxygen Demand Removal Across Hydraulic Retention Time Iterations

The average COD of incoming sewage feed was 202 (± 21.0), 191 (±24.3), and 238 (±53.8) mg/L during the 24-, 16-, and 8-hour HRT iterations, respectively. These results are shown in figure 3.3. There was no statistically significant difference between the mean COD sewage feed at the 24-hour, 16-hour, and 8-hour HRTs (P-value: 0.231).

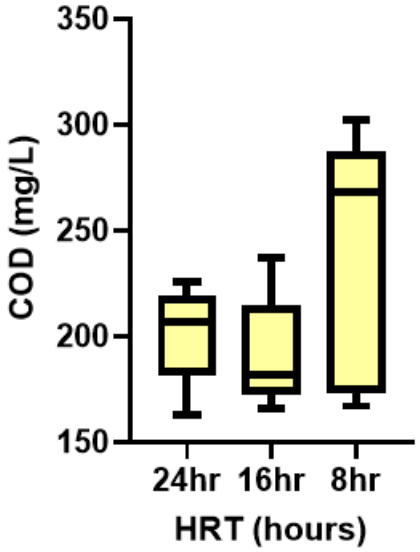

Figure 3.3: COD of sewage used to feed the reactors at each iteration of the experiment. For the 24-hour HRT, samples were collected in a 26-day span, between 7/6/2023 and 8/1/2023. For the 16-hour HRT, samples were collected in a 35-day span, between 10/26/2023 and 11/29/2023. For the 8-hour HRT, samples were collected in a 9-day span, from 2/17/2023 to 2/26/2023. N=5 for all HRTs.

As seen in figure 3.4, during the 24-hour HRT, the average COD in the effluent of reactors A, B, and C across the five testing events were 67.7 (±4.8), 61.4 (±4.1), and 66.7 (±2.0) mg/L. The coefficient of variation (CV) values for all HRTs are listed in table 3.1. Chemical oxygen demand was reduced by an average of 68% during these experiments. There was no statistically significant difference in mean removal between reactors at this HRT (P-value: 0.060).

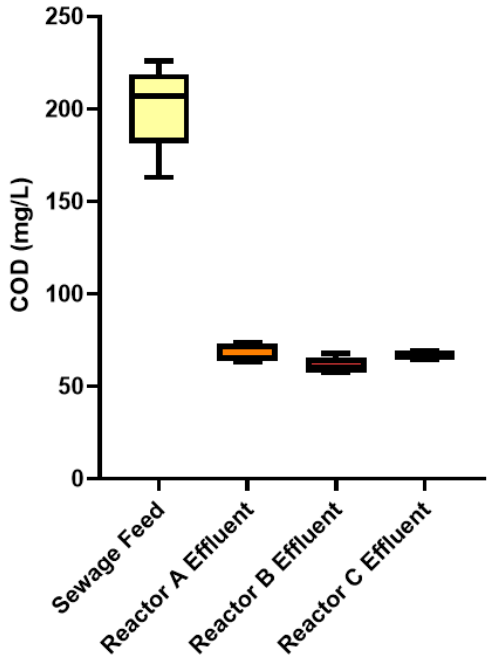

Figure 3.4: COD reduction during the 24-hour HRT experiment. N=5 for all effluents and N=5 for sewage feed. Samples were collected in a 26-day span, between 7/6/2023 and 8/1/2023.

Table 3.1: COD and CV summary for sewage feed and treated effluent at 24-, 16-, and 8-hour HRTs.

Sample Type	Average COD at 24-hour HRT (mg/L)	%CV	Average COD at 16-hour HRT (mg/L)	%CV	Average COD at 8-hour HRT (mg/L)	%CV
Sewage Feed	202	10%	191	13%	238	23%
Reactor A Effluent	67.7	7%	72.1	12%	66.6	5%
Reactor B Effluent	61.4	7%	64.4	11%	67.1	10%
Reactor C Effluent	66.7	3%	61.8	11%	71.9	10%

Note. For the 24-hour HRT, samples were collected in a 26-day span, between 7/6/2023 and 8/1/2023. For the 16-hour HRT, samples were collected in a 35-day span, between 10/26/2023 and 11/29/2023. For the 8-hour HRT, samples were collected in a 9-day span, from 2/17/2023 to 2/26/2023.N=5.

The effluent during the 16-hour HRT experiment, depicted in figure 3.5, had COD values of 72.1 (±8.9), 64.4 (±7.1), and 61.8 (±6.9) mg/L for reactors A, B and C,

respectively. The CVs were 12%, 11%, and 11% for these effluent COD values. The COD was reduced from 191 to an average of 66.1 mg/L, a 65% reduction. There was no statistically significant difference between the mean COD of all three reactors (P-value: 0.131).

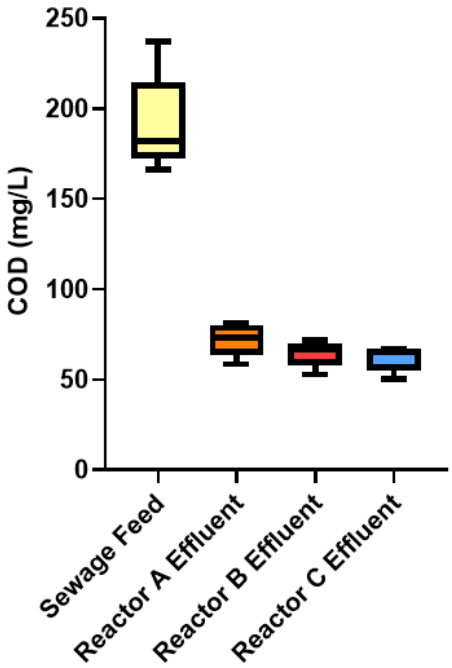

Figure 3.5: COD reduction during the 16-hour HRT experiment. N=5 for all effluents and sewage feed. Samples were collected in a 35-day span, between 10/26/2023 and 11/29/2023.

For the 8-hour HRT, seen in figure 3.6, the average COD value of effluent samples from reactors A, B, and C were 71.9 (±7.5), 66.6 (±3.6), 67.1 (±6.4) mg/L, with CV values of 5%, 10% and 10%, respectively. Chemical oxygen demand fell from 238 to an average of 68.5, a 71% drop in COD from sewage feed to treated effluent. There was no statistically significant difference between the mean COD of all three reactors (P-value: 0.341).

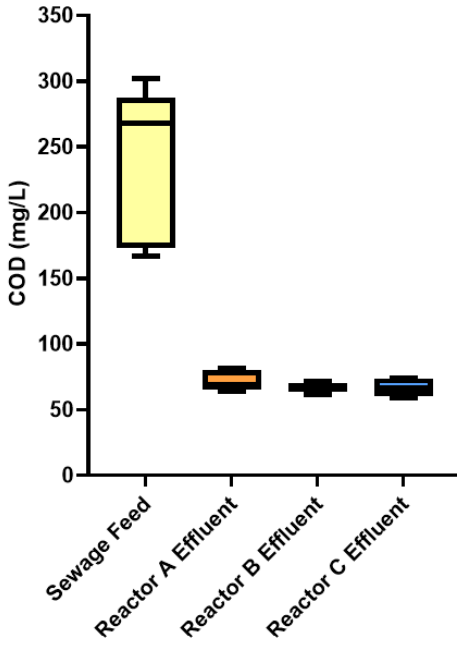

Figure 3.6: COD reduction during the 8-hour HRT experiment. N=5 for all effluents and sewage feed. Samples were collected in a 9-day span, from 2/17/2023 to 2/26/2023.

There was no statistical difference between the COD of the reactor effluents across all three HRTs. The P-value for the Brown-Forsythe and Welch ANOVA test for this comparison, which assumes a Gaussian distribution but does not assume equal standard deviations, was 0.0926.

3.2 Comparison of Ammonia Content Across Hydraulic Retention Time Iterations

As seen in figure 3.7, the ammonia content in the effluent of the A, B and C reactors during the 24-hour HRT was not statistically different (P-value: 0.364). There was an average of 0.05 (±0.01) mg/L of ammonia in the reactor effluent at the 24 HRT. During this iteration of the experiment, there was an average of 19 (±5.3) mg/L ammonia in the sewage after primary settling feed. There was a 99.7% reduction of ammonia.

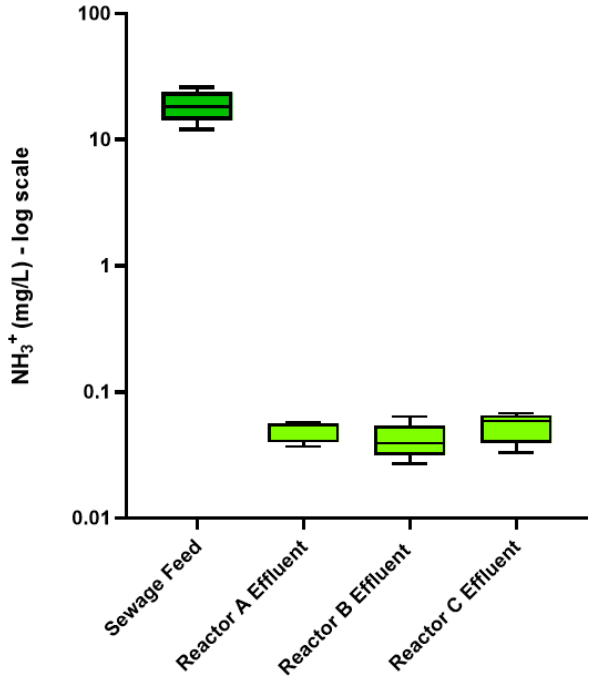

Figure 3.7: Concentration of ammonia in reactor feed during the 24-hour HRT phase of the experiment. N=5 for all reactors and sewage samples. Samples were collected in a 26-day span, between 7/6/2023 and 8/1/2023.

As seen in figure 3.8, there was no statistical difference between the ammonia content of the A, B and C reactor effluents in the 16-hour experimental iteration (P-value: 0.461). However, ammonia content was much higher than in the 24-hour effluents (0.05 mg/L). An average of 7.4 (±4.2) mg/L ammonia was present after treatment for the 16-hour HRT. The incoming sewage feed contained 21.5 (±5.7) mg/L ammonia. There was an average of 65.6% removal of ammonia from primary sewage at the 16-hour HRT.

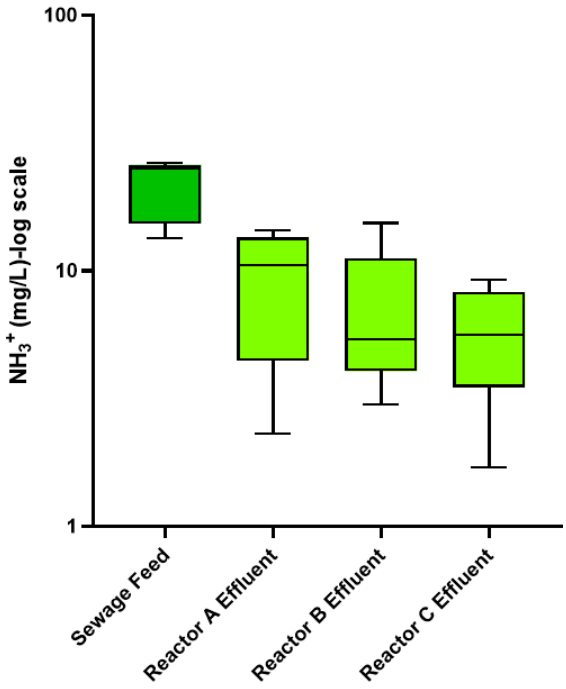

Figure 3.8: Concentration of ammonia in the reactor's effluent compared to the feed concentration during the 16-hour HRT experiment. N=5 for all reactor effluents and sewage. Samples were collected in a 35-day span, between 10/26/2023 and 11/29/2023.

In contrast to the 24-hour and 16-hour effluent results, the 8-hour HRT ammonia in the effluents from the A, B and C reactors varied a bit more (statistically different, p-value: 0.022) with the effluent level in C slightly higher than A which was slightly higher than B, as seen in figure 3.9, averaging 9.1 (±2.3), 7.0 (±1.1) and 10.6 (±1.1) mg/ L, respectively. The average ammonia concentration in the sewage feed was 18.98 (±4.5) mg/L, while the average effluent ammonia concentration was 8.9 (±2.1) mg/L. There was an average of 53.1% ammonia removal from primary sewage at the 8-hour HRT.

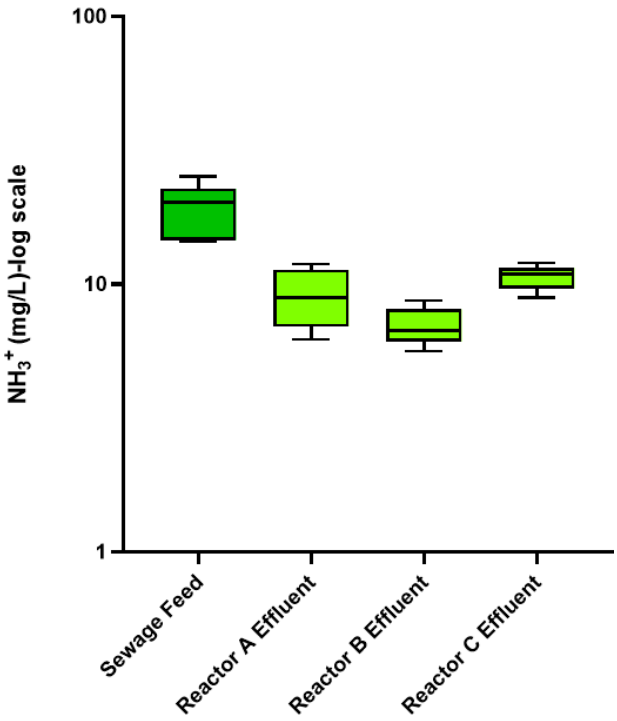

Figure 3.9: Concentration of ammonia in the reactors' effluent compared to the feed concentration during the 8-hour HRT experiment. N=5 for all reactor effluents and sewage. Samples were collected in a 9-day span, from 2/17/2023 to 2/26/2023.

The coefficients of variation (CV), listed in table 3.2, were higher in the 16-hour effluents compared to the 24-hour. The average CV for the 16-hour effluents was 55.7% compared to 25.7% during the 24-hour HRT. However, the average CV for the reactor effluents during the 8-hour HRT was 17.4%, lower than the 24-hour and 16-hour CV. The CV for the sewage feed was 28.13%, 27.24%, and 23.62% during the 24-hour, 16-hour, and 8-hour HRTs respectively. All statistical comparison P-values used to determine statistical differences are listed on table 3.3.

Table 3.2: Coefficients of variation of ammonia concentration in each reactor's effluent and system sewage feed during each of the three HRT runs (24-, 16-, and 8-hour).

HRT	Sewage Feed	Reactor A	Reactor B	Reactor C
24-hour	28.13%	17.97%	32.78%	26.21%
16-hour	27.24%	52.41%	67.00%	47.76%
8-hour	23.62%	25.12%	16.38%	10.65%

Note. For the 24-hour HRT, samples were collected in a 26-day span, between 7/6/2023 and 8/1/2023. For the 16-hour HRT, samples were collected in a 35-day span, between 10/26/2023 and 11/29/2023. For the 8-hour HRT, samples were collected in a 9-day span, from 2/17/2023 to 2/26/2023.N=5 for each reactor and sewage feed.

Table 3.3: P-values for ammonia concentration data set comparisons using the Brown-Forsythe and Welch ANOVA test.

Comparison	24-hour Effluents (A, B, C)[a]	16-hour Effluents (A, B, C)[a]	8-hour Effluents (A, B, C)[a]	24- vs 16- vs 8-hour[a]	16-vs8-hour[b]
P-value	0.364	0.461	0.022*	0.0022	0.2433
Statistically Significant?	No	No	Yes	Yes	No

Note. P-value summaries: *= $P \leq 0.05$, **=$P \leq 0.01$, ***=$P \leq 0.001$. N=15.[a] N=15. [b] N=10

3.3 Comparison of Phosphorus Content in Activated Sludge Across HRT Iterations

Although not the main focus of this work, the concentration of phosphorus was measured in the sludge to demonstrate the workings of the bench scale system. Phosphorus enters the system through the sewage feed and is accumulated in the sludge as bacteria store phosphorus in the form of ortho-phosphate. The phosphorus steadily climbed over the 5 days of sampling for the 24-and 16-hour HRT experiments in each of the aerated sludge tanks. Figure 3.10 shows the phosphorus concentration over time in reactor sludge at the 24-hour HRT, while figure 3.11 shows the phosphorus concentration over time at the 16-

hour HRT. However, the phosphorus content in the 8-hour HRT sludge remained the same or fell across the 5 sampling events, as seen in figure 3.12.

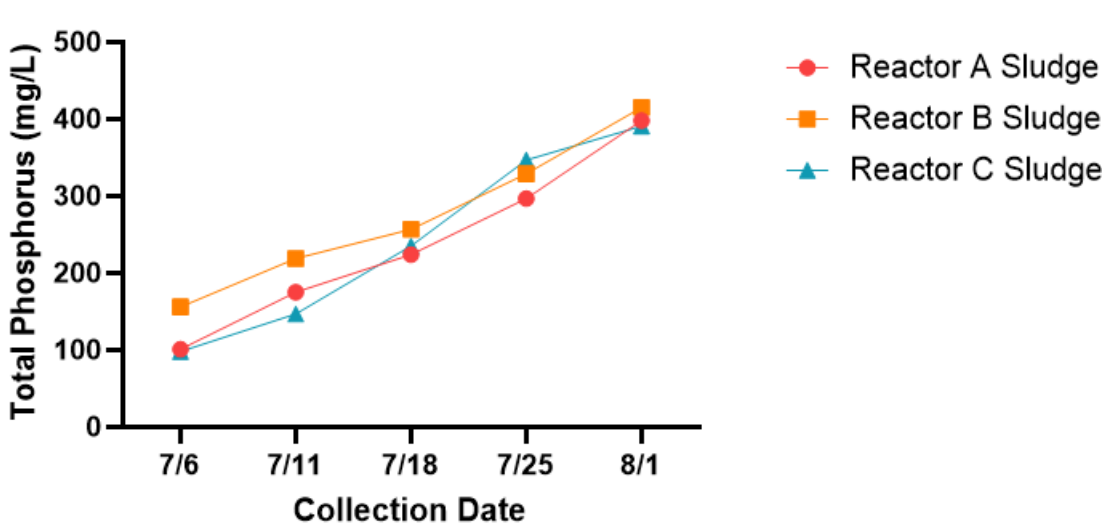

Figure 3.10: The accumulation of phosphorus in the reactor systems' sludge during the 24-hour HRT experiment from the beginning of stability until the end of the testing period, a total of 26 days. N=3 for each reactor's sludge on each testing day.

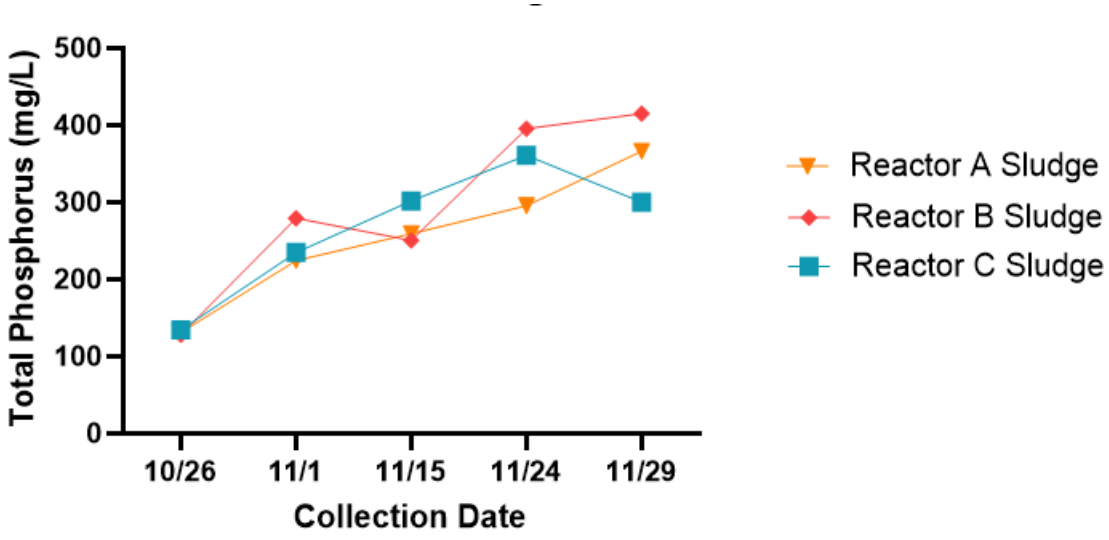

Figure 3.11: The phosphorus accumulation in the reactor systems' activated sludge during the 34-day testing period for the 16-hour HRT experiment. N=3 for each reactor's sludge on each testing day.

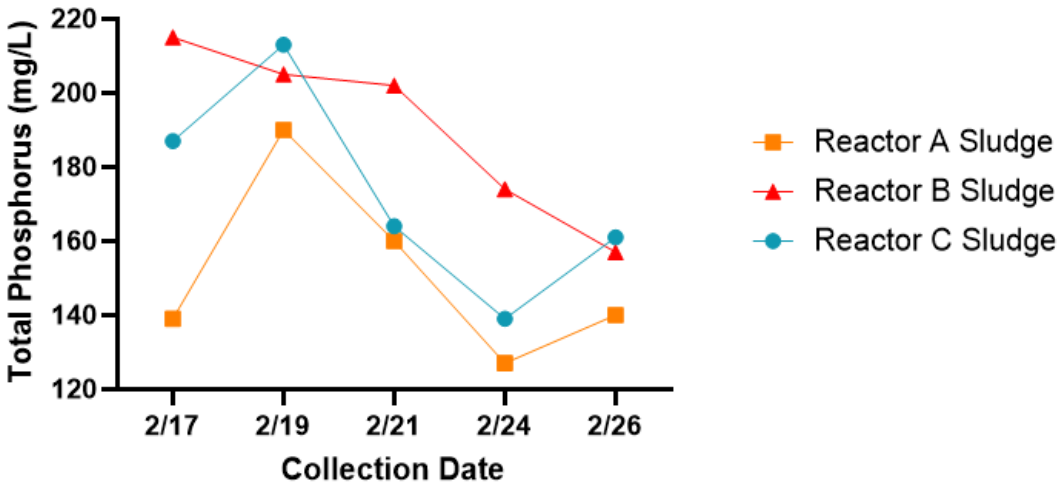

Figure 3.12: The concentration of phosphorus in the reactor systems' sludge during the 8-hour HRT testing period, spanning 9 days. N=3 for each reactor's sludge on each testing day.

The average phosphorus concentration in the incoming sewage feed was 9.8 mg/L and was not statistically different across the three HRT experiments. An ordinary one-way ANOVA, assuming Gaussian distribution and equal standard deviations, was used to determine statistical difference. A P-value of 0.0538 was computed. The rate of accumulation of phosphorus in the reactor sludge at the three tested HRTs is listed in table 3.4.

Table 3.4: The starting and ending phosphorus concentration of each reactor's sludge and the testing period span.

HRT	Average Starting Phosphorus Concentration (mg/L)	Average Ending Phosphorus Concentration (mg/L)	Sample Collection Period (days)	Phosphorus Accumulation Rate (mg/L*day)
24-hour	118	401	26	11
16-hour	131	229	34	6.7
8-hour	180	153	9	-3.1

Note. N=9. The values for all three reactors at the beginning and end of the experimental period were averaged.

The 24-hour iteration experiment took place over a 26-day period. Phosphorus concentration in aeration sludge rose an average of 11 mg/L per day. In the 16-hour iteration of the experiment, spanning 34 days, the average rise in concentration was 6.7 mg/L per day. The 8-hour HRT experiment had a sampling period of 9 days, during which there was no accumulation of phosphorus in the activated sludge of the reactor systems.

3.4 Effects of Hydraulic Retention Time on Coliphage Attachment to Particles

3.4.1 Comparison of Coliphage Removal Across Hydraulic Retention Time Iterations

The coliphage concentration in the sewage after primary settling, collected fresh every day from the ELWWTP and used as feed to the reactors, did not vary significantly across HRT iteration experiments. The average somatic coliphage concentration of the sewage feed was 2588 plaque forming units (pfu)/mL. Although not statistically different (p-value: 0.491), the average concentration of somatic coliphage was lower in the 16-hour HRT sewage 2464 (±895) pfu/mL compared to the 24-hour HRT which was 2948 (±1031) pfu/mL, and higher than the 8-hour HRT concentration, 2352 (±356) pfu/mL.

The average F-specific coliphage concentration was 2778 pfu/mL in the sewage feed for all experiments. The average pfu/mL for the f-specific coliphage during the 16- and 8- hour HRT experiments, was not significantly different (p-value: 0.695). At the 24-, 16-, and 8-hour HRTs the average concentration was 2418 (±1401), 2972 (±817), and 2944 (±1123) pfu/mL respectively. The box plot distribution of coliphage concentrations in the sewage feed at each HRT can be seen in figures 3.13 and 3.14.

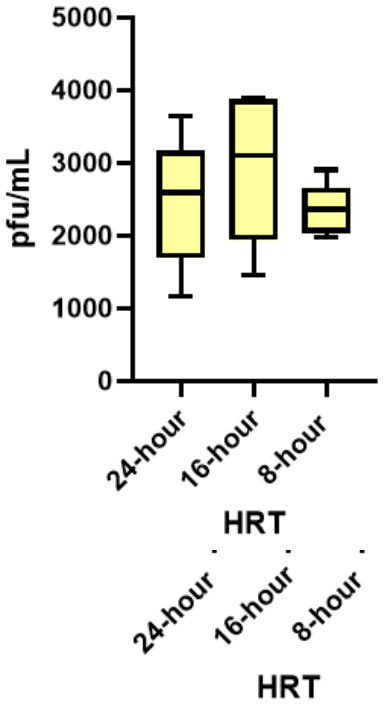

Figure 3.13: Somatic coliphage concentration of sewage feed at each HRT. N=5 for each HRT

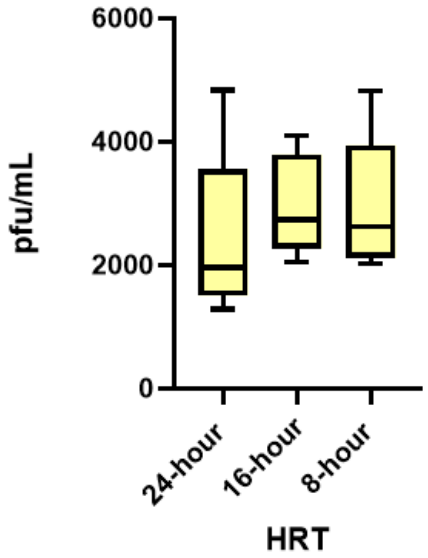

Figure 3.14: F-specific coliphage concentration of sewage feed at each HRT. N=5 for each HRT.

A comparison between somatic coliphage concentrations in the sewage feed and the effluent at different HRTs for each of the reactors A, B and C can be seen in figure 3.15. There was no statistically significant difference between reactor A, B, and C's performance in the 24- and 16-hour HRTs, p-values of 0.078 and 0.437 respectively. At the 8-hour HRT however, reactor A performed differently than B and C (p-value: 0.017). Figure 3.15 shows the removal across HRTs using the average somatic concentration in sewage feed, N=15.

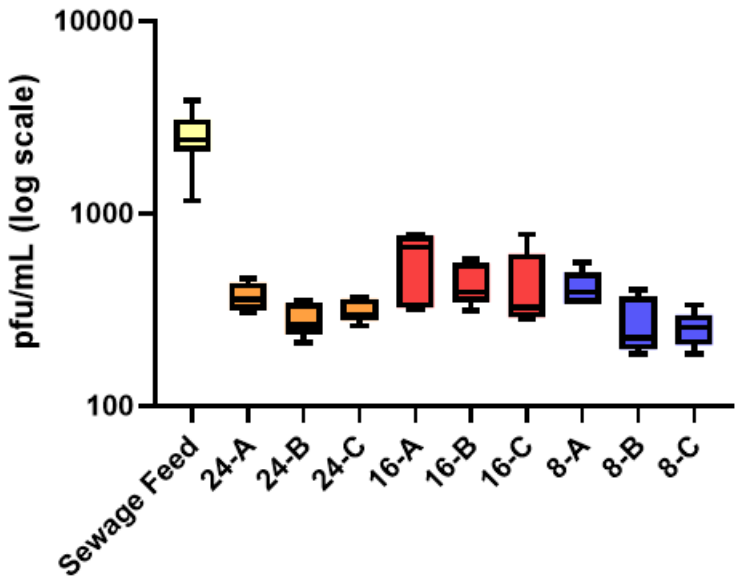

Figure 3.15: Somatic coliphage concentrations in the sewage feed and in each reactor's effluent at all three HRTs. For this graph, yellow designates the sewage feed. Orange, red, and blue correspond to the 24-, 16-, and 8- hour HRTs, respectively. N=15 for the sewage feed, it is averaged across all three HRTs. N=5 for reactor effluents at each HRT.

The individual removal efficiencies for somatic coliphage of reactors across HRTs varied between 80 and 89%. The average removal of somatic coliphage was 87, 84, and 87% at the 24-, 16-, and 8-hour HRTs, respectively. There was no statistical difference between removal efficiencies of reactors at the 24- and 16- hour HRTs. However, there was a difference between reactor somatic coliphage removal performance at the 8-hour HRT. All p-values for these comparisons are found in the paragraph above. Although statistically different, the removal efficiencies between reactors at this HRT varied no more than 6%. The removal efficiencies are summarized in table 3.5. Table 3.5 shows the individual removal efficiency percentages of each reactor at all HRTs using the average f-specific concentration of sewage feed during that iteration of the experiment.

Table 3.5: Average somatic coliphage concentration of each reactor's effluent at each HRT. N=5. Average sewage concentration for that HRT iteration was used to calculate removal efficiency.

Reactor	Average concentration in the sewage feed	Average effluent concentration (pfu/mL)	Removal efficiency
24-hour			
A	2464	372.2	85%
B	2464	283.8	88%
C	2464	314.8	87%
Average	2464	323	87%
16 hour			
A	2948	571.2	80%
B	2948	438.4	85%
C	2948	427.4	86%
Average	2948	479	84%
8 hour			
A	2352	403.4	83%
B	2352	272.8	88%
C	2352	253.8	89%
Average	2352	310	87%

Note. Sewage feed here is not averaged across the three HRTs. It is the average of the 5 readings taken during each HRT, compared to the graphic above, which averages all 15 results.

The individual removal efficiency of f-specific coliphage by the reactors varied from 94-97%. There was no statistical difference in removal of f-specific coliphage across HRTs, the p-value for this comparison was 0.138. There was no statistical difference between reactors at each HRT, p-values for these comparisons were 0.857, 0.626, and 0.734 for the 24-, 16-, and 8-hour HRTs, respectively. However, there was a higher removal efficiency at the 24-hour HRT compared to the 16- and the 8-hour HRTs. Figure 3.16 shows the removal across HRTs using the average f-specific concentration in sewage feed, N=15. Table 3.6 shows the individual removal efficiency percentages of each reactor at all HRTs using the average f-specific concentration of sewage feed during that iteration of the experiment.

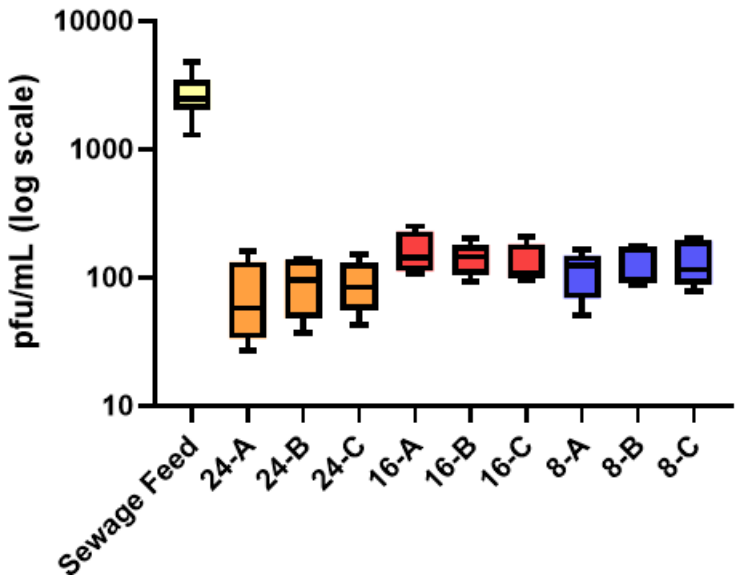

Figure 3.16: F-specific coliphage concentrations in the sewage feed and in each reactor's effluent at all three HRTs. N=15 for sewage feed, N=5 for each reactor's effluent at each HRTs. Orange, red, blue corresponds to 24-, 16-, and 8- hour effluents.

Table 3.6: Average f-specific coliphage concentration of each reactor's effluent at each HRT. N=5 for effluent concentration and sewage feed. Average sewage concentration for that HRT iteration was used to calculate removal efficiency.

Reactor	Average concentration in the sewage feed	Average effluent concentration (pfu/mL)	Removal efficiency
24-hour			
A	2418	78.0	97%
B	2418	93.4	96%
C	2418	91.6	96%
Average	2418	87.7	96%
16 hour			
A	2972	165.2	94%
B	2972	143.4	95%
C	2972	134.2	95%
Average	2972	147.6	95%
8 hour			
A	2944	112.4	96%
B	2944	124.6	96%
C	2944	136.6	95%
Average	2944	124.5	96%

Note. Sewage feed here is not averaged across the three HRTs. It is the average of the 5 readings taken during each HRT, compared to the graphic above, which averages all 15 results.

Because the reactors were determined to operate as replicates, only one reactor's effluent was used for the particle separation experiment. As reactor A did not perform similarly to reactors B and C in the 8-hour HRT when it came to somatic coliphage removal, reactor C's effluent was used for the particle separation analysis. The average concentration of somatic and f-specific coliphage in the sewage feed did not vary more than 15% from the mean concentration across HRTs. Somatic and f-specific coliphage concentrations in the reactor effluents did not vary more than 50% from the mean. Because of this, the effect of HRT on coliphage attachment to particles in section 3.4.3 below is described in terms of percent association.

3.4.2 Control Coliphage Preparation Removal by Filters of Various Pore Sizes

The concentrations of the purified suspension of coliphage did not change post filtration through filters with pore sizes of 100, 20, 3 and 0.45 um. Figure 3.17 shows the pfu/mL in the unfiltered suspension vs the filtered suspensions of PhiX174, a somatic coliphage. The average pfu/mL of each filtrate was 40.7, 36.8, 37.8, and 42.7 pfu/ mL and the unfiltered suspension was 38.5 pfu/mL listed in table 3.7

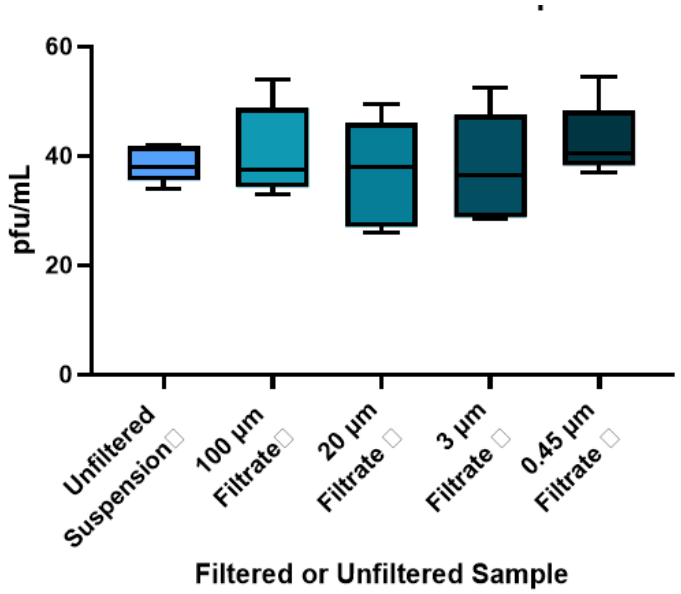

Figure 3.17: The purified PhiX174 concentration in the unfiltered suspension of coliphage and the filtrate of each filter pore size. N=5 for each sample type.

Table 3.7: Average coliphage concentration (pfu/mL) in the purified unfiltered suspension of somatic and f-specific coliphage and in the filtrate from each filter pore size.

Suspension Type	Unfiltered (pfu/mL)	100 µm Filtrate (pfu/mL)	20 µm Filtrate (pfu/mL)	3 µm Filtrate (pfu/mL)	0.45 µm Filtrate (pfu/mL)
PhiX174 (Somatic Coliphage)	38.5 ±3.3	40.7 ±8.4	36.8 ±9.8	37.8 ±10.1	42.7 ±6.8
MS2 (F-Specific Coliphage)	27.3±5.6	24.3±6.1	27.9±5.4	22.3±3.1	29.1±4.3

Note. Averages were computed using an N=5. Thirty mL of the purified coliphage was run through each filter size in parallel.

Figure 3.18 shows the pfu/mL of the unfiltered and filtered purified suspension of MS2 an f-specific coliphage. The concentration of coliphage did not vary significantly between the unfiltered suspension and the filtrate collected after each filter pore size. The average pfu/mL of each sample is listed in table 3.7 above. The average pfu/mL of each filtrate was 24.3, 27.9, 22.3, and 29.1 pfu/ mL and the unfiltered suspension was 27.3 pfu/mL. There was no statistically significant

difference between the coliphage in the unfiltered purified suspensions of PhiX174 and MS2 when compared to the filtrate of each suspension through the different filter pore sizes. An ordinary ANOVA was used to compute statistical difference on the PhiX174 data sets, as all were normally distributed. A Kruskal-Wallis ANOVA test was used on the MS2 data sets because the 100 µm filtrate data were not normally distributed. Table 3.8 summarizes the P-value of the statistical comparisons.

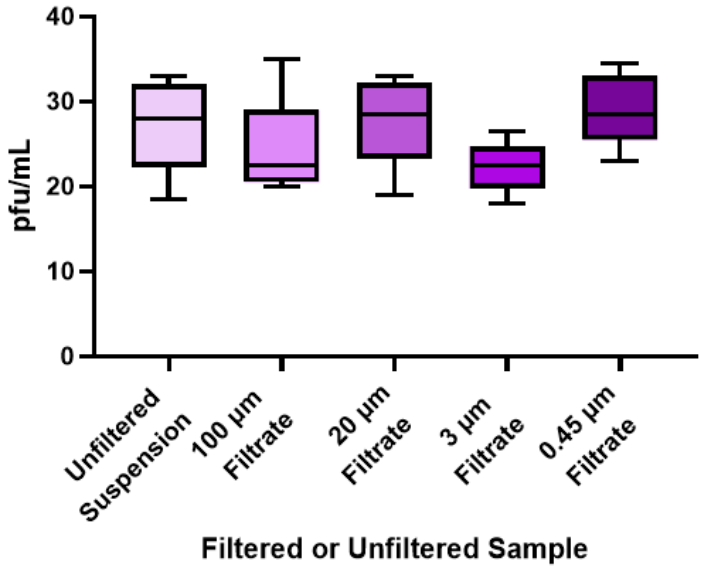

Figure 3.18: The *MS2* concentration in the unfiltered purified suspension of coliphage and the filtrate from each filter pore size. N=5 for each sample type.

Table 3.8: ANOVA P-values for the analysis of statistically significant differences between filtered and unfiltered suspensions of coliphage.

Suspension Type	Statistically Significant Difference between Filtered vs Non-Filtered Suspensions?	P-Value
PhiX174 (Somatic Coliphage)	No	0.7825
MS2 (F-Specific Coliphage)	No	0.1943

Note. PhiX174 comparison used an ordinary ANOVA. MS2 comparison was computed using a Kruskal-Wallis ANOVA test. Differences in method are due to normality of data assumptions.

3.4.3 Coliphage Attachment to Particles Across Hydraulic Retention Time Iterations

There was no statistically significant difference between the particle association of somatic coliphage across HRTs, ELWWTP effluent, and sewage feed at the 100 µm filter. Table 3.9 lists the percentage coliphage associated with particles of 100 µm or greater for each sample type. The percent somatic coliphage associated to particles of 100 µm size or greater was 8.6, 8.4, and 2.2% in the 24-, 16-, and 8-hour HRT, respectively. In the ELWWTP effluent (7-hour HRT) and the sewage feed there was a 4.2 and an 11.0% association of somatic coliphage to particles of this size. The association profile at this particle size or greater is shown in figure 3.19. A Kruskal-Wallis test was used to determine statistically significant differences because not all data were normally distributed.

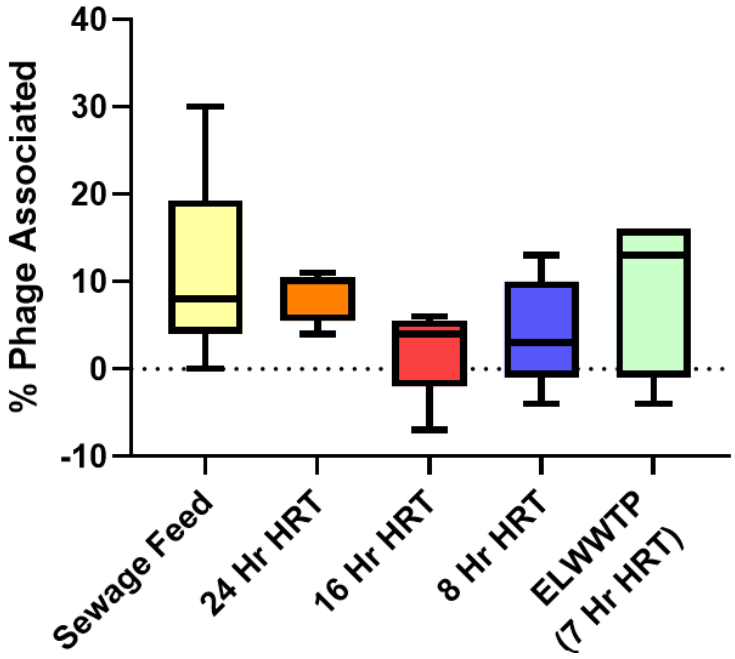

Figure 3.19: The association profile of somatic coliphage to particles of 100 µm size or greater across reactor effluent, ELWWTP effluent, and sewage feed. N=5 for reactor effluents (24-, 16- and 8- Hr HRT). N=5 for ELWWTP. N=15 for sewage feed.

For f-specific coliphage there was a statistically significant difference between the 24-hour HRT compared to the association profiles of 16- and 8-hours reactor effluents, ELWWTP effluent, and sewage feed. The association of coliphage to particles was much higher in the 24-hour HRT effluent than in all other samples. The percentage f-specific coliphage associated with particles of 100 µm particle size or greater are listed in table 3.9 and the profile is shown in figure 3.20. The percent f-specific coliphage associated to particles of 100 µm size or greater was 63.6, -1, and 6.2% in the 24-, 16-, and 8-hour HRT, respectively. In the ELWWTP effluent (7-hour HRT) and the sewage feed there was a 2.0 and an 8.0% association of f-specific coliphage to particles of this size.

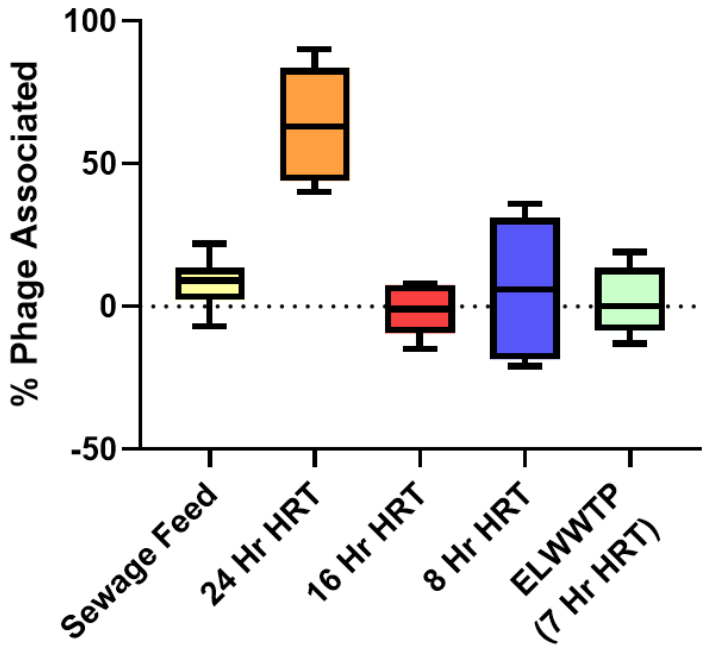

Figure 3.20: The association profile of f-specific coliphage to particles of 100 µm size or greater across reactor effluent, ELWWTP effluent, and sewage feed. N=5 for reactor effluents (24-, 16- and 8- Hr HRT). N=5 for ELWWTP. N=15 for sewage feed.

Table 3.9: The percentage coliphage associated to particles of 100 µm or greater in each sample type.

Sample Type	% Somatic Coliphage Associated	% F-Specific Coliphage Associated
24-hour HRT Effluent	8.6	63.6
16-hour HRT Effluent	8.4	-1
8-hour HRT Effluent	2.2	6.2
ELWWTP	4.2	2.0
Sewage Feed	11.0	8.0

There was no statistically significant difference between the percent somatic coliphage associated to particles at the 20 µm filter pore size across the HRT effluents, the ELWWTP effluent, and the sewage feed. The percentage association increased in all samples compared to the 100 µm filter pore size. The association profile is shown in figure 3.21. The percent somatic coliphage associated to particles of 20 µm size or greater was 9.8, 6.4, and 8.8% in the 24-, 16-, and 8-hour HRT, respectively. In the ELWWTP effluent (7-hour HRT) and the sewage feed there was a 4.2 and an 9.8% association of somatic coliphage to particles of this size.

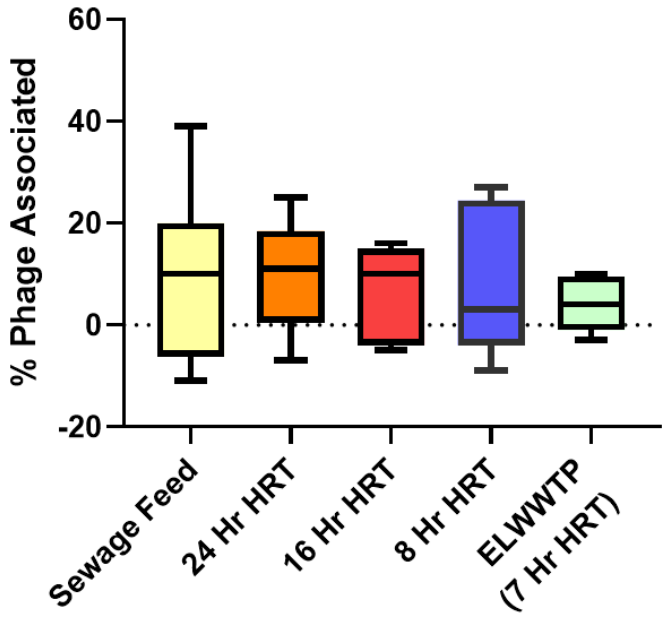

Figure 3.21: The association profile of somatic coliphage to particles of 20 µm size or greater across reactor effluent, ELWWTP effluent, and sewage feed. N=5 for reactor effluents (24-, 16- and 8- Hr HRT). N=5 for ELWWTP. N=15 for sewage feed.

There was a statistically significant difference between the f-specific coliphage associated with particles 20 µm or greater in size when comparing all sample types. Additionally, there was a difference between the association profile to particles of this size when comparing reactor effluents at the three HRTs. There was a 66% association to particles at the 24-hour HRT. This decreased to 11% in the 16-hour HRT effluent and further to 2.2% in the 8-hour HRT. The attachment profile for this particle size for all samples is shown in figure 3.22. The percentage of somatic and f-specific coliphage associated to particles of 20 µm or greater for each sample type is summarized in table 3.10.

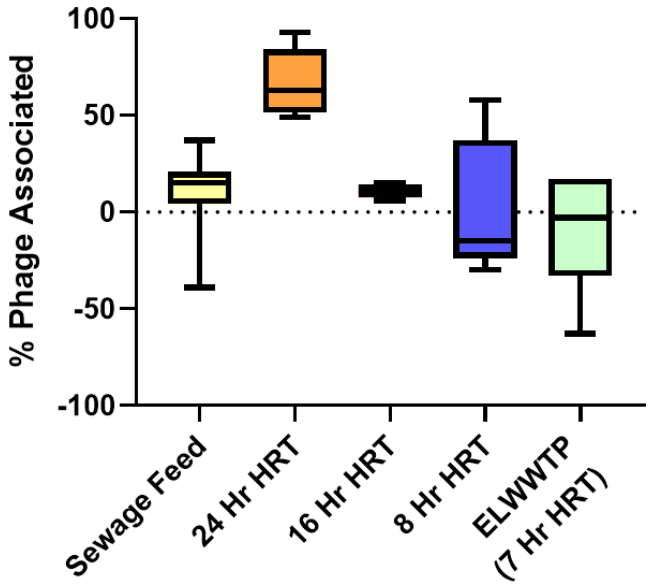

Figure 3.22: The association profile of f-specific coliphage to particles of 20 µm size or greater across reactor effluent, ELWWTP effluent, and sewage feed. N=5 for reactor effluents (24-, 16- and 8- Hr HRT). N=5 for ELWWTP. N=15 for sewage feed.

Table 3.10: The percentage coliphage associated to particles of 20 µm or greater in each sample type.

Sample Type	% Somatic Coliphage Associated	% F-Specific Coliphage Associated
24-hour HRT Effluent	9.8	66.8
16-hour HRT Effluent	6.4	11.0
8-hour HRT Effluent	8.8	2.2
ELWWTP	4.2	-7.0
Sewage Feed	9.8	11.1

As seen in figure 3.23, there was a statistically significant difference between the association of somatic coliphage to particles of 3 µm size or greater when comparing reactor effluent (24-, 16-, and 8-hour HRT) with ELWWTP effluent and the sewage feed. However, there was no difference when comparing reactor effluents across HRTs. The percent somatic coliphage associated to particles of 3 µm size or greater was 31.0, 17.8, and 28.0% in the 24-, 16-, and 8-hour HRT, respectively. In the

ELWWTP effluent (7-hour HRT) and the sewage feed there was a 9.4 and an 34.5% association of somatic coliphage to particles of this size.

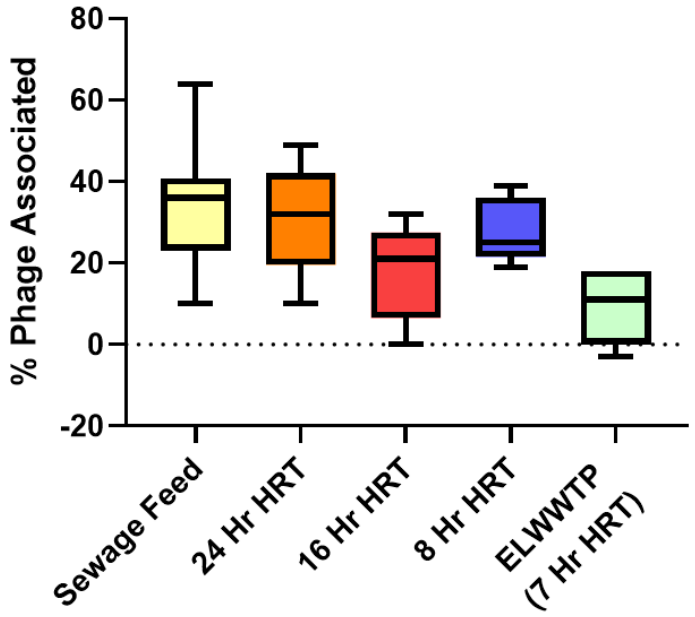

Figure 3.23: The association profile of somatic coliphage to particles of 3 µm size or greater across reactor effluent, ELWWTP effluent, and sewage feed. N=5 for reactor effluents (24-, 16- and 8- Hr HRT). N=5 for ELWWTP. N=15 for sewage feed.

There was a statistically significant difference between the association of f-specific coliphage to particles of 3 µm or greater when comparing reactor effluent from the 24-hour HRT to the 16-hour HRT, 8-hour HRT, and the ELWWTP effluent. This follows the trend seen in larger filter pore sizes: as HRT decreases, so does the association of f-specific coliphage to particles of that filter pore size or greater. There is no significant difference between the attachment profile of the 16-hour vs the 8-hour effluents. The percentage association at the 24-hour HRT surpasses the association in the sewage feed. The attachment profile is shown in figure 3.24 and

table 3.11 summarizes the average percent associated coliphage, somatic and f-specific, at this filter pore size.

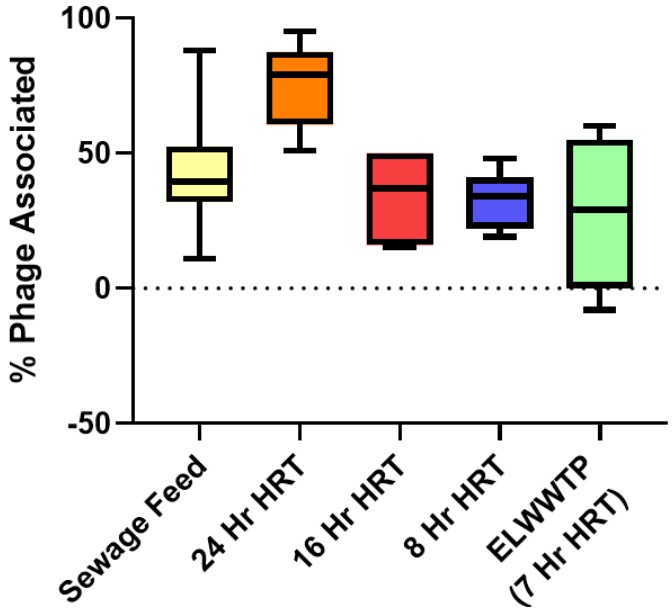

Figure 3.24: The association profile of f-specific coliphage to particles of 3 µm size or greater across reactor effluent, ELWWTP effluent, and sewage feed. N=5 for reactor effluents (24-, 16- and 8- Hr HRT). N=5 for ELWWTP. N=15 for sewage feed.

Table 3.11: Average percent association of somatic and f-specific coliphage at the 3 µm filter pore size across sample types.

Sample Type	% Somatic Coliphage Associated	% F-Specific Coliphage Associated
24-hour HRT Effluent	31.0	75.0
16-hour HRT Effluent	17.8	33.8
8-hour HRT Effluent	28.0	32.0
ELWWTP	9.4	27.8
Sewage Feed	34.5	42.3

As seen in figure 3.25, there was a statistical difference for the somatic coliphage attachment to particles of 0.45 µm size or greater across the 24-hour, 16-hour and 8-hour HRT effluents. There is a downward trend indicating that as HRT decreases, so does the association of somatic coliphage to particles of 0.45 µm size or greater. The

association was 66.6, 51.4, and 47.2% in the 24-, 16-, and 8-hour HRT, respectively. In the ELWWTP effluent (7-hour HRT) and the sewage feed there was a 28.0 and a 62.1% association of somatic coliphage to particles of this size.

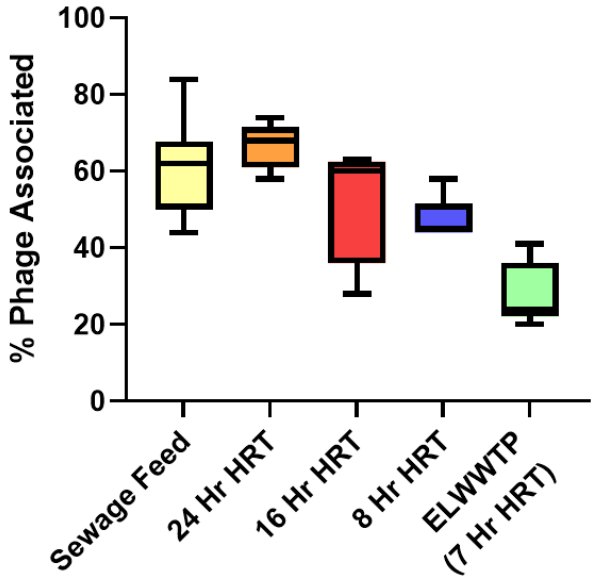

Figure 3.25: The association profile of somatic coliphage to particles of 0.45 µm size or greater across reactor effluent, ELWWTP effluent, and sewage feed. N=5 for reactor effluents (24-, 16- and 8- Hr HRT). N=5 for ELWWTP. N=15 for sewage feed.

In figure 3.26, there was a statistically significant difference when comparing the 24-hour HRT to the 16- and 8-hour HRTs. There was not a statistically significant difference between the 16-hour and the 8-hour HRT effluent association Table 3.12 summarizes the percentage association of somatic and f-specific phage with particles of 0.45 µm size or greater.

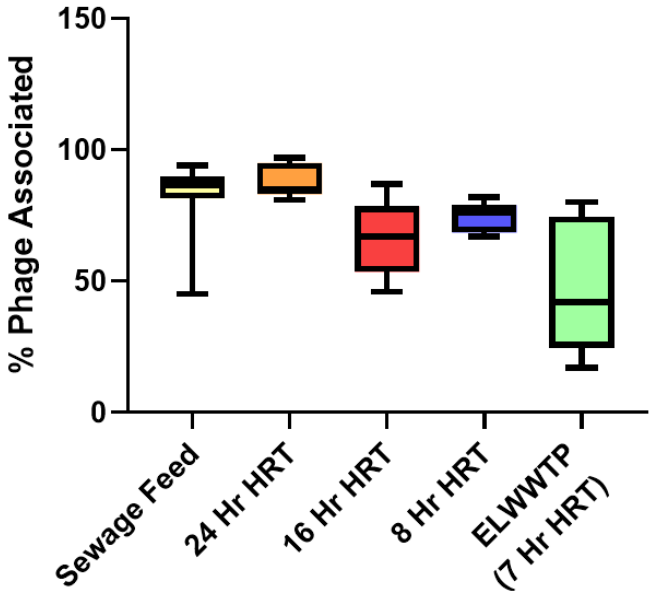

Figure 3.26: The association profile of f-specific coliphage to particles of 0.45 µm size or greater across reactor effluent, ELWWTP effluent, and sewage feed. N=5 for reactor effluents (24-, 16- and 8- Hr HRT). N=5 for ELWWTP. N=15 for sewage feed.

Table 3.12: Average percent association of somatic and f-specific coliphage at the 0.45 µm filter pore size across sample types.

Sample Type	% Somatic Coliphage Associated	% F-Specific Coliphage Associated
24-hour HRT Effluent	66.6	88.2
16-hour HRT Effluent	51.4	66.2
8-hour HRT Effluent	47.2	74.2
ELWWTP Effluent	28.0	48.0
Sewage Feed	62.08	83.3

Table 3.13 contains a summary of all particle association percentages at each filter pore size and at all HRTs for efficient comparison. In summary, it was found that as HRT decreased coliphage association to particles also decreased. This trend was most evident in somatic coliphage when considering the 0.45 µm pore size. However, this trend was immediately evident with f-specific coliphage at the larger (100, 20, and 3 µm) pore sizes, and less so at the 0.45 µm pore size. A summary of all statistical

comparisons made for this section and their corresponding P-values can be found on table 3.14.

Table 3.13: Summary of coliphage association percentages across sample types and HRTs.

Coliphage	**Somatic**				**F-Specific**			
	Filter pore size (µm)							
	100	**20**	**3**	**0.45**	**100**	**20**	**3**	**0.45**
Primary Sewage (n=15)	11.0	9.8	34.5	62.1	8.0	11.1	42.3	83.3
HRT (hour)								
24 (n=5)	8.4	9.8	31.0	66.6	63.6	66.8	75.0	88.2
16 (n=5)	2.2	6.4	17.8	51.4	-1.0	11.0	33.8	66.2
8 (n=5)	4.2	8.8	28.0	47.2	6.2	2.2	32.0	74.2
ELWWTP 7 (n=5)	8.6	4.2	9.4	28.0	2.0	-7.0	27.8	48.0

Table 3.14: Statistical comparisons used and their results when analyzing particle attachment profiles of somatic and f-specific coliphage across bench scale HRTs, ELWWTP effluent, and reactor sewage feed.

Comparison	**ANOVA Test**	**P-value**	**Statistical Difference?**
≥ 100 µm Particle-Somatic Attachment Profile -All Samples	Kruskal-Wallis	0.3427	No
≥ 100 µm Particle-Somatic Attachment Profile -Reactor Effluent	Kruskal-Wallis	0.3093	No
≥ 20 µm Particle-Somatic Attachment Profile -All Samples	BFW	0.9047	No
≥ 20 µm Particle-Somatic Attachment Profile -Reactor Effluent	BFW	0.9051	No
≥ 3 µm Particle-Somatic Attachment Profile -All Samples	BFW	0.0051	Yes**

Table 3.14 (cont'd)

≥ 3 µm Particle-Somatic Attachment Profile -Reactor Effluent	BFW	0.2173	No
≥ 0.45 µm Particle-Somatic Attachment Profile -All Samples	Kruskal-Wallis	0.0008	Yes***
≥ 0.45 µm Particle-Somatic Attachment Profile -Reactor Effluent	Kruskal-Wallis	0.0148	Yes*
≥ 100 µm Particle-F-specific Attachment Profile -All Samples	BFW	0.0003	Yes***
≥ 100 µm Particle-F-specific Attachment Profile -Reactor Effluent	BFW	0.0010	Yes***
≥ 20 µm Particle-F-Specific Attachment Profile -All Samples	BFW	0.0042	Yes**
≥ 20 µm Particle-F-Specific Attachment Profile -Reactor Effluent	BFW	0.0088	Yes**
≥ 3 µm Particle-F-specific Attachment Profile -All Samples	BFW	0.0103	Yes*
≥ 3 µm Particle-F-specific Attachment Profile -Reactor Effluent	BFW	0.0013	Yes**
≥ 0.45 µm Particle-F-specific Attachment Profile -All Samples	Kruskal-Wallis	0.0023	Yes**

Table 3.14 (cont'd)

≥ 0.45 μm Particle-F-specific Attachment Profile - Reactor Effluent	BFW	0.0285	Yes*

Note. P-value summaries: *= P ≤ 0.05, **=P ≤ 0.01, ***=P ≤ 0.001. N=15. Brown-Forsythe and Welch ANOVA (BFW).

3.5 Effects of Hydraulic Retention Time on Bacterial, Fungal, and Viral Communities

3.5.1 Bacterial Functional Genes

The normalized reads of bacterial functional genes related to the first two steps of nitrification, *amo* and *hao,* decreased between the 24- and 16- hour HRTs, but less so from the 16- to 8-hour HRT. This is consistent with the effluent ammonia content. There was an increase in ammonia between the 24- (0.05 mg/L) and 16-hour (7 mg/L), HRTs but a much smaller increase between the 16- and 8- (8.9 mg/L) HRT. The expression of *amo* (A, B, and C) fell from 1.94 EFPKG (number of fragments per kb of effective gene length per genome equivalent) at the 24-hour HRT to 0.572 and 0.566 EFPKG at the 16- and 8-hour HRTs, respectively. This is a normalization method used by Kbase to compare genetic potential across different samples. This method normalizes across library size, target gene size, and predicted average genome size. Similarly, the EFPKG corresponding to *hao* fell from 0.415 in the 24-hour HRT to 0.160 and 0.142 in the 16- and 8-hour HRTs.

While the fragment count for these functional genes decreased, the count for the genes associated with the third step of nitrification, NO_2^- to NO_3^-, increased. The *narG*/*nxrA* functional gene group is responsible for the interconversion of NO_2^- and NO_3^- in the last step of nitrification and the first step of denitrification. The EFPKG for this gene group increased from 2.700 at the 24-hour HRT to 3.707 and 4.236 in

the 16- and 8-hour HRTs, respectively. Table 3.15 summarizes the fragment count of each of the genes mentioned above.

Table 3.15: Functional gene normalized fragment count across HRTs.

Functional Gene	Average 24-hour EFPKG	Average 16-hour EFPKG	Average 8-hour EFPKG
amoA	0.501	0.167	0.168
amoB	0.463	0.161	0.154
amoC	0.975	0.244	0.244
hao	0.415	0.160	0.142
narG/nxrA	2.700	3.707	4.236

Note: EFPKG is a normalization metric for paired-end libraries used in Kbase. It quantifies the number of fragments per kb of effective gene length per genome-equivalent. N=3 for each HRT.

The bacteria containing these functional genes were mapped using the Kaiju app in Kbase. This app generated a normalized score for functional gene reads, different from the fragment counts (EFPKG) used above, corresponding to various nitrifying bacteria. The normalized score scales raw counts to sequencing depth, gene length, and DNA composition. The higher the normalized score, the higher the amount of that gene found in a certain bacterium compared to another. This score can be used to compare functional gene reads across samples.

In the 24-hour HRT, the main nitrifiers containing the *amoA* gene were *Nitrosomonas* and *Nitrospira*. The normalized read score was higher for *Nitrosomonas.* This trend holds in the 16-hour HRT results, although the normalized score for each bacterium is lower, meaning that there was less of that gene in the sample. At the 8-hour HRT, there was a small amount of *amoA* reads corresponding to *Nitrospira*. The *amoA* read

count in *Nitrosomonas* remained the same. Similarly, the *amoB* gene was primarily found in *Nitrosomonas* across all HRTs. *Nitrospira* did exhibit some *amoB* reads during the 24-hour HRT, but was not found in large quantities at the 16- and 8-hour HRTs. *Nitrosomonas, Nitrospira, and Nitrosospira* were all found to have the *amoC* functional gene at the 24-, 16-, and 8-hour HRTs. *Nitrosomonas* bacteria again dominated the read count for this functional gene. However, the score dropped between the 24- and 16-hour HRT but did not change much between the 16- and 8-hour HRTs.

The *hao* functional gene exhibited a similar pattern. *Nitrosomonas* dominated the read count for this functional gene at all HRTs. However, the score dropped between the 24- and 16-hour HRTs. *Nitrospira* was found to contain large amounts of this functional gene in the 24-hour and 16-hour HRTs, but was not found to contain *hao* at the 8-hour HRT.

Nitrospira dominated the read count for the *narG*/*nxrA* across all HRTs. It was the only bacterium found to contain this functional group at the 24-hour HRT, where there was a significant amount of nitrate in the reactor effluent, 18.1 mg/L. The amount of *narG*/*nxrA* found in *Nitrospira* decreased at the 16-hour HRT and did not change at the 8-hour HRT. *Reyranella* appeared at the 16- and 8-hour HRTs, with its normalized score staying relatively consistent across the two shorter HRTs. *Janibacter* appeared as another bacterium containing *narG*/*nxrA* reads, but only at the 8-hour HRT. The normalized reads for functional genes related to nitrification and the bacterium they were found in are summarized on table 3.16.

Table 3.16: Normalized read scores for functional genes related to nitrification and the bacterial genomes that were found in across HRTs.

Gene	Genus	24-hour HRT Score	16-hour HRT Score	8-hour HRT Score
amoA	*Nitrosomonas*	0.155	0.037	0.04
	Nitrospira	0.025	0.001	-
amoB	*Nitrosomonas*	0.259	0.089	0.089
	Nitrospira	0.013	-	-
amoC	*Nitrosomonas*	0.198	0.059	0.068
	Nitrospira	0.178	0.007	0.003
	Nitrosospira	0.006	0.003	0.002
hao	*Nitrosomonas*	0.257	0.11	0.105
	Nitrospira	0.069	0.003	-
	Candidatus	0.003	-	-
	Methylocystis	-	0.002	0.001
narG/nxrA	*Nitrospira*	0.376	0.099	0.155
	Reyranella	-	0.019	0.016
	Janibacter	-	-	0.031

Note. N=3 for each functional gene.

The percent relative abundance of *Nitrospira* and *Nitrosomonas,* the two main nitrifying bacteria, changed with HRT. *Nitrospira* had a higher percent relative abundance compared to *Nitrosomonas* at all HRTs. The percent *Nitrospira* dropped from 5% at the 24-hour HRT to 1.3% in the 16- and 8-hour HRTs. The percent relative abundance of *Nitrosomonas* dropped from 0.9% in the 24-hour HRT to 0.5% and 0.4% in the 16- and 8-hour HRT, respectively. Table 3.17 summarizes these results.

Table 3.17: Percent relative abundance of *Nitrospira* and *Nitrosomonas* in shotgun sequencing samples across all HRTs.

HRT	Bacterium	Percent Relative Abundance
24-hour	*Nitrosomonas*	0.9
	Nitrospira	5
16-hour	*Nitrosomonas*	0.5
	Nitrospira	1.3
8-hour	Nitrosomonas	0.4
	Nitrospira	1.3

Note. N=3 at each HRT.

3.5.2 Nitrifying Fungi

The genera with more than 1 percent relative abundance are listed in table 3.18. *Aspergillus, Batrachochytrium, Spizellomyces, Lobosporangium, Synchytrium, Linderina, Sordaria, and Rhizophagus* were the dominant genera in this environment. Although *Sordaria* only appeared above 1% relative abundance in the 16- and 8-hour HRTs.

Table 3.18: Genera of fungi and percent relative abundance found in the 24-, 16-, and 8-hour HRT shotgun sequencing reads.

Genera	24-hour HRT	16-hour HRT	8-hour HRT
Aspergillus	4%	4%	5%
Batrachochytrium	3%	3%	3%
Spizellomyces	3%	3%	3%
Lobosporangium	3%	2%	2%
Synchytrium	2%	2%	2%
Linderina	2%	2%	2%
Rhizophagus	2%	2%	1%
Sordaria	NS	2%	2%

Note. N=3 for each genus.

Of the nitrifying fungi listed in the literature review of this work, only *Aspergillus flavus* was found in the sludge samples sequenced. And as seen in table 3.19, the percent relative abundance of this fungi did not change much with HRT.

Table 3.19: Percent relative abundance of *Aspergillus flavus* in sludge samples across HRTs.

HRT	Sequencing Sample One	Sequencing Sample Two	Sequencing Sample Three
24-hour	0.01%	0.02%	0.01%
16-hour	0.008%	0.01%	0.01%
8-hour	0.01%	0.01%	0.02%

3.5.3 Viral Community

A similar approach was taken when characterizing the sludge viral community in reactors at all three HRTs. Virions do not nitrify, but they affect the nitrification process by infecting bacteria and fungi that do. The dominant DNA viral families remained the same across HRTs with two exceptions. The relative percentage of *Siphoviridae* increased with HRT, and the *Gokushovirinae* family appeared only in the 8-hour HRT samples. Table 3.20 summarizes the dominant families at each HRT.

Table 3.20: The percent relative abundance of dominant viral families in reactor sludge samples at the 24-, 16-, and 8-hour HRT.

Family	24-hour HRT	16-hour HRT	8-hour HRT
Myoviridae	38%	39%	36%
Siphoviridae	30%	32%	38%
Nucleocytoviricota	17%	17%	13%
Podoviridae	4%	3%	3%
Schitoviridae	0.9%	0.8%	0.9%
Ackermannviridae	0.7%	0.7%	0.7%
Herelleviridae	0.7%	0.7%	0.5%
Autographiviridae	0.7%	0.6%	0.7%
Gokushovirinae	-	-	2%

Note. N=3 for each family.

Found under each family of virus, there were several different bacteriophages. The bacteria targeted by the dominant bacteriophages (above 1% relative abundance within each family group) in the samples collected are summarized below on table 3.21.

Table 3.21: The bacteria targeted by the bacteriophage found in each viral family in reactor sludge samples.

Family	Target Bacteria
Myoviridae	*Escherichia sp., Salmonella sp., Bacillus sp., Aeromonas sp., Prochlorococcus sp., Faecalibacterium sp., Agrobacterium sp. Synechococcus sp.*
Siphoviridae	*Streptococcus sp., Bacillus sp., Mycobacterium sp., Gordonia sp. Microbacterium sp.*
Nucleocytoviricota	*
Podoviridae	*Escherichia sp., Erwinia sp., Pseudomonas sp., Sinorhizobium sp., Xanthomonas sp., Ralstonia sp., Bordatella sp., Phormidium sp. Serratia sp.*
Schitoviridae	*Agrobacterium sp., Ruegeria sp., Dinoroseobacter sp., Roseobacter sp., Vibrio sp., Eriwinia sp., Sulfitobacter sp., Salmonella sp., Xanthomonas sp., Sinorhizobium sp., Acinetobacter sp., Pseudomonas sp.*
Ackermannviridae	*Virbio sp., Agrobacterium sp., Sinorhizobium sp., Rhizobium sp., Ralstonia sp., Aeromonas sp., Erwinia sp., Enterobacter sp.*
Herelleviridae	*Bacillus sp., Enterococcus sp., Staphylococcus*
Autographiviridae	*Pseudomonas sp., Ralstonia sp., Mesorhizobium sp., Pelagibacter sp.,*
Gokushovirinae	-

Note. Nucleocytoviricota infect a wide spectrum of eukaryotes (Wu et al. 2024), the primary targets of the phage in the activated sludge samples collected here were green algae and amoebas.

CHAPTER 4: DISCUSSION

4.1 Effects of Hydraulic Retention Time on Nitrification and Phosphorus Accumulation

The ammonia concentration in reactor effluent increased as HRT decreased, as seen in figures 3.7-3.9. The initial concentration of ammonia in the reactor effluent at 24-hour HRT was 0.05 mg/L. This increased to an average of 7.0 mg/L in the 16-hour HRT and 8.9 mg/L in the 8-hour HRT. This is consistent with previous studies that found that nitrifying bacteria grow slowly and require intense aeration and long HRTs to retain a foothold in conventional activated sludge systems (CAS) (Li et al. 2007). This phenomenon may not only apply to CAS but has been observed in other types of activated sludge systems, but not all. A study in 2009 by Whang et al. found that in a mixed batch reactor operated at DO levels higher than 0.52 mg/L, longer HRT combined with higher mixed liquor suspended solids concentration increased nitrification. While the studies by Li et al. (2007) and Whang et al. (2009) support the results presented here, a study in 2013 using a CAS bench scale reactor found that although nitrification decreased initially when HRT was lowered from 30- to 10-hours, the nitrification rate re-stabilized to pre-shortening levels (Li et al. 2013). This system was run for 260 days, almost ten times longer than the maximum run length of this study, 36 days. This may indicate that the nitrification rate might have increased had the reactors been run for longer, allowing the slow growing nitrifier populations a chance to restabilize in the aerated sludge. Additionally, the findings of this study do not extend to all activated sludge systems. The study by Cydzik-Kwiatkowska and Wojnowska-Baryła (2015) found that a 90% ammonia nitrification efficiency was attainable at 10-, 13-, and 19-hour HRTs when running an aerobic granular sequencing batch reactor.

Although not the focus of this study, the accumulation of phosphorus in reactor activated sludge was characterized across HRTs. Phosphorus is another recoverable material of interest. While phosphorus was accumulated in reactor sludge during the 24- and 16-hour HRTs, this was not the case during the 8-hour HRT. The 8-hour HRT iteration was the shortest of the three, lasting only 9 days compared to 34 and 26 days for the other two HRTs. This may not have allowed enough time for the phosphorus to accumulate to the levels seen at the longer HRTs. Additionally, the 8-hour HRT had the shortest residence time. It is possible that the increase in flow disrupted the phosphorus accumulation activity within the activated sludge. So this suggests that recovery of more ammonia at shorter HRTs also means recovery of phosphorus in the effluent.

4.2 Effects of Hydraulic Retention Time on Coliphage Attachment to Particles

There are no previous studies characterizing virus-particle attachment profiles with changing HRTs. However, the findings in this study are consistent with studies that have characterized attachment percentages in sewage and municipal WWTP effluent. More than 50 years ago, Wellings et al. (1976) found a similar attachment profile when examining the sewage entering a small municipal wastewater treatment plant. This was done by collecting influent and effluent samples and assaying them before and after sonication. Wellings et al. (1976) targeted animal viruses supported by buffalo green monkey broth and reported that 24-81% and 90-100% of the virus in the plant influent and effluent was particle associated, respectively. The results obtained from the reactors in this study are like the findings by Wellings et al. (1976), 62% of the somatic coliphage in the primary sewage were found to be associated to particles of 0.45 μm or larger, compared to 83.3% of f-specific coliphage. These results are seen in figures 3.25 and

3.26 for somatic and f-specific coliphage, respectively. The coliphage-particle attachment in bench scale reactor effluent was less drastic in the study presented in this thesis, with the maximum association seen in the 24-hour HRT effluent. As summarized in table 3.12, 66.6% of somatic coliphage and 88.2% of f-specific coliphage in this study were associated to particles of 0.45 μm or larger. This difference could be attributed to different viral targets and a bench scale operation. The focus of this study was coliphage, virus that infects *E. coli* bacteria.

In another early study on full-scale wastewater treatment plants, Gerba et al. (1978) found that 1-24% f-specific coliphage in effluent directly following activated sludge treatment was particle associated but did not specify what HRTs the treatment plants operated at, which may explain some of the discrepancy. Because bench scale reactors were used in this study, a direct comparison is difficult as often full-scale reactors do not perform similarly to bench scale wastewater system even if operating at the same HRTs. Yet, as summarized in table 3.11, 27.8% of f-specific coliphage attached to particles of 3 μm or larger in the ELWWTP effluent, operated at an average of 7-hour HRT during sample collection. Discrepancies could also be due to differences in host bacteria. This study employed ATCC#700891 host for f-specific coliphage, while Gerba et al (1978) used ATCC #15597. It is possible that the host used in that study supports a smaller variety of f-specific coliphage than the one used in these experiments.

Particle attachment was always lower in ELWWTP effluent compared to bench scale reactor effluent, except in somatic coliphage association to particles ≥ 100 μm seen in figure 3.19. The ELWWTP ran its hydraulic retention time at an average of 7-hours during the sampling period, lower than the lowest HRT tested in the reactors (8-hours). It

is unknown whether this lower association is due to a lower retention time, or that the ELWWTP is a full-scale operation. To compare reactor performance to full scale treatment plants, other facilities running similar retention times would need to be tested.

It was established that the somatic and f-specific coliphage concentrations in the ELWWTP sewage, used for the reactor feed, were not statistically different across the three HRT experiments at bench scale, as seen on figures 3.13 and 3.14. The concentration of f-specific coliphage in the reactor effluent of this study did not vary significantly across HRTs. More than half of the f-specific coliphage in the effluent of the 24-hour HRT was associated to particles of 100 μm or greater, seen in figure 3.20. There is a steep drop-off in association at the 16- and 8- hour HRTs, and significant association (above 20%) is not seen in these effluents until the 3 μm filter pore size (figure 3.24). Approximately 33% of f-specific coliphage associated with particles of 3 μm or greater at these HRTs, compared to 75% association with particles of this size or greater at the 24-hour HRT. There was less of a difference in performance when looking at percent association of f-specific coliphage to particles of 0.45 μm or greater (figure 3.26 and table 3.12). This suggests that shorter HRT could significantly decrease the amount of virus (similar to f-specific coliphage) associated to particles of 3 μm or greater but have a smaller effect on association to smaller particles.

The somatic coliphage concentration in reactor effluent was found to be slightly statistically different across HRTs, as seen in figure 3.15. However, the average concentrations were similar: 323, 479, and 310 pfu/mL for the 24-, 16-, and 8-hour HRTs experiments. This was mostly due to a change in the influent somatic coliphage concentration during the 16-hour HRT iteration of the experiment. Average percent

association of somatic coliphage to particles in the 24-hour HRT effluent was higher at all filter pore sizes compared to the 16- and 8-hour HRT effluents, as seen in table 3.13. The drop-off in association at the lower HRTs was not as steep as with f-specific coliphage.

Somatic and f-specific coliphage differ in their infection mechanism and morphological characteristics (Monis, 2015 and Skraber et al. 2004). F-specific coliphage include filamentous and icosahedral morphologies and are between 6 to 27 nm in size. Their isoelectric points vary from pH 2-7 (Heffron and Mayer 2021, Kuzmanovic et al. 2003, Singh et al. 2022). Somatic are primarily icosahedral and elongated icosahedral are between 25-90 nm in size. Their isoelectric points range from 3-7.8 (Heffron and Mayer 2021, Singh et al. 2022). Their ability to attach and their attachment mechanism are affected by their morphology, size, and isoelectric point (Templeton et al. 2008, Gerba et al 1976). This may be the reason for the difference in the attachment of the somatic and f-specific phage.

Although the association was consistently higher in the 24-hour HRT effluent, the largest difference in association across HRTs was seen at the 0.45 μm filter pore size. This implies that the shortening of HRT is effective at decreasing somatic coliphage association to all particles within the 180 to 0.45 μm size range, but especially to the smaller particle sizes in that range. The percent association of somatic coliphage to particles in the 24-hour HRT effluent was similar to the association profile of somatic coliphage in the influent sewage. Therefore, the resulting hypothesis is that somatic coliphage enters the system associated, then dissociates, and reassociates the longer it mingles with sludge in the aeration column. A similar hypothesis is possible with f-

specific coliphage, although the percent association is even higher in the 24-hour HRT effluent than in the influent sewage.

This study did not focus on animal viruses or human enteric viruses. Although somatic and f-specific are similar to some human enteric viruses in size, shape, and isoelectric point, human enteric viruses may perform differently, and further studies are needed to determine their attachment characteristics. Additionally, sewage after primary settling was used in the particle separation experiment and the smallest filter pore size was 0.45 μm. Therefore, only coliphage association to particles of ~180 μm to 0.45 μm was characterized. As seen in figure 1.1, coliphage can associate to bacteria. Not all bacteria would be filtered out using a 0.45 μm filter pore size. In order to determine the amount of coliphage that could be associated with biological contaminants a 0.22 μm filter pore size should be used. Hejkal et al. (1978) found a threefold increase in virus concentration when fecal homogenate was sonicated and passed through a 0.22 μm filter, suggesting most virus may associate with particles of 0.22 μm or greater.

There is also an operational limitation in this study. The particle separation assay was performed on bench scale reactor effluent and performance may differ from full-scale operations. The ELWWTP effluent assayed was treated using an average of 7-hour HRT, lower than the shortest HRT tested in this study, and its particle association profile for both somatic and f-specific coliphage follow the trend of less association at shorter HRT. These findings are preliminary, however, and this effluent was not compared to effluent from a full-scale WWTP that operates at a longer HRT. Further analysis using effluent from another WWTP operating at longer aeration HRT is necessary to determine whether the trend also applies to full-scale operations.

4.3 Effects of Hydraulic Retention Time on Bacteria, Viral, and Fungal Communities

As expected with the drop in nitrification at lower HRTs, the functional genes related to ammonia oxidation, *amo* and *hao*, were not as prevalent in sludge samples taken from the aeration column of the two lower HRTs (16- and 8-hour). However, there was an increase in the prevalence of the *nxr* (nitrite oxidoreductase) functional gene at the lower HRTs. The *nxr* complex is responsible for oxidizing nitrite to nitrate in the final step of nitrification. However, the *narG/nxrA* functional group is also related to nitrate and nitrite conversion in the first step of denitrification (Ma et al. 2019). This indicates that as nitrification slowed at shorter HRTs, denitrification increased. This is consistent with the study by Cydzik-Kwiatkowska and Wojnowska-Baryła (2015) that found a decrease in denitrification in a granular sequencing batch reactor at longer HRTs. Furthermore, studies have been conducted showing the aerobic denitrification capabilities of certain bacteria and fungi, singularly or in consortia (Xi et al 2022, Zhang et al. 2023, Zuo et al. 2023). *Trichoderma* sp., *Penicillium* sp., and *Fusarium* sp. have all been found to aerobically denitrify (Zhang et al. 2023). The percent relative abundance of both *Trichoderma* sp., *Penicillium* sp. increased in the sludge samples collected from the shorter HRTs. However, further analysis using percent relative abundance fluctuations compared to decreased nitrification and increased denitrification is necessary in order to determine potential symbiotic relationships or denitrification/nitrification capabilities. Ammonia oxidizing bacteria like *Nitrosomonas, Nitrospira,* and *Nitrosospira* were found in the sludge samples of this study. Previous studies that found these were the main AOBs found in engineered environments like WWTPs (Daimes et al. 2016). *Nitrosomonas* was the main AOB found in all the sludge samples sequenced across HRTs. This is consistent

with studies demonstrating *Nitrosomonas* as the dominant AOB at short and long HRTs (Li et al. 2013). The majority of the *amo* and *hao* functional gene reads correspond to *Nitrosomonas. Nitrospira* also exhibited the *amoA, amoC,* and *hao* functional genes at the 24- and 16-hour HRTs but was phased out at the 8-hour HRT while *Nitrosomonas* remained. The smallest portion of the *amoC* reads corresponded to *Nitrosospira,* and the normalized read score decreased with HRT. This may mean that *Nitrosospira* does not play as integral role in nitrification at these HRTs. At a difference to Li et al. (2013) the results of this study did not indicate that *Nitrobacter* replaced *Nitrospira* at lower HRTs. The relative percentage of *Nitrobacter* remained stable at 0.02% during all HRTs and the relative percentage of *Nitrospira* decreased with HRT. At a difference to Park and Noguera (2004), the main *Nitrosomonas* species in the reactor sludge was *N. ureae*, not *N. oligotropha* or *N. europae*, although *N. oligotropha* was always the second dominant species. Their chemostat was operated at 8.5 mg/L DO and fed synthetic wastewater, while mine were operated at a range of 6-8 mg/L and was fed fresh sewage from the local WWTP. These differences in method may have promoted the growth of different bacterial populations, causing the differences in results.

Some species of *Nitrospira* are known to be comammox bacteria, catalyzing the full transformation of ammonia to nitrate, and contain all three related enzymes (*amo, hao,* and *nxr*) (Daimes et al. 2016). This is consistent with the findings of this study, where *Nitospira* was the main exhibitor of the *narG/nxrA* functional group at all HRTs and was found to contain *amo* and *hao* functional genes at the 24-hour and 16-hour HRTs. It seems, however, that comammox *Nitrospira* were mostly phased out at the 8-hour HRT since only a small amount of *amoC* reads corresponded to *Nitrospira*. When looking at

only *Nitrospira*‘s nitrite oxidizing capability, it was the only dominant genus at the 24-hour HRT. However, it was joined by *Reyranella* at the 16-hour HRT, and both *Reyranella* and *Janibacter* at the 8-hour HRT. This diversification in nitrate oxidizing bacteria may be related to the increase in denitrification at lower HRTs.

Of the nitrifying fungi discussed in the literature review section of this work, only *Aspergillus flavus* was found in the sludge samples submitted for shotgun sequencing. The relative percentage of *A. flavus* did not change with HRT in the same way *Nitrosomonas* and *Nitrospira* relative percentage did. There were no quantifying assays run on these samples.. It is possible that fungi are not as prevalent in suspended growth environments like this one and may play a larger role in nitrification in attached growth processes, where microbial growth occurs on stone or plastic media (EPA, 2004). It is also possible that more fungi could have been found if sequencing samples had been taken by scraping the aeration column walls instead of just the middle of the tank, which swirled constantly by aeration and could have disrupted fungal structures. It is also possible that the DNA extraction procedure from the Qiagen DNeasy PowerSoil kit was not efficient in fungal DNA extraction.

There was only one bacteria-phage pair identified in the literature review of this work. The phage, phiNF-1, is a double stranded DNA phage (Quiros et al. 2023). It was not found when looking at the sample shotgun sequencing results processed through the Kaiju app on Kbase. It may be that the phage is not present in the samples, since its DNA would have been extracted using the DNeasy PowerSoil kit by Qiagen, or the sequence does not exist in the library used to process the samples and would not have been identified even if present. This was a limitation mentioned in several studies, including

Wang et al. (2018). Looking at the broader viral population, the dominant DNA viral families remained the same across HRTs with two exceptions. The relative percentage of *Siphoviridae* increased from 30%, to 32%, to 38% in the 24-, 16-, and 8-hour HRTs, respectively. *Siphoviridae* and *Myoviridae* are the two dominant viral communities in the human gut (Tian et al. 2024). Being that these were reactors processing real sewage from a WWTP, it would make sense that these viruses would be present. However, why the relative percent abundance of *Siphoviridae* increased as HRT decreased is unclear. The influent sewage to the reactors was not sequenced, therefore it is not known whether the percent relative abundance of *Siphoviridae* increased in the reactor feed, but this may be the reason for the change in population. The second exception was the emergence of the *Gokushovirinae* family only in the 8-hour HRT samples. *Gokushovirinae* are a family of viruses found in a plethora of ecosystems worldwide, including the human gut, and little is known about them (Kirchberger and Ochman, 2020). However, they are distantly related to bacteriophage phiX174, a somatic coliphage and the control used for the particle separation experiments in this study (Lee et al. 2022). The increase in abundance of these two viral families could be due to cyclical changes in the WWTP virome, consistent with the study by Wang et al. (2018). Additionally, the minute changes in viral families and fungal genera could support the studies by Wang et al. (2018) and Saunders et al. (2016), which postulate that there is a core microbial community in WWTPs with similar biological processes. Wang et al. (2018) found that there were shared viral clusters across ten WWTPs around the world. Similarly, Saunders et al. 2016 found that there was a core community of bacteria shared by 13 Danish WWTPs.

As seen on table 3.21, the range of host bacterium under each viral family found in the samples collected in this study is broad. This may explain why there is little difference between the dominant families across HRTs. The hypothesis posed based on this work is: since these viruses can infect a wide range of hosts, they are able to survive at similar percent relative abundances regardless of HRT. This might suggest that dominance of one viral family versus another is based more on kinetics than on amounts of a specific bacterium within the sludge, at least in the more abundant families.

The types of bacteria that each dominant family infects are listed below in table 4.1. Further analysis using percent relative abundance fluctuations and quantitative PCR data compared to decreased nitrification at lower HRTs is necessary to determine potential symbiotic relationships and nitrification capabilities of or between fungi and bacteria. Continuation of this research by isolating of viruses infecting *Nitrosomonas* and *Nitrobacter* could be the next step in a speculatively complex path to "phage therapy" for engineered environments.

4.4 Conclusion

This study established that a decrease in hydraulic retention time in a bench scale activated sludge wastewater treatment system increases the recoverable ammonia and reduces the occlusion of coliphage by association to particles between 180 and 0.45 μm in treated effluent. Additionally, the experiment must be broadened to human enteric viruses, as the coliphage tested in this work are surrogates, and may perform differently.

It sets the groundwork to identify possible fungal symbiotes that work in conjunction with nitrifying bacteria to oxidize ammonia in activated sludge. There are few studies identifying bacteriophage pairs for nitrifying bacteria. An extension of this work could be

the isolation and sequencing of another bacteria-phage pair to further control nitrification and ammonia recovery.

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