# Understanding Microbial Agents and Exposures through the Collection and Production of Urine-Derived Fertilizers

by

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# Abstract

Source-separated urine is rich in nutrients and provides numerous benefits, including: offsetting energy requirements at wastewater treatment plants; offsetting energy required to produce nitrogen and phosphorus fertilizers; reducing the environmental impact of fertilizer production; and providing an alternative source of fertilizer. Source-separated urine can contain chemical and biological contaminants that need to be managed prior to its use as a fertilizer. Bacteria, viruses, and extracellular nucleic acids, if present in fertilizer, all have the potential to impact the environment being fertilized and consumers of fertilized products. Thus, it is important to understand their behavior and fate in urine and urine-derived products. Information about the fate of chemical and biological contaminants can help inform appropriate treatment technologies that transform urine into useful products while mitigating public and environmental health exposures.

This dissertation is focused on microbiological contaminants that may impact public and environmental health. The presence of polyomavirus, a urinary tract virus, was evaluated in stored urine in which urea had been hydrolyzed and the solution pH was around 9.0. Polyomavirus infectivity measured through tissue culture assays was compared to its genome integrity measured through qPCR assays. The virus infectivity was also compared to two surrogate viruses, the bacteriophages MS2 and T3. The infectivity of polyomavirus decreased rapidly in stored urine within 1.1 - 11 hours, compared to surrogate virus infectivity, which remained stable for 3 - 5 weeks. In contrast, polyomavirus genomes were stable for more than 3

weeks despite this loss of infectivity. This led us to look at the fate of extracellular DNA, which may carry antibiotic resistance genes, in hydrolyzed urine. DNA transformation, integrity, and conformation were evaluated using transformation assays, qPCR assays, and gel electrophoresis. Based on filtered and pasteurization experiments, the loss in transformation efficiency correlated to plasmid linearization and appeared to be microbially driven, likely from organisms smaller than 0.22 μm or enzymes larger than 100 kDa. Collectively, these results indicate that the microbial activity of hydrolyzed urine reduced viral infectivity and the transformation of extracellular DNA, decreasing both the risk of exposure to infectious polyomavirus and spread of plasmid- associated antibiotic resistance genes. Finally, urine-diverting toilets, which are used to collect urine separate from other wastes, were compared to conventional flush toilets in terms of virus exposure. Virus-laden droplets were detected at a higher frequency outside the conventional institutional high- flush toilet compared to a urine- diverting toilet, indicating an added benefit of urine- diverting toilets. We conclude that the conditions of hydrolyzed urine reduce the potential risk of polyomaviruses and plasmid- associated antibiotic resistance gene transfer, and that using urine- diverting toilets can reduce one's exposure to viruses from flushing events. Because storage is a common pretreatment before other fertilizer conversion technologies, this work demonstrates that microbial risks may be low and further advances the possibility of recovering urine for beneficial reuse.

# Chapter 1.
# Introduction

Human activities influence carbon, nitrogen, and phosphorus biogeochemical cycles which can impact the planet in both positive and negative ways. Human activities typically introduce nitrogen and phosphorus into the environment in forms that can have profound impacts on aquatic and terrestrial ecosystems. These nutrients come from a range of sources, including: fertilizer runoff from agricultural lands; nutrient infiltration into groundwater, municipal wastewater effluents; and stormwater runoff into receiving waters. Wastewater treatment plants located in nutrient-sensitive watersheds have stringent guidelines to reduce nitrogen and phosphorus in effluents, as even small amounts of these elements can cause potential eutrophication and harmful algal blooms. These outcomes can have a negative impact on both public health when they occur in surface waters that serve as drinking water sources, and on the ecological health of downstream ecosystems (Smith et al. 1998). Engineering solutions have been implemented in an attempt to mitigate these problems; however, the problem of excess nutrient cycling through effluents and emissions persists. This dissertation focuses on alternative approaches to wastewater management through repurposing nutrients as a way to positively influence nutrient cycles.

Achieving efficient nutrient management is becoming increasingly necessary and has motivated innovative solutions of wastewater treatment that allow nutrients to be repurposed as a resource rather than a waste. Under the conventional paradigm, large amounts of resources,

(including energy) are required to remove nitrogen and phosphorus from municipal wastewater. For example, enhanced centralized nitrogen removal at advanced wastewater treatment plants can produce excellent effluent water quality but consume approximately 50% more energy than conventional treatment technologies (Programme 2012; Maurer et al. 2003). Similarly, the energy required to create fertilizers for food production is also significant. Conventional nitrogen fertilizer production uses the Haber–Bosch process, which fixes atmospheric nitrogen to produce usable nitrogen fertilizer. While this process has revolutionized the food production system, it requires high amounts of energy and thus has a large carbon footprint (45 MJ/kg of nitrogen) (Maurer et al. 2003). Energy is also required to recover non-renewable phosphorus fertilizers. Mining and extracting phosphorus are energy intensive processes (requiring 29 MJ/kg of phosphorus) (Maurer et al. 2003). Even still, phosphorus is projected to be depleted within 50-100 years (Smil 2000). Increasingly, more complex extraction methods are required to recover the remaining phosphorus from a depleting resource and to remove heavy metals that contaminate the phosphate rock (Mew 2016). Taken together, the challenges associated with creating conventional forms of nitrogen and phosphorus fertilizers are not sustainable into the future.

The dual interest in reducing the energy requirements at wastewater treatment plants and identifying alternative and renewable fertilizer sources has led some to separate urine from the sewage (i.e., urine diversion) and repurpose it for its use as a fertilizer. Urine contributes the majority of the nitrogen, phosphorus, and potassium that are found in domestic wastewater but contributes less than 1% of the wastewater volume (Larsen & Gujer 2001; Otterpohl 2002; Maurer et al. 2006; Lienert et al. 2007; Meinzinger & Oldenburg 2009). Separating this concentrated stream of nutrients allows for its conversion into a useful fertilizer or fertilizer

precurors. Meanwhile, preventing nutrients from ever entering the wastewater streams would reduce the degree to which nutrient removal technologies need to be deployed at treatment plants and should reduce the mass of nutrients released to surface waters (Wilsenach & van Loosdrecht 2006; Jimenez et al. 2015). Urine diversion offers additional advantages beyond nutrient recovery. For example, pharmaceuticals are found in high concentrations in urine (60-70% of excreted pharmaceutical residues) (Lienert et al. 2007) and could be managed more efficiently by treating in urine than as diluted constituents in sewage. Four out of five flushes are typically used for urinating events (Berndtsson 2006; Wilsenach & van Loosdrecht 2004). Typically, urine diverting toilets require minimal flushing, so a significant reduction in potable water needed for flushing can be achieved with urine diversion.

While there are many benefits to recovering and repurposing nutrients in urine, several questions remain that require research before urine diversion can become a reality. Urine typically contains a number of chemical and biological contaminants that need to be managed prior to its use as a fertilizer. Varying technologies need to be evaluated to ensure a safe and useful product that can supplement current fertilizer needs. A marketable product that gives consumers an economic incentive to purchase them is critical as environmental benefits alone will not make urine-derived fertilizers viable in the market. Infrastructure for safe and sufficient collection onsite and safe and easy transport of fertilizer products to the location of use must be designed, constructed, and implemented. Public perception about the use of human urine and the "real" risks compared to the "perceived" risks must be addressed, accounted for, and communicated clearly to users.

This dissertation focuses on investigating the microbial contaminants in unprocessed urine that could pose risks in urine-derived fertilizers. In Chapter 3, the infectivity of a urinary

tract virus, polyomavirus, is evaluated over time in stored urine and compared to other surrogate viruses that are commonly used in studies on the environmental fate of viruses. Persistence of infectious virus measured through tissue culture methods was compared to persistence of genes measured through molecular methods. We saw that polyomavirus genes persisted for a longer period of time in urine than the infectious virus (Goetsch et al. 2018), which lead us to investigate the fate of other nucleic acids present in urine. Chapter 4 thus focuses on the fate of extracellular DNA in urine with a specific emphasis on the transformation of antibiotic resistance genes by soil bacteria. Chapters 3 and 4 focus on the fate of microbial contaminants as urine is stored and converted to fertilizer and thus focuses on the potential exposure of fertilizer handlers and consumers to biological contamiants in food fertilized by urine derived fertilizers. Chapter 5 focuses on another potential area of exposure to microbial contaminants: at the source of urine collection in public restrooms. Release of bacteriophages in droplets were measured from a urine-diverting toilet and compared to a high-flush institutional (non urine-diverting) toilet. The measured release of these viruses were combined with literature values of urinary and enteric virus concentrations to compare exposures presented by toilets.

This dissertation will advance our understanding of how to safely close the nutrient cycle by assessing potential exposure to biological contaminants during urine diversion and fertilizer production. Historically we have not considered microbial contaminants in urine as an important public health issue. This work will contribute to future risk assessments for consumers of eating food fertilized with urine-derived fertilizers and workers who handle urine and urine-derived fertilizers. This work also highlights the need for more research on urinary tract viruses and other types of pathogens that are present in urine. Finally this work demonstrates that more research is

needed to identify the biological agents that degrade extracellular DNA and to identify the environments where these agents are active.

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# Chapter 2.
# Background

## 2.1 Converting source separated urine into fertilizer

To be useful, fertilizer products must be high in nutrient content, be easy to use and transport, and contain minimal contaminants that introduce risks to public and environmental health when applied to the environment. Several aspects of urine make this challenging, including the odor and volatilization of ammonia in stored hydrolyzed urine, the storage capacity and transport necessary for large volumes of source-separated urine, and the microbial and chemical contaminants that are present in source-separated urine. A number of treatments and technologies have been developed to address one or several of these issues at the same time.

Storing urine in a sealed container is a simple way to convert fresh urine into fertilizer. The purpose of storage is to first allow urea in the urine to hydrolyze. Urea hydrolysis, which is facilitated by urease enzymes present in bacteria, converts urea to ammonia. The resulting high ammonia concentrations and high pH in the urine is harnessed for its sanitizing effect on microorganisms present in urine. The World Health Organization recommends storing urine for more than 6 months before use as a fertilizer to allow time for the microorganisms to be inactivated (WHO 2006). In addition to extended storage after hydrolysis, urine can also be sanitized through heat pasteurization. A drawback of using storage or pasteurization to produce fertilizer is that the large volumes of urine can be difficult to store and transport from the place of collection to the place of application.

One approach to decreasing urine volume is to precipitate nutrients out of the urine as minerals. Struvite ($NH_4MgPO_4$ $6H_20$), for example, is produced through chemical precipitation by adding a magnesium source. The resulting solid phosphorus fertilizer is easier to transport (Wilsenach, Schuurbiers, and van Loosdrecht 2007; Etter et al. 2011; Ronteltap, Maurer, and Gujer 2007). This approach, however, does not recover most of the nitrogen in the urine. Other approaches involve reducing the volume of liquid fertilizer by removing water through pressure (e.g., reverse osmosis), heat (e.g., distillation), or freezing (freeze-thaw cycles) (Maurer, Pronk, and Larsen 2006).

Some methods prevent the loss of nitrogen by ammonia volatilization (*i.e.*, nitrogen stabilization). The hydrolysis of urea by urease can be inhibited by acidifying the urine (Hellström, Johansson, and Grennberg 1999), increasing the pH of the urine (Randall et al. 2016; Dutta 2012), or by adding hydrogen peroxide at the beginning of collection (Zhang et al. 2013). Once hydrolyzed, urine can also be stabilized by aerobic treatment (Udert and Wächter 2012), in which ammonia is converted to nitrate and organic nitrogen. Likewise, reducing the pH can prevent nitrogen loss through ammonia offgassing. These methods have varying energy and material inputs that should be considered and compared.

### 2.2 Microbial risks of urine-derived fertilizer

Unprocessed urine has microbiological contaminants that can cause risks to human health and can impact the soil, plants, and water environments. Pathogens can be present in source-separated urine due to fecal matter contamination as well as urinary tract infections. In terms of human pathogens, *Clostridia*, *Salmonella enterica*, *Mycobacterium tuberculosis, Staphylococcus, Lactobacillus,* and fecal streptococci have all been detected in source-separated urine (Chandran,

Pradhan, and Heinonen-Tanski 2009; Vinnerås et al. 2008; Hoglund et al. 1998; Karak and Bhattacharyya 2011; Lewis et al. 2013).

Ideally, pathogenic organisms should be inactivated or removed before urine is applied as a fertilizer. Indicator organisms and surrogates have been used to determine contaminant fate through some urine treatment processes, which include pasteurization by storage and precipitation of struvite (Chandran, Pradhan, and Heinonen-Tanski 2009; Vinnerås et al. 2008; Hoglund et al. 1998; Wohlsager et al. 2010; Höglund et al. 2002; Decrey et al. 2011). *Escherichia coli, Salmonella enterica*, and MS2 coliphage, for examples, are enteric indicator organisms or phages that have been studied through fertilizer production. Most research on microorganism fate has focused on storage as a treatment, and results suggest the majority of indicator organisms are inactivated within a week of storage, although *Salmonella enterica* was more persistent (Chandran, Pradhan, and Heinonen-Tanski 2009). Decrey et al. investigated the fate of human virus surrogate ϕX174 and *Ascaris suum* eggs through struvite precipitation and determined that air-drying with increased temperatures aids in inactivating both microorganisms (Decrey et al. 2011).

The organisms studied thus far do not represent all microorganisms in source-separated urine. Lahr et al. enumerated total bacterial cells in fresh urine and urine-derived fertilizers and demonstrated that a number of bacteria survive the high ammonia and high pH environment of stored urine (Lahr et al. 2016). 16S sequencing of the bacterial community showed consistent changes in different urine samples through urine storage. Furthermore, there were groups of organisms in the stored urine fertilizer with associated opportunistic bacteria, but deeper sequencing is required to identify these organisms at the species level (Lahr et al. 2016).

### 2.2.1 Viral risks in urine

Viruses can cause a range of human illnesses including respiratory infections, gastrointestinal illnesses, and others. Most research on viruses in source separated urine has focused on enteric viruses or viruses that are shed in the feces and tend to cause gastrointestinal illnesses. Adenovirus, rotavirus, and norovirus genes have been detected in source-separated urine, however their infectivity states have not been assessed (Bischel et al. 2015). Viruses are also present in urine without fecal contamination. Zika virus, for example, has been detected in the urine of infected individuals (Gourinat et al. 2015) and is known to cause microcephaly in newborns of infected mothers (Calvet et al. 2016). Similarly, cytomegalovirus (CMV) is shed in the urine of infected individuals and is a risk to infants of infected mothers as the virus can cause hearing and vision loss, as well as other developmental disabilities when fetuses are exposed in utero (Stagno et al. 1986).

Polyomaviruses have received relatively little attention in source-separated urine research, despite the fact that they infect the urinary tract (Monini et al. 1995) and can be excreted in urine (Urbano et al. 2016; Antonsson et al. 2010). This non-enveloped, double-stranded DNA virus readily infects a large percentage of the public asymptomatically (Dorries 1998), but can cause severe diseases in immune-compromised individuals (Bofill-Mas, Pina, and Girones 2000). Polyomavirus primary infection often occurs during childhood, likely before the age of 5 years (Shah, Daniel, and Warszawski 1973; Knowles 2001). While most people are infected, not everyone may be excreting polyomaviruses continuously (Ling et al. 2003).

There are several members of the polyomavirus family that are excreted in urine. BK polyomavirus (BKPyV) has been found at concentrations of $10^7$-$10^{10}$ gene copies per mL in the fresh urine of infected renal transplant patients (Randhawa et al. 2004) and 100-$10^4$ gene copies

per mL in the urine of healthy individuals (Urbano et al. 2016). BKPyV is known to cause severe diseases in the liver and kidney, primarily in immune-compromised individuals. It can also cause hemorrhagic cystitis and nephritis in organ and bone marrow transplant recipients (Reploeg, Storch, and Clifford 2001; Arthur, Dagostin, and Shah 1989). JC polyomavirus (JCPyV) is also found in the fresh urine of healthy individuals at levels $10^4$–$10^7$ gene copies per mL of urine (Urbano et al. 2016) and is known to cause progressive multifocal leukoencephalopathy, a rare but fatal viral disease that causes inflammation of the white matter in brain tissue (Astrom, Mancall, and Richardson 1958; Padgett et al. 1971).

The structural elements of virus particles help provide stability in the environment. The viral genome is protected from the environment in a capsid that is comprised of proteins. Some viruses also have a lipid envelop outside the capsid. These structural elements can protect the virus from chemical and physical environmental conditions that could cause inactivation (Lucas 2010). Polyomavirus has a unique structural element of disulfide bonds in its protein capsid that is typically thought to enhance virus stability (Walter and Deppert 1975).

Research on viruses in the environment tends to utilize culture-based methods to detect infectious viruses of human health concern. In circumstances where these methods are unavailable, researchers rely on molecular techniques to detect nucleic acids of the virus. They also study the infectivity of culturable strains similar to the virus of interest (i.e. surrogates). As discussed above, most research thus far on microorganisms in urine have focused on enteric organisms that may not fully describe the risk that source-separated urine could pose. Additional research that includes culture based assays and molecular tools of index and model organisms are required for a better understanding of their fate in the environment.

### 2.2.2 Antibiotic resistance risk in urine

Antibiotic resistance in the environment and the concern of anthropogenic activities contributing to this resistance has gained much recent attention (Pruden, Arabi, and Storteboom 2012; Vikesland et al. 2017). Antibiotic resistant bacteria can adversely affect human health through more serious infections and decreased efficacy of antibiotics (Ventola 2015). Bacteria can obtain resistance through the direct uptake of DNA that confers this resistance (antibiotic resistance genes (ARGs)), through mutations, or through environmental selective pressures (Ashbolt et al. 2013). Soil environments where manure and other treated wastewater products have been applied are particular areas of interest since these applied products contain antibiotics, antibiotic resistant bacteria, and ARGs (Heuer, Schmitt, and Smalla 2011; Storteboom et al. 2010).

Previous work suggests that numerous ARGs are present in fresh and hydrolyzed urine (Bischel et al. 2015). Preliminary metagenomic analyses on these urine samples suggest the relative abundances of various ARGs can decrease, increase, or stay the same with extended storage (Desta, in preparation). Aminoglycoside resistance genes, for example, decreased after storage, while beta-lactamase resistance genes were no longer detected in stored samples. Quinolone and macrolide resistance genes did not change in relative abundance, whereas some tetracycline and bacitracin resistance genes increased in relative abundance with storage. These genes may persist and some may even flourish as urine is converted to fertilizer (Desta, in preparation). Impacts from the release of ARGs to the environment remain to be determined, yet there is general consensus that ARG release to the environment should be minimized.

DNA in urine released to the environment may undergo many fates. Extracellular DNA can be degraded by nucleases, chemically modified, sheared, or stabilized by binding to mineral

surfaces or humic substances (Nielsen et al. 2007). Exposure to high temperatures can lead to single-stranded and fragmented DNA molecules (Bauer et al. 2003). DNases are found in most microbial habitats (Blum, Lorenz, and Wackernagel 1997; Novitsky 1986; Paul et al. 1989; Turk et al. 1992), and the majority of the nuclease activity in soil is likely from bacteria. Blum et al. suggested that nucleases associated with the active microbiological community are responsible for most of the observed degradation of DNA in nonsterile soil (Blum, Lorenz, and Wackernagel 1997). Although disinfection processes in drinking water and wastewater treatment are designed to remove pathogenic organisms, ARGs could retain biological activity, remaining available for recipient cells in exposed environments (Dodd 2012). The same could be true for treatments used to prepare urine-derived fertilizers.

ARGs are commonly monitored in environmental samples with qPCR. Although qPCR does detect specific DNA sequences in genes related to antibiotic resistance in organisms, the genes detected may not be expressed, may have mutated and thus no longer confer resistance, may be present in an inactivated cell or present outside of a cell, or may not be part of complete resistance genes (Luby et al. 2016). Other studies have used markers to track the transfer of antibiotic resistance by using plasmids, integrons and transposons (Nandi et al. 2004; Binh et al. 2008; Musovic et al. 2014; Klümper et al. 2015). In each of these methods, the qPCR technique does not assess the transferability of the ARG to other bacteria in the environment.

Even when genes are present as extracellular DNA, the genetic material can be picked up by other bacteria via transformation. Transformation assays assess this ability of bacteria to take up and incorporate extracellular DNA. For a bacterial cell to take up this extracellular DNA, the recipient cell must be competent. At least 80 strains of bacteria are naturally competent, including strains found in the soil bacteria genera *Azotobacter* and *Acinetobacter* (Lorenz and

Wackernagel 1994). Natural transformation studies using strains from these genera have determined transformation efficiencies in lab scale microcosms of groundwater and biofilms growing in river environments (Lorenz, Reipschl, and Wackernagel 1992; Williams et al. 1996). A better understanding is needed on how DNA that contains ARGs persists through fertilizer production, and on how ARG data collected with conventional qPCR methods relates to the actual transferability of ARGs in the environment.

### 2.2.3 Other exposure risks associated with source-separated urine and toilet flushing

Thus far, risks associated with source-separated urine have focused on converting collected urine into fertilizer and applying urine-derived fertilizers (Höglund, Stenström, and Ashbolt 2002). These health risks can come from exposures to pathogenic organisms by direct and indirect sources, including direct exposure from accidental ingestion during fertilizer processing or application, or indirect exposure from ingestion of contaminated drinking water, breathing in aerosols from fertilizer application, or consumption of food fertilized with urine-derived fertilizers.

Exposures to infectious organisms can also occur as the urine is collected for fertilizer production. For example, toilet flushing can generate infectious aerosols and droplets that can be harmful to human health (Darlow and Bale 1959). Likewise, bathroom surfaces such as door handles, flush handles of toilets, etc. have been connected with the transmission of enteric viruses (Gallimore et al. 2008).

In terms of exposure from flushing, several studies have examined the contamination of surfaces by enteric and indicator viruses and bacteria after toilet flushing. In one study, bacteriophage MS2 and *E.coli* were seeded into household toilets and the microorganisms remained in the bowl after multiple flushes (Gerba, Wallis, and Melnick 1975). The researchers

concluded that the microorganisms had adsorbed to the porcelain surface of the toilet bowl and were gradually eluted from the flushing mechanism. Droplets produced by flushing harbored both the bacteria and viruses. Bacteria and viruses were detected on surfaces in the bathrooms after flushing, indicating a possible transmission route (Gerba, Wallis, and Melnick 1975). Jessen found that after seeding different types of toilets (cistern-fed, gravity-flow, and mains-fed pressure-valve), bacteria were present several minutes after the first flush (Jessen 1955). As expected, with increasing flushing energy, the amount of bioaerosols generated increased (Jessen 1955). In another study using spiked bacteriophage MS2 and *Serratia marcesens*, the first flush reduced viable microorganisms, but the microorganisms persisted on toilet bowl surfaces and in the bowl water (Barker and Jones 2005). These studies confirm that microorganisms excreted into toilets can persist on the toilet bowl surface and in the bowl water, and are disseminated into the air with toilet flushing.

The most significant toilet plume airborne infection risks are likely from viruses (Johnson et al. 2013). Norovirus accounts for most of the nonbacterial gastroenteritis outbreaks worldwide, has a low infectious dose, and can be transmitted by aerosols (Atmar and Estes 2006; Teunis et al. 2008). Some coronaviruses are also shed in feces and are believed to be transmitted through aerosols (Hung et al. 2004; Liu et al. 2004; Booth et al. 2005; Olsen et al. 2003). Nonenteric viruses that are shed in urine have not been extensively studied or monitored in bathroom settings.

Culture-independent techniques that rely on PCR are commonly used to measure pathogens in bioaerosols, including toilet aerosol studies (Peccia and Hernandez 2006). RT-PCR and qPCR were used to detect and measure norovirus, enterovirus, rhinovirus, rotavirus, and Torque teno virus from numerous surfaces and in collected air samples in offices and a hospital.

The surfaces with the most viruses were door handles, followed by toilet flushing handles, toilet seats, and toilet covers (Verani, Bigazzi, and Carducci 2014).

A limited number of urine-diverting toilet technologies exist in Europe and the U.S., and more are under development. In the U.S., the Wostman EcoFlush and the WC Dubbletten are available but are configured differently. The Wostman toilet has an S-bend for odor control during urine collection, while the Dubbletten has a plastic membrane in the urine-collecting bowl instead to prevent odors. The urine diverting toilets use less water to flush (Wostman, 1 L; Dubbletten, 0.5 L) in comparison to standard public toilets that use 11- 26 L per flush and newer dual-flush toilets that use no more than 4.8 L per flush (USEPA). Urine-diverting toilets typically collect urine in a drain that is separated from the water reservoir where feces are collected. The urine drains immediately from the toilet and into a separate piping system. Feces and toilet paper typically go in the back bowl filled with water, similar to more conventional toilets (Figure 2-1). This reservoir is drained with a flush mechanism, much like a traditional toilet, and the drain is typically connected to the sewer system. Because of the different toilet configurations, exposures to urine-borne and fecal borne microorganisms may be different. However these exposures have not yet been evaluated and are important to be considered for future toilet use.

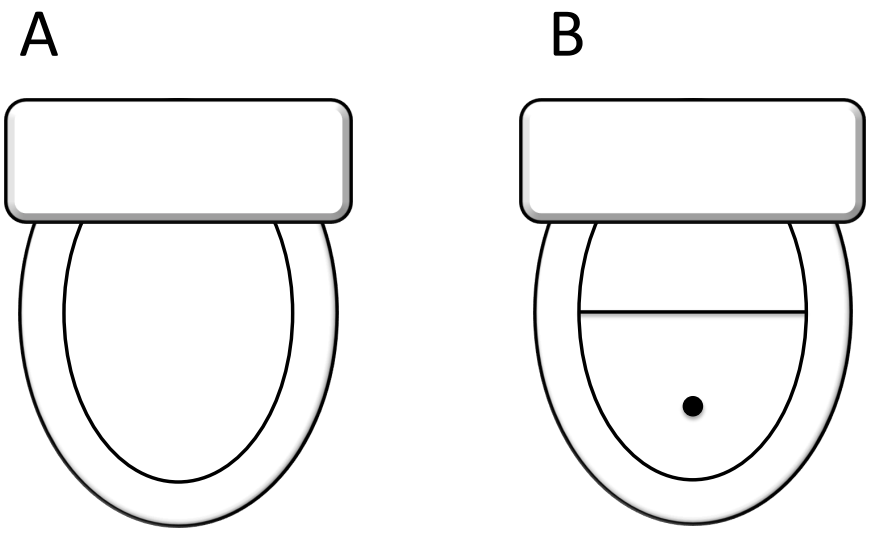


**Figure 2-1. Schematic of toilet bowls for (A) conventional toilets and (B) urine-diverting toilets. Urine-diverting toilet bowls are separated into a front bowl where urine is collected and the back bowl that is connected to the existing sewer plumbing.**

## 2.3 Conclusions

These research gaps focus on exposures to pathogens found in urine through collection via toilets and throughout processing into fertilizer. The fate of microbial contaminants not yet studied in urine (*i.e.*, urinary viruses and extracellular DNA) throughout storage need to be better understood. The determined fates can help inform more comprehensive risk assessments of using urine-derived fertilizers. Beyond the exposure of unmanaged microbial contaminants in urine, human health could also be impacted by exposures to pathogens from toilet flushing. Urinary pathogens have historically not been evaluated in flushing, and their potential for transmission should be explored. The exposures from source-separating toilets have also not been evaluated or compared to existing toilet infrastructure.

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# Chapter 3.

# Fate of the Urinary Tract Virus BK Human Polyomavirus in Source-Separated Urine

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## 3.1 Introduction

Enteric viruses that arise from fecal contamination have long been known to be of serious concern for public health. Viruses that infect the urinary tract of humans can also be shed in high quantities yet their concentrations and fate in the environment are unclear. Zika virus, for example, is excreted in urine (Gourinat et al. 2015) and can cause microcephaly in newborn children of infected mothers (Calvet et al. 2016). Similarly, cytomegalovirus (CMV) is shed in the urine of infected individuals and is a risk to infants of infected mothers as the virus can cause hearing and vision loss and other developmental disabilities (Stagno et al. 1986).

Polyomaviruses are another class of emerging pathogens that commonly infect the urinary tract of humans (Monini et al. 1995; Urbano et al. 2016; Antonsson et al. 2010). These non-enveloped, dsDNA viruses readily infect a vast majority of the public asymptomatically (Dorries 1998; Kean et al. 2009), but can also cause severe diseases in immunocompromised individuals (Bofill-Mas et al. 2000). Primary infection occurs in childhood, and the viruses persist for the entire life of the individual, mainly in epithelial cells in the kidneys and urinary tract and leukocytes in the blood (Antonsson et al. 2010; Dorries 1998; Bofill-Mas et al. 2000;

Ling et al. 2003; Dolei et al. 2000; Dorries et al. 1994). BK polyomavirus (BKPyV) and JC polyomavirus (JCPyV) are most commonly found excreted in urine (Knowles 2006; Bialasiewicz et al. 2009). The excretion of BKPyV by healthy individuals is asymptomatic, but in transplant patients, replication can cause severe disease (Reploeg et al. 2001; Bressollette-Bodin et al. 2005; Hirsch et al. 2002). JCPyV and BKPyV concentrations have been reported as high as $10^{10}$ gene copies/mL in the urine of sick individuals, with healthy adults typically excreting lower concentrations (Randhawa et al. 2004; Urbano et al. 2016).

Despite the potential for abundant polyomavirus gene copies in excreted urine, its transmission pathways have not yet been fully determined. Respiratory and fecal-oral routes of transmission have been proposed for BKPyV (Goudsmit et al. 1982; Monaco et al. 1998; Bofill-Mas et al. 2001; Rachmadi et al. 2016), and urine may play a role (Bofill-Mas 2017). Ingestion of contaminated water and food has been implicated as an exposure route (Bofill-Mas et al. 2001), indicating the potential significance of polyomavirus transmission via the environment.

The need to better understand polyomavirus transmission by urine is underscored by the growing trend of diverting urine from the waste stream and capturing nutrients in urine-derived fertilizers. Urine diversion can provide several environmental benefits, including a sustainable source of phosphorus (Larsen & Gujer 2001; Maurer et al. 2006; Meinzinger & Oldenburg 2009), reduction in costs and pollution associated with wastewater treatment (Wilsenach & van Loosdrecht 2006; Jimenez et al. 2015), a potential reduction of water usage (USEPA n.d.), and more efficient treatment of contaminants. Despite the benefits of diverting urine, biological contaminants need to be managed before urine can be reused. Biological contaminants in urine are mitigated with a number of treatment technologies, including long-term storage for several months, pasteurization, or by nutrient precipitation (*e.g.,* struvite) (WHO 2006; Larsen et al.

2013; Ronteltap et al. 2007).

When urine is stored in sealed containers to inactivate biological contaminants, the urea in urine is hydrolyzed, resulting in high pH (~9) and an increase in aqueous ammonia concentrations (2000-8000 mg N/L) (Udert et al. 2006; Höglund et al. 2000). This transition to hydrolyzed urine can occur within a few hours or days depending on urease enzyme activity in the urine. The high pH and high aqueous ammonia levels have a biocidal impact on indicator organisms (Chandran et al. 2009; Warren 1962; Schonning & Stenstrom 2004).

Research on biological contaminants in source-separated urine has primarily focused on the presence and fate of enteric pathogens (Höglund, Ashbolt, et al. 2002; Chandran et al. 2009; Decrey et al. 2011; Hoglund et al. 1998; Höglund, Stenström, et al. 2002; Vinnerås et al. 2008; Wohlsager et al. 2010). Many enteric viruses are single-stranded RNA (ssRNA) viruses, so ssRNA viral surrogates are often used to predict enteric virus fate in urine. Inactivation of the ssRNA bacteriophage MS2, for example, correlated well with aqueous base (*e.g.* $NH_3$, $OH^-$) activity, suggesting that inactivation is caused by transesterification of the ribose in RNA (Decrey et al. 2014). Other ssRNA viruses are susceptible to ammonia activity, whereas the single-stranded DNA (ssDNA) bacteriophage ΦX174, the double-stranded RNA (dsRNA) reovirus, and the double-stranded DNA (dsDNA) human adenovirus and bacteriophage T4 were not susceptible to the same transesterification inactivation pathway (Decrey et al. 2016). These results suggest that although common enteric ssRNA viruses are susceptible to the conditions in hydrolyzed urine, viruses commonly found in the urinary tract (polyomavirus, cytomegalovirus, etc.) may be stable in the high aqueous ammonia concentrations found in hydrolyzed urine and could therefore pose risks in urine-derived fertilizers.

To identify the potential transmission risks that polyomavirus may pose in source-separated urine and urine-derived fertilizer production, we tracked the presence and fate of human polyomavirus in fresh and hydrolyzed urine using molecular and culture-based methods. We compared these results to the behavior of common bacteriophage surrogates in an effort to better understand how well surrogate infectivity predicts environmental virus fate and how capsid characteristics may influence inactivation in environmental matrices.

## 3.2 Materials and Methods

### 3.2.1 Urine collection and characterization

Urine was collected from men and women at nine public events in Vermont, Massachusetts, and Michigan, USA. After collection, urine was stored in sealed containers at room temperature to minimize ammonia off-gassing. Specific urine samples were also pasteurized by heating to 80° C for at least 3 minutes. Nutrient content (total ammonia nitrogen, total phosphorus, total nitrogen) of hydrolyzed urine was evaluated using Standard Methods (4500-$NH_3$ F; 4500-P J and 4500- P E; 4500-N C and 4500-$NO_3^-$ C) (APHA; AWWA; WEF 2005). Details on collection location, event type, number of donors, and characteristics of the urines used in this study are provided in Table 1. Experiments with urines are described by the collection event (A-I), followed by the treatment the urine has undergone (fresh, hydrolyzed, pasteurized), and the length of time after that treatment. For example, urine collected from a rural festival in Vermont (event A; Table 3-1) and used for an experiment 10 months after it underwent hydrolysis was referred to as "A – hydrolyzed 10 months."

**Table 3-1. Characteristics of urines used in experiments. Standard deviations are reported for > 2 measurements.**

| Urine Label | Collection Location | Event Type | No. of Donors | Total Phosphorus (mg P/L) | Total Nitrogen (mg N/L) | Total Ammonia Nitrogen |
|---|---|---|---|---|---|---|

| | | | | | | (mg N/L) |
|---|---|---|---|---|---|---|
| A | Vermont | Rural Festival | 300 | 410±1 | 4700±170 | 5000±260 |
| B | Massachusetts | Male rest stop | >100 | 240±24 | 4400±170 | 4300±210 |
| C | Vermont | Combination of parade and festival | >300 | 400±9 | 7100±430 | 5700±60 |
| D | Michigan | University | 200 | 850±210 | 7400±270 | 5600±200 |
| E | Michigan | Outdoor Theater | 80 | 490±4 | 6300±230 | 5600±250 |
| F | Michigan | Outdoor Theater | 60 | 320 ±1 | 4600±230 | 4800±640 |
| G | Michigan | University | 10 | 700±10 | 9700±1000 | 6300±110 |
| H | Vermont | Urine Community Collection | >100 | 450±1 | 6400±400 | 5800±320 |
| I | Vermont | Urine Community Collection | >100 | 460±10 | 4900±600 | 6300±100 |

### 3.2.2 Bacteriophage and virus strains

Bacteriophages used as surrogates for human viruses included *Escherichia coli* non-enveloped bacteriophages T3, MS2, and Q $\beta$ . Bacteriophage T3 (38.2 kbp) is a dsDNA virus that has dimensions of 50 × 20 nm in size and was used as a surrogate for polyomaviruses, which are dsDNA viruses. Bacteriophages MS2 (3.6 kbp) and Q $\beta$ (4.2 kbp) are both ssRNA viruses and 25 nm in diameter, but Q $\beta$ has disulfide bonds in its capsid, and MS2 does not. These two

viruses were studied to help explain differences observed in the inactivation kinetics of BKPyV and T3.

The BK polyomavirus Dunlop variant was used to study the fate of BKPyV. This genetic variant was chosen since it can be propagated at a high titer (~$10^8$ IU (infectious unit) $mL^{-1}$) to provide maximal experimental sensitivity and range. BKPyV (dsDNA) has a 5.1 kbp genome and is approximately 45 nm in diameter (Krumbholz et al. 2009).

Bacteriophages MS2 and T3 were propagated in their *E. coli* hosts (ATCC 15597 and 11303). The viruses were extracted from cellular material with a chloroform extraction method (Agency 2001) and purified with an Econo Fast Protein Liquid Chromatography system (BioRad, USA) equipped with a HiPrep Sephacryl S-400 column (GE, USA). The purified virus fraction was concentrated with 100 kDa Amicon ultracentrifugal filters and filter-sterilized with 0.22 $\mu$ m polyethersulfone (PES) membrane filters (Millipore, USA). The final MS2 and T3 stocks (~$10^{11}$ PFU $mL^{-1}$) were stored in phosphate buffer (5 mM $NaH_2PO_4$ and 10 mM NaCl, pH 7.5) at 4 °C. Bacteriophage Q $\beta$ was propagated in its *E. coli* host (ATCC 15597) and purified similar to MS2 and T3, except the protein chromatography step was excluded. The Q $\beta$ stocks (~$10^{11}$ PFU $mL^{-1}$) were stored in phosphate buffer at 4 °C for immediate use in infectivity experiments. The bacteriophages were enumerated by the double layer plaque assay (Agency 2001). Briefly, aliquots of each virus were serially diluted, and 100 $\mu$ L of final serial dilutions were combined with 100 $\mu$ L of an overnight culture of their respective *E. coli* hosts and 5 mL of soft agar. Plaques were enumerated after overnight incubation at 37 °C.

BKPyV was propagated in Vero and 293TT cells using previously published methods (Broekema & Imperiale 2012; Jiang et al. 2009). Briefly, Vero and 293TT cells were grown to 70% confluency, infected with BKPyV crude lysate at MOI 0.1 IU $cell^{-1}$, and incubated at 37 °C

for three weeks (Vero cells) or 10 days (293TT cells). Virus lysates were purified over density CsCl gradient centrifugation, and the collected virus fraction was dialyzed overnight in buffer (10 mM HEPES, 1mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, pH 7.9).

Infectious BKPyV was enumerated in renal proximal tubule epithelial (RPTE) cells with immunofluorescence assays (IFAs) (Broekema & Imperiale 2012). When RPTE cells in the wells of 24 well plates reached 70-80% confluency, serial dilutions of the BKPyV virus samples were added to the cells. Following a one-hour infection at 4° C, the cells were further incubated at 37° C for two days. Cells were then fixed with 4% paraformaldehyde for 20 minutes, washed three times with phosphate-buffered saline (154 mM NaCl, 5.6 mM $Na_2HPO_4$, 1.06 mM $KH_2PO_4$, pH 7.4, PBS), washed with a 0.1% Triton detergent solution, rinsed with PBS, and then washed with a 5% goat serum blocking solution. To identify infected cells, the cells were treated first with a 1:200 dilution of antibody pAB416 (Harlow et al. 1986) in 5% goat serum, and then with a 1:200 dilution of polyclonal goat anti-mouse IgG FITC antibody (Sigma) in 5% goat serum. The virus titer was determined by counting the individual fluorescent cells. At least nine random fields of view with at least five positive cells each were averaged to obtain the titer values (IU $mL^{-1}$) in each well. Duplicate wells were prepared for each sample and their titer values were averaged (Jiang et al. 2009).

### 3.2.3 Virus inactivation in urine and buffer

All inactivation experiments were conducted at room temperature in the dark. T3 bacteriophage was spiked into 10-50 mL of hydrolyzed urine (n=3) at concentrations of $10^8 – 10^9$ PFU $mL^{-1}$ to evaluate the inactivation rate of the human dsDNA virus surrogate. Bacteriophages MS2 and Q $\beta$ were spiked into 10 mL of hydrolyzed urine (n=2) to evaluate the inactivation rates of the bacteriophages with (MS2) and without (Q $\beta$ ) disulfide bridges in the

protein capsid. Both surrogates were spiked into hydrolyzed urine at an initial concentration of $10^8 – 10^9$ PFU $mL^{-1}$. Infectious particles were quantified over time with plaque assays.

In the BKPyV inactivation experiments, 50 $\mu$ L of stock BKPyV was spiked into 1 mL of urine or buffer at a concentration of $5\times10^5$- $1\times10^6$ IU $mL^{-1}$. At various time points, 50 $\mu$ L aliquots were removed and spiked into 450 $\mu$ L of tissue culture media (renal epithelial growth medium, REGM). The samples were then frozen at -80° C until enumerated with the tissue culture assays. Infectious BKPyV could be detected at concentrations as low as $3 \times 10^2$ IU $mL^{-1}$.

The BKPyV stock was spiked into buffers, hydrolyzed urine, pasteurized urine, and filtered urine to evaluate the role of solution characteristics on inactivation rates. Pasteurization consisted of heating urine to 80° C for at least 3 minutes. Urine was filtered through a 0.22 $\mu$ m PES syringe filter. BKPyV inactivation was also measured in buffer solutions with various pH and ammonia concentrations (Table S2). These included phosphate carbonate buffers with and without ammonia (147 mM, consistent with levels found in hydrolyzed urine) and adjusted to pH 7 and 9, described as PC7, PC9, AmPC7, and AmPC9 (Decrey et al. 2016).

### 3.2.4 Polyomavirus qPCR conditions

Endogenous BKPyV DNA concentrations in collected urine samples and Dunlop BKPyV DNA concentrations in spiked urine were evaluated using qPCR (conditions described in Table S1). DNA was extracted from all urine samples (100-300 $\mu$ L) for qPCR analysis with Maxwell Total Viral Nucleic Acid Extraction kits (Promega) using the Maxwell 16 instrument (Promega). Primers (5’ to 3’) specific for endogenous BKPyV were selected to target the large T-antigen (152 bp; forward: AAGGAAAGGCTGGATTCTG; reverse: TGTGATTGGGATTCAGTG (Bennett et al. 2015)) and primers specific for the Dunlop BKPyV strain were designed to target

the VP2 protein region of the Dunlop strain (900 bp; forward: ATTTCCAGGTTCATGGGTGCT; reverse: AGGCAACATCCATTGAGGAGC). The 10 $\mu$L reactions included 5 $\mu$L 2X Biotium Fast-Plus EvaGreen Master Mix, 0.5 $\mu$M primers, 0.625 mg/mL bovine serum albumin (BSA), and 1 $\mu$L of DNA template (0.5 to 5 ng). Standard curves were prepared between $10^1$-$10^8$ gene copies $mL^{-1}$. All efficiencies were greater than 70%, and $R^2$ values were greater than 0.99.

### 3.2.5 Experiments to study polyomavirus sorption

Control experiments were conducted to determine if the measured decreases in BKPyV concentrations were due to the sorption of viruses to particles found in hydrolyzed urine. In these experiments, 50 $\mu$L of stock BKPyV was spiked into 1 mL of urine at a concentration of $5\times10^5$-$1\times10^6$ IU $mL^{-1}$ and briefly mixed. The solution was then incubated for various amounts of time to allow particles in the urine to settle. At predetermined times, two 50 $\mu$L aliquots were removed, including one from the top of the sample to avoid settled particles and one of the mixed sample. All aliquots were spiked into 450 $\mu$L of tissue culture media (REGM). Infectious BKPyV levels in the supernatant were compared to levels in the mixture.

### 3.2.6 Experiments to study polyomavirus genome degradation

To assess BKPyV genome stability, 50 $\mu$L of stock BKPyV was spiked into 1 mL of hydrolyzed urine at a concentration of $5\times10^5$-$1\times10^6$ IU $mL^{-1}$. 50 $\mu$L aliquots were removed from the mixture at different time points up to 27 days and added to 450 $\mu$L of tissue culture media (REGM). The samples were stored at -80° C until viral DNA extraction, and then qPCR was conducted on a 900 bp region of the genome (Table S1).

To estimate the reaction rate constants for the entire BKPyV genome, we first extrapolated the concentrations measured for the 900 bp amplicon to the entire 5.1 kbp genome with the following relationship (Pecson et al. 2011):

$$log\frac{N}{N_0} = log\frac{n}{n_0} \times \frac{genome\ size}{amplicon\ size} \qquad \text{(Equation 1)}$$

where $\frac{N}{N_0}$ is the extrapolated concentration of the entire genome and $\frac{n}{n_0}$ is the measured concentration of the 900 bp amplicon. First order rate constants for the BKPyV genome were estimated by conducting linear regressions of $\ln(N/N_0)$ versus time. This extrapolation assumes that the reactions in the genome are uniformly distributed.

The ability of the BKPyV Dunlop strain qPCR method to detect small decreases of the 900 bp gene copy concentrations in hydrolyzed urine was tested by diluting the spiked urine solutions by 10% and 20% (i.e. 0.9× and 0.8×) with nuclease free water. The undiluted, 0.9× and 0.8× samples were extracted five times each, and then the 900 bp targets were quantified with qPCR. The measured gene copies in the 0.9× and 0.8× samples were compared to the measured gene copies in the undiluted sample.

### 3.2.7 Experiments to study polyomavirus capsid disassembly

To evaluate protein capsid stability in hydrolyzed urine, BKPyV proteins were separated with SDS-PAGE gels, and the VP1 capsid proteins were visualized by western blotting (Jiang et al. 2009). Specifically, 50 $\mu$L of stock BKPyV was spiked into 1 mL of hydrolyzed urine at a concentration of $5\times10^5$-$1\times10^6$ IU $mL^{-1}$. Immediately after adding the virus to the urine and then again after 24 hours, 80 $\mu$L aliquots were removed from the mixture. To preserve the disulfide bond configuration of BKPyV in the urine samples, 80 $\mu$L aliquots were combined with 1.6 $\mu$L of freshly prepared 1 M *N*-ethylmaleimide (Sigma), diluted in 100% ethanol. This mixture was

incubated on ice for 45 minutes and stored at -80 °C until viral proteins from the BKPyV capsid were separated using 8% SDS-PAGE gels. In the SDS-PAGE analysis, 80 $\mu$ L sample aliquots were added to 20 $\mu$ L of reducing buffer (250 mM Tris-HCl pH 6.8, 50% glycerol, 10% SDS, 250 mM dithiothreitol, 0.025% bromophenol blue) or 20 $\mu$ L nonreducing buffer (250 mM Tris-HCl pH 6.8, 50% glycerol, 10% SDS, 0.025% bromophenol blue). Samples in reduced conditions were diluted 1:20 to have similar signal strength as non-reduced samples for western blot visualization. After buffer addition, all samples were heated to 42° C for 5 minutes and resolved on an SDS-PAGE gel. Under non-reducing conditions, the capsid proteins will enter the gel only if the disulfide bridges were broken in the experiments. Control samples were prepared in reduced conditions to evaluate the total amount of VP1 proteins in the samples. After separation, VP1 proteins were wet-transferred to a nitrocellulose membrane in buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 60 V overnight. Membranes were blocked in 2% nonfat dry milk diluted in 0.1% Tween 20 prepared in phosphate buffer (PBS-T), stained with 1:2000 VP1 primary antibody diluted in 2% nonfat dry milk solution, stained with 1:2000 anti-mouse HRP secondary antibody (Amersham) diluted in 2% nonfat dry milk solution, and washed with PBS-T.

### 3.2.8 Viability of bacteria in urine

Bacteria with intact and damaged cell membranes in duplicate urine samples were quantified using BacLight “Live/Dead” stain (Molecular Probes) according to the manufacturer’s protocol. Viable (fluorescent green) cells were counted and compared to cells with damaged membranes (fluorescent red) using fluorescence microscopy and averaging counts over 10 random fields of view.

### 3.2.9 Data Analysis

Virus inactivation was modelled with first order kinetics. Rate constants *k* were calculated with linear regressions of natural log concentration and time using the Data Analysis plugin for Microsoft Excel. The reported errors correspond to 95% confidence intervals. The $T_{90}$ values, or time required for 90% reduction in infectivity or gene copies, were calculated as the reciprocal of the first order rate constants.

Student T-tests were used to compare bacteria live/dead ratios, changes in the 900 bp gene copy concentrations after storage in hydrolyzed urines, and to test the sensitivity of our DNA extraction and qPCR assays. ANOVA multiple linear regression analysis was used to compare the inactivation kinetics of two model viruses, MS2 and Q $\beta$ , and to assess the impact of urine pasteurization and filtration on virus inactivation kinetics. *p*-values are provided for all statistical analyses.

## 3.3 Results and Discussion

### 3.3.1 Polyomavirus concentrations in urine and urine-derived fertilizers

Infectious polyomaviruses present in urine cannot be enumerated due to a lack of a compatible tissue culture for studying urine isolates. Consequently, BKPyV DNA concentrations in the collected urine before and after hydrolysis and pasteurization were enumerated by qPCR. The endogenous BKPyV DNA concentration in freshly collected source-separated urine A prior to hydrolysis was $7.0 \times 10^5$ gene copies $mL^{-1}$ (Figure 3-1) which is consistent with reported concentrations in urine of healthy individuals ($5 \times 10^0 - 1.24 \times 10^8$ gene copies $mL^{-1}$) (Urbano et al. 2016). This data is based on one fresh urine sample, as it is difficult to collect large fresh urine samples since hydrolysis can happen quickly. The average BKPyV gene copy concentration in hydrolyzed urines A, B, C was $3.8 \times 10^6$ gene copies $mL^{-1}$ and $1.2 \times 10^7$ gene

copies $mL^{-1}$ in pasteurized urines A, B, C (Figure 3-1) These data suggest that the polyomaviruses, or at least the polyomavirus DNA, survive the harsh conditions of hydrolyzed urine storage and pasteurization.

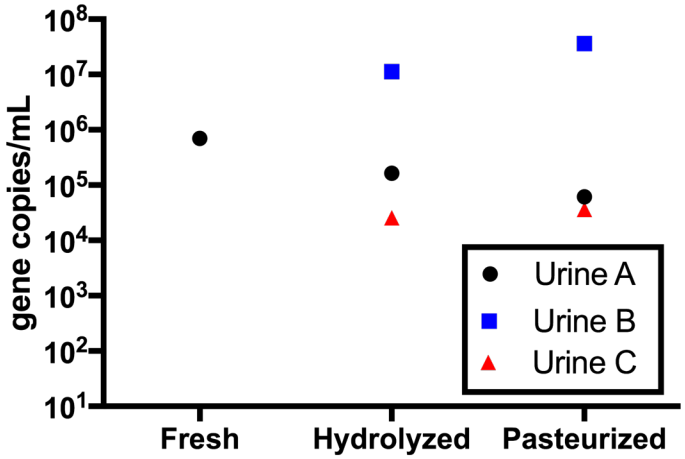


**Figure 3-1. BKPyV gene copy (152 bp) concentrations detected by qPCR in urine A (fresh, hydrolyzed for 10 months, and pasteurized after hydrolyzed for 10 months) and urine B and C (hydrolyzed 1 month and after 10 months, respectively, and pasteurized after the same amount of hydrolysis time). Fresh urine was only available for urine A due to rapid hydrolysis in urines B and C.**

**Table 3-2. First order rate constants and $T_{90}$ values for BKPyV and bacteriophages T3, Qβ, and MS2 in hydrolyzed urines. 95% confidence intervals are reported for the linear regression analyses.**

| | BKPyV | | T3 | | Q $\beta$ | | MS2 | |
|---|---|---|---|---|---|---|---|---|
| | dsDNA – disulfide bonds | | dsDNA | | ssRNA – disulfide bonds | | ssRNA | |
| Urine (hydrolysis time) | k ($hour^{-1}$) | $T_{90}$ (hours) | k ($hour^{-1}$) | $T_{90}$ (hours) | k ($hour^{-1}$) | $T_{90}$ (hours) | k ($hour^{-1}$) | $T_{90}$ (hours) |
| D (< 1 week) | | | $1.7 \times 10^{-3} \pm 3.1 \times 10^{-4}$ | 590 | | | | |
| E (4 months) | 0.67±0.97* | 1.5 | $2.2 \times 10^{-3} \pm 9.5 \times 10^{-4}$ | 450 | | | | |
| E (8 months) | | | | | | | | |
| E (9 months) | 0.28 ±0.51* | 3.6 | | | 0.16±0.33 | 6.3 | 0.11±0.011 | 9.1 |
| E (15 months) | | | | | | | | |
| F (4 months) | | | $9.0 \times 10^{-4} \pm 5.0 \times 10^{-4}$ | 1100 | | | | |
| F (8 months) | 0.40 ±3.1* | 2.5 | | | | | | |
| F (11 months) | 0.90±0.41 | 1.1 | | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| G (11 months) | $4.7 \times 10^{-3}$ $\pm 7.6 \times 10^{-3}$* | 210 | | | | |
| H (3 months) | | | | 0.13±0.033 | 7.7 | 0.11±0.019 | 9.1 |
| H (9 months) | 0.17±0.060 | 5.9 | | | | |
| I (2 months) | 0.45±0.048, | 2.2 | | | | |
| I (2 months), pasteurized | 0.26±0.045 | 3.8 | | | | |
| I (2 months), filtered | 0.31±0.037 | 3.2 | | | | |
| I (10 months) | 0.16 ±0.037 | 6.3 | | | | |
| I (10 months), pasteurized | 0.078±0.16* | 13 | | | | |
| I (10 months), filtered | 0.079±0.21* | 13 | | | | |
| I (11 months) | 0.094±0.030 | 11 | | | | |
| I (11 months), pasteurized | 0.045±0.027 | 22 | | | | |
| I (11 months), filtered | 0.036±0.009 | 28 | | | | |

* Indicates not statistically different from zero

### 3.3.2 BKPyV and bacteriophage T3 inactivation in hydrolyzed urine

The gene copy concentrations measured by qPCR do not necessarily correspond to the concentrations of infectious viruses. Although infectivity assays for the polyomaviruses found in urine are not possible at this time, certain polyomavirus genome variants, such as BKPyV Dunlop can be enumerated *in vitro*. BKPyV Dunlop is a rearranged variant of the archetype that is present in humans. This variant differs in the non-coding control region of the genome and is easily grown in cell culture models (Broekema et al. 2010). The variant viral particles are structurally identical to the viruses found in urine. BKPyV Dunlop was spiked into various hydrolyzed urine samples, and inactivation kinetics were then measured. Source-separated urine characteristics vary depending on the donors' age, nutrition, amount of physical exercise, etc. (Rose et al. 2015). We therefore utilized a number of source-separated urines collected from different regions of the United States and stored for different amounts of time to capture these variations.

Control experiments confirmed that the unspiked urine samples contained no culturable BKPyV. In the spiked hydrolyzed urine samples, BKPyV inactivation rates ranged from $4.7\times 10^{-3}$ – 0.90 $hour^{-1}$, corresponding to $T_{90}$ values from 1.1 – 210 hours (Figure 3-2 and Table 3-2). Most of the urine samples exhibited rapid inactivation rates (E, F, H, and I), with $T_{90} < 11$ hours, but BKPyV was much more stable in urine G. Urine G did not differ from the other urine samples in its ammonia concentration (Table 3-1), but was collected from fewer donors than urines E, F, H, and I.

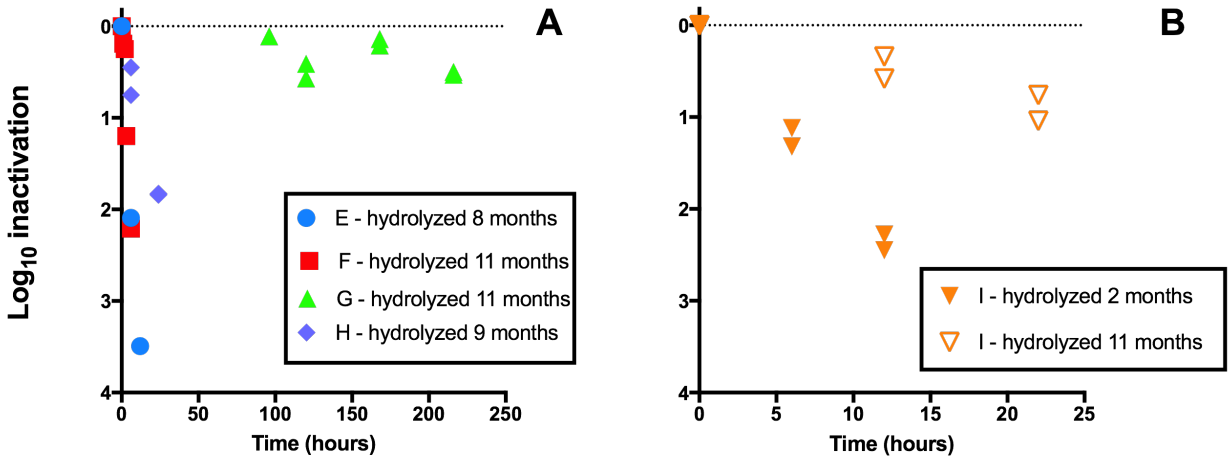


**Figure 3-2. Infectivity of BK polyomavirus in hydrolyzed urine samples (E - I) measured over time. Initial infective virus concentrations in urine were $5 \times 10^5$-$1 \times 10^6$ IU (infectious units) $mL^{-1}$. Experiments were conducted until the detection limit was reached ($3 \times 10^2$ IU $mL^{-1}$). Left panel A shows variability in kinetics across different urine aged between 8-11 months. Right panel B shows kinetic changes with hydrolysis time.**

In addition to different urine samples exhibiting different inactivation rates, the same collected urine sample hydrolyzed for different amounts of time exhibited different BKPyV inactivation rates (Figure 3-2B and Table 3-2). Our previous work demonstrated that the bacterial community changes as the urine is stored (Lahr et al. 2016). Other work has shown that microbial activity can play a role in virus inactivation, particularly for DNA viruses that experience slower abiotic inactivation or in matrices with higher microbial activity (Decrey & Kohn 2017). We therefore hypothesized that the microbial activity was influencing inactivation, and that the variation of inactivation kinetics was due to different microbial activities in the urine samples.

Microbial activity was thus evaluated in urine samples that exhibited slow and fast virus inactivation rates (urines G – hydrolyzed 11 months and I – hydrolyzed 2 months) with BacLight viability staining. The average urine I ratio (0.66) was larger than the average urine G ratio (0.085, $p = 0.0194$). Interestingly, the higher live/dead bacteria ratio was measured in the sample

that inactivated the virus at a significantly faster rate. This supported our hypothesis that microbial activity played a role in the different observed polyomavirus inactivation rates.

Other dsDNA viruses are stable in human excreta and animal manure with high ammonia and high pH (Decrey et al. 2016). T4 (dsDNA), ΦX174 (ssDNA), and rhesus rotavirus (dsRNA) are stable in urine with $T_{90}$ values of 12.5 days, 7 days (Decrey & Kohn 2017), and 35 days at 20° C (Höglund, Ashbolt, et al. 2002), respectively, while human adenovirus (dsDNA) is less stable, with $T_{90}$ values ranging from 2-24 hours (Decrey & Kohn 2017). We used another dsDNA virus bacteriophage T3 to confirm that our observation was not unique to the urine samples tested. The dsDNA bacteriophage T3 was spiked into various urine samples, and the concentration of infectious T3 particles was measured over time. T3 was much more stable than BKPyV in hydrolyzed urine (Table 3-2), with an average first order rate constant of $1.6\times 10^{-3}$ $hour^{-1}$ and an average $T_{90}$ equal to 630 hours (n = 3). T3 is also stable in other aquatic environments, remaining infectious in a wide pH range (5-9.2) and in wastewater matrices for at least 48 hours (Jończyk et al. 2011; Ye et al. 2016). T3 and BKPyV exhibited very different inactivation kinetics despite having the same genome type. This suggests that the inactivation mechanism for BKPyV is different than the inactivation mechanism for bacteriophage T3. We next sought to determine why BKPyV was susceptible to the conditions of hydrolyzed urine.

### 3.3.3 Attributes of polyomavirus leading to inactivation

Inactivation of non-enveloped viruses can be due to damage to the capsid structure or damage to the genome (Ward et al. 1986; Roy et al. 1981; Kim et al. 1980; Brien & Newman 1979). To assess if inactivation in the urine was due to degradation of the polyomavirus genome, a 900 bp region of the BKPyV genome was monitored by qPCR as the virus was incubated in hydrolyzed urine I. The 900 bp amplicon covered ~20% of the BKPyV genome, and controls

confirmed that unspiked urine did not contain the amplicon sequence. After 27 days we detected no significant decrease in gene copies based on both linear regressions of the entire data set and a student T-test of the gene copy concentrations at time = 0 and time = 27 days. Our qPCR assay could effectively detect a 20% decrease in the initial gene copy concentration of BKPyV (student T-test, p=0.0062); this means that the reaction rate constant for the 900 bp amplicon in urine was $< 0.0083$ day$^{-1}$ ($T_{90}$>120 days). Extrapolating this rate constant to the entire genome using equation (1) results in a genome rate constant $k < 0.047$ day$^{-1}$ and a $T_{90}$ value greater than 21 days. For comparison, the infectivity $T_{90}$ for this same urine sample was 6.3 hours (Table 3-2). These data verify that reactions in the dsDNA polyomavirus genomes are not responsible for virus inactivation in the hydrolyzed urine.

To investigate if capsid disassembly plays a role in polyomavirus inactivation, western blots were employed to monitor the disulfide bonds that provide stability to the protein capsid structure. VP1 is the major structural protein in the polyomavirus capsid. The capsid is composed of 72 pentamers of this protein (Rayment et al. 1982) connected with inter- and intrapentameric disulfide bridges (Walter & Deppert 1975). The presence of disulfide bridges sets polyomavirus apart from the other dsDNA viruses that have been tested in hydrolyzed urine and human excreta at combined high pH and ammonia levels. To investigate the stability of the capsid structure, SDS-PAGE western blots were conducted on BKPyV proteins after the viruses were stored in urine. If the disulfide bonds are intact, the virus is unable to enter the SDS-PAGE gel. Results confirmed protein structural changes following incubation in hydrolyzed urine (Figure 3-3). Immediately after BKPyV was added to urine, minimal VP1 pentamers, dimers, or monomers were visible; therefore, most of the BKPyV particles were intact and too large to enter the non-reducing SDS-PAGE gel. After BKPyV was incubated in hydrolyzed urine for 24 hours

and inactivated, VP1 monomers were detected; therefore, the BKPyV capsid was disassembling. Control samples at time = 0 and after 24 hours, in which the VP1 protein disulfide bonds were reduced experimentally prior to electrophoresis, confirmed that the increase in signal observed in Figure 3-3 was due to disassembly of BKPyV in urine and not due to different amounts of added virus capsids. These results, coupled with the relatively slow T3 inactivation kinetics and the relatively slow genome reaction kinetics, suggest that something about the capsid structure of BKPyV renders it susceptible to disassembly and rapid inactivation in hydrolyzed urine.

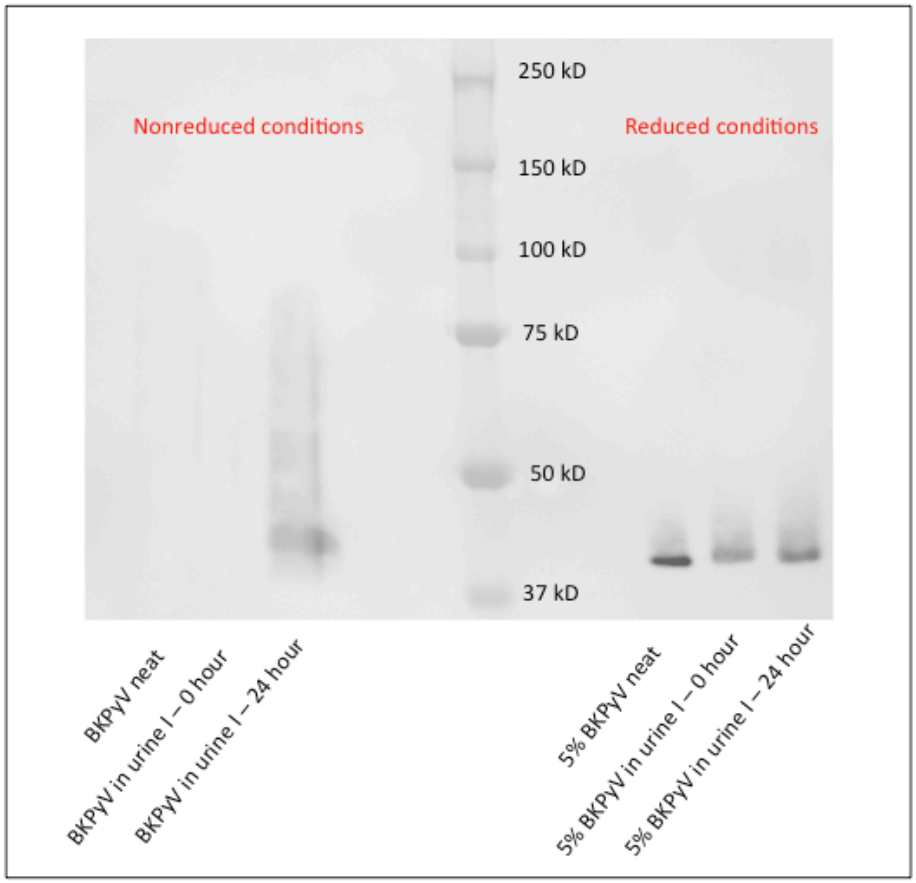


**Figure 3-3. Western blot of BKPyV proteins separated under non-reducing (left) and reducing (right) conditions. BKPyV proteins were analyzed after addition to buffer (neat), immediately after addition to hydrolyzed urine (0 hour) and after incubating for one day in hydrolyzed urine (24 hour). The reduced samples were diluted to 5% of the experimental concentration to allow visualization on the same gel as the non-reduced samples. The BKPyV VP1 monomer size is 42 kD.**

To explore the role of disulfide bonds when viruses are inactivated in hydrolyzed urine, two related model viruses (MS2 and Q $\beta$ ) were added to hydrolyzed urine, and infectivity was measured over time. These two ssRNA viruses have similar RNA sequences (up to 80% similarity in the replicase subunit) and capsid size (25 nm), but differ in that the capsid proteins of Q $\beta$ are connected with disulfide bridges. Bacteriophage Q $\beta$ inactivated at a significantly faster rate in hydrolyzed urine than MS2 ($p$ = 0.00105) (Table 3-2 and Figure S1). The RNA genome of Q $\beta$ is longer than MS2 (4.2 kbp vs. 3.6 kbp), and based on a model developed by Decrey et al., we compared the expected ssRNA transesterification rates in Q $\beta$ and MS2 based on their genome sizes (Decrey et al. 2014). The $k_{\{NH3\}}$ for Qβ was predicted to be 1.17× larger than the $k_{\{NH3\}}$ for MS2. Our inactivation rate constant for Q $\beta$ was 1.32× larger than that of MS2. Consequently, if mechanisms beyond RNA transesterification inactivate Qβ, such as disruption of the capsid structure, these mechanisms are minor. Additional viruses that contain disulfide bonds will need to be studied in hydrolyzed urine to better define the role these bonds play in virus inactivation.

### 3.3.4 Hydrolyzed urine characteristics that influence BKPyV inactivation rates

To understand why different hydrolyzed urine samples exhibited different rates of BKPyV inactivation, we explored which characteristics of the hydrolyzed urine contributed to BKPyV inactivation. We first hypothesized that the BKPyV particles adsorbed to large particulates in the urine and then settled out rapidly before aliquots were collected for culturing. Control experiments were therefore conducted in which particulates in the urine were allowed to settle and inactivation of BKPyV was measured in the supernatant. The rate constant with particulates (0.40±0.12 $hour^{-1}$) was not statistically different from the rate constant without

particulates (0.39±0.12 hour$^{-1}$, $p = 0.90$). Sorption to particulates and settling was therefore ruled out as a significant contributing factor in the observed inactivation rates.

We next tested the impact of the high pH and ammonia levels in the hydrolyzed urine samples, as these conditions are biocidal to many organisms, including RNA viruses (Decrey et al. 2014). Linear regressions conducted on BKPyV concentrations over time in buffers with pH and ammonia levels similar to hydrolyzed urine were not significantly different from zero (n=3). This demonstrated that the BKPyV was not losing infectivity due to the elevated pH and high ammonia concentrations in hydrolyzed urine.

Finally, we tested the role of microbial activity. Microorganisms can contribute to virus inactivation in some environments (Nasser et al. 2002; Ming Yi Deng & Cliver 1992; Decrey & Kohn 2017). To evaluate if the microorganisms present in hydrolyzed urine impact the infectivity of BK polyomavirus, BKPyV was added to hydrolyzed urine, hydrolyzed urine that was recently pasteurized, and hydrolyzed urine that was recently filtered through filters with 0.22 $\mu$ m pores. ANOVA multiple linear regression analyses suggested that BKPyV was inactivated at slower rates when urine I (hydrolyzed 11 months) was either pasteurized or filtered (Table 3-2, Figure 3-4, $p = 0.0014$ for pasteurized urine, $p = 9.7 \times 10^{-5}$ for filtered urine). This experiment was repeated in urine I (hydrolyzed 2 months) and urine I (hydrolyzed 10 months) with similar results (Table 3-2). Inactivation was not completely prevented after filtration and pasteurization; therefore, additional unknown factors contributed to BKPyV inactivation in the hydrolyzed urine.

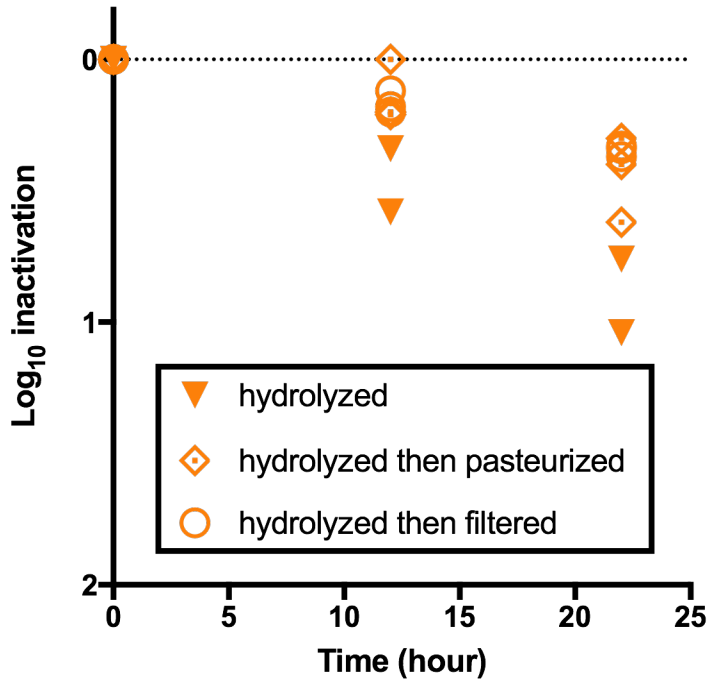


**Figure 3-4. Infectivity over time of polyomavirus BKPyV spiked into urine I samples that had been hydrolyzed for 11 months, pasteurized, and filtered. Initial BKPyV concentrations were $5\times10^5$-$1\times10^6$ IU $mL^{-1}$. Experiments were conducted until the detection limit was reached ($3 \times 10^2$ IU $mL^{-1}$).**

This work demonstrates that the urinary tract virus BKPyV is rapidly inactivated in most hydrolyzed urine samples. It is therefore likely that short-term storage of urine (< 1 month) is adequate for mitigating risks associated with polyomavirus. We ruled out the role of elevated ammonia and pH levels in BKPyV inactivation and provided evidence for the role of microbial activity. The specific inactivation mechanism most likely involves capsid damage, since the DNA was not degraded over long periods of time and the capsid proteins disassembled. We hypothesize that the disulfide bonds in BKPyV make it more susceptible to inactivation in the hydrolyzed urine because the dsDNA bacteriophage T3 was very stable. Inactivation experiments with MS2 and Qβ were not able to support this hypothesis due to the rapid RNA transesterification reactions that inactivated both viruses.

Our results are a reminder that positive qPCR measurements do not indicate the presence of infective viruses. BKPyV DNA genes persisted for months, but infective viruses persisted for

only hours to days. Employing bacteriophage surrogates to predict pathogen behavior is ideal for viruses that are not culturable; however, our results underscore the fact that bacteriophages are often inappropriate surrogates for human viruses even when they contain the same genome type. Using the inactivation behavior of bacteriophage T3 to predict polyomaviruses would greatly overestimate the BKPyV risk posed by urine-derived fertilizer.

It is, of course, impractical to test the survivability of every human virus in every environmental condition with culture assays, especially when a number of human viruses do not have readily available culture systems (e.g., human norovirus). Instead, the environmental virology community should aim to understand how the chemical, structural, and biological characteristics of viruses impact their environmental fate. This requires studying the mechanistic fate of a broad range of viruses in various environmental conditions. Our results suggest that the capsid of BKPyV is disassembled in hydrolyzed urine samples despite the common assumption that disulfide bridges have a stabilizing effect on protein structures. It remains to be seen whether this effect influences the stability of other important human viruses with disulfide bonds in capsid proteins including HIV, Hepatitis B, and Hepatitis C (Ivanov et al. 2007; Wynne et al. 1999; Blanchard et al. 2002). Once we identify the protein characteristics that drive inactivation in hydrolyzed urine and other environmental matrices, we will be able to select more appropriate process surrogates for specific human viruses.

Finally, microbial activity appears to contribute to the BKPyV inactivation, although the specific mechanism by which microorganisms inactivate the virus remains unclear. Viral inactivation in environmental matrices often depends on a variety of factors, and the components leading to inactivation can vary from sample to sample. The varied inactivation rates observed amongst the urine samples may be due to the prokaryotic and eukaryotic levels and communities

in the urine samples. Our earlier work revealed that bacterial communities in several collected urine samples converge to have similar structures at the 16S level when stored (Lahr et al. 2016). Identifying the specific prokaryotic and eukaryotic organisms responsible for the inactivation of viruses in urine samples and other environmental matrices will be necessary to more systematically evaluate the risks that they pose.

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# Chapter 4.
# A mechanistic study of DNA persistence, conformation changes, and transformation ability in source-separated urine

## 4.1 Introduction

Collecting and treating source-separated urine for fertilizer use is a promising technology that addresses several environmental challenges. The majority of nitrogen and phosphorus found in wastewater is from urine (Larsen & Gujer 2001; Otterpohl 2002; Maurer et al. 2006; Lienert et al. 2007; Meinzinger & Oldenburg 2009). Thus, nitrogen and phosphorus could be recovered from urine before being diluted into the rest of the wastewater stream, offsetting energy intensive traditional fertilizer production processes (Maurer et al. 2003). The recovery of these nutrients can reduce the need for nutrient removal at wastewater treatment plants (Wilsenach & van Loosdrecht 2006; Jimenez et al. 2015) and prevent nutrients from ending up in waterways, where they can cause harmful algal blooms and eutrophication (Smith et al. 1998). To harness these benefits, urine is collected separately before entering the wastewater stream. This source-separated urine can be converted into fertilizer through multiple methods. The simplest process is by storing urine for long periods of time (WHO 2006). As urine is stored in a sealed container, the nitrogen present in fresh urine as urea is hydrolyzed to ammonia and the pH rises to ~9, resulting in an environment that can be difficult for some bacteria to survive.

Stored urine still contains microorganisms and extracellular DNA. In terms of viral DNA, the dsDNA genome of a urinary tract virus, BK polyomavirus, persisted in hydrolyzed urine for more than three weeks despite the virus' loss of infectivity within several hours (Goetsch et al.

2018). Previous work suggests that bacteria persist and remain active during the storage of urine (~ $10^8$ – $10^9$ cells per mL of urine), and the bacterial communities change throughout storage (Lahr et al. 2016). Urinary tract bacterial infections are often treated with antibiotics, and this can lead to an enrichment of antibiotic resistant organisms in urine (Foxman 2010). In fact, antibiotic resistance genes have been detected in source-separated urine collected for fertilizer use. Bischel et al. detected *sul1*, a sulfonamide resistance gene, in bacterial extracts from urine sampled from urine storage tanks throughout eThekwini, South Africa (Bischel et al. 2015). It is worth noting that as bacteria, viruses, and other microorganisms in urine are inactivated, they can degrade and release their nucleic acids. Consequently, ARGs present in source-separated urine may be present both within organisms and as extracellular DNA.

Bacteria can obtain antibiotic resistance through vertical gene transfer where genetic information is passed to daughter cells, or through horizontal gene transfer where organisms that do not express resistance obtain genetic information from their environment. Horizontal gene transfer can occur through three mechanisms: conjugation, where cell to cell contact allows the transfer of genetic information; transduction, where bacteriophages transfer genetic information into host cells; and transformation, where organisms take up and express DNA from the surrounding environment (Thomas & Nielsen 2005; Levy & Marshall 2004). In the case of transformation, the DNA from the environment is extracellular, and thus not incorporated in viable or infective organisms.

Both culture-based and molecular methods are commonly applied to study the presence and transfer of ARGs. Culture methods detect the presence of culturable resistance but many resistant bacteria are not culturable or are susceptible to a viable but nonculturable state (Oliver 2005). Molecular methods like quantitative PCR detect sections of resistance genes that may or

may not be in viable bacteria. In fact, Chang et al. demonstrated that when plasmid DNA was treated with $UV_{254}$, the rate constants for plasmid transformation loss as detected from qPCR were 2-7x larger than the rate constants measured with transformation assays. In other words, these methods that detect DNA do not capture the gene's ability to be transformed (Chang et al. 2017). Thus multiple methods are required to understand antibiotic resistance gene fate in the environment.

Due to the observed persistence of DNA in urine and the presence of antibiotic resistance genes detected with qPCR, there is a need to better understand whether genes present in urine fertilizers can spread antibiotic resistance when the fertilizer is applied in the environment. This study evaluates the fate of DNA in hydrolyzed urine and its potential to be transferred and expressed by competent bacteria. Plasmid DNA was added to hydrolyzed urine, and its ability to transform *Acinetobacter baylyi* was evaluated over time. We employed *A. baylyi* as a model organism because it is common in soils, is highly competent for natural transformation (Young et al. 2005; Vaneechoutte et al. 2006), and is relevant for opportunistic infections (Chen et al. 2008). This plasmid-bacteria system has relatively high transformation rates, and this allowed us to measure up to a 99% loss in transformation efficiency. As plasmids lost their ability to transform resistance to *A. baylyi,* qPCR was used to measure gene concentration, and gel electrophoresis was used to assess plasmid DNA conformation. We also studied the impact of urine chemistry and microbiology on the DNA fate. This study provides insight into how the conditions found in hydrolyzed urine change extracellular DNA and may result in reducing the risk of spreading antibiotic resistance genes.

## 4.2 Materials and Methods

### 4.2.1 Urine collection and characterization

Urine was collected from over 100 female and male donors in Vermont. The >100 L of collected urine was stored at room temperature in a sealed plastic container. All hydrolyzed urine used in this study was stored between 5 months and 1 year. Total ammonia nitrogen levels were measured with Standard Method 4500 - $NH_3$ (APHA; AWWA; WEF 2005). Measured ammonia concentrations (6,000 ± 100 mg N /L ammonia; standard deviation for $n \geq 2$) were consistent with concentrations reported in previous studies (Udert et al. 2006; Höglund et al. 2000).

### 4.2.2 General experimental approach

DNA stability was tested in a number of experimental solutions, including: hydrolyzed urine; hydrolyzed urine that was filtered through 0.22 μm membrane filters (PES, Dot Scientific); hydrolyzed urine that was filtered through centrifugal ultrafilters with 100 kDa pores (Amicon Ultra centrifugal filter units, Millipore-Sigma); hydrolyzed urine that was filtered through 0.22 μm membrane filters and heated to 75 °C in a water bath for 10 minutes; and nuclease free water. Plasmid pWH1266 was used as a model DNA molecule and was spiked into each solution at a final plasmid DNA concentration of 7-8 ng/μL, as measured with a Qubit 2.0 fluorometer (Invitrogen). The spiked solutions were stored in the dark at room temperature for the duration of the experiments. Aliquots were removed immediately after DNA was added to the solutions and after two subsequent incubation times that ranged from 10 to 48 hours. Plasmid DNA in the aliquots was tested for transformation efficiency using transformation assays, for gene concentrations by qPCR, and for plasmid DNA conformation by gel electrophoresis. Aliquots collected for qPCR and gel electrophoresis were frozen and stored at -20 °C until they

were analyzed. Aliquots collected for transformation efficiency measurements were assayed immediately.

### 4.2.3 Model transformation system

We employed plasmid pWH1266 as a model DNA molecule and studied its transformation of *Acinetobacter baylyi*. Plasmid pWH1266 is a construct of plasmids pBR322 and pWH1277. pBR322 is an *Escherichia coli* plasmid that includes a tetracycline and ampicillin resistance gene (Hunger et al. 1990; Cooksey et al. 1985; McNicholas et al. 1992). pWH1277 is an *A. lwoffi* plasmid, and its sequence was not available prior to this study. We conducted the primer-walking method with Sanger sequencing to obtain the sequence of the pWH1277 region in pWH1266 (provided in Appendix B). *A. baylyi* is a naturally competent bacterium known to take up pWH1266 and express resistance to tetracycline and ampicillin when transformed.

### 4.2.4 Plasmid extraction

Plasmid DNA pWH1266 was propagated in *E.coli* strain TOP10, which was kindly provided from Dr. Chuanwu Xi at the University of Michigan. Frozen cultures of *E. coli* were inoculated on LB broth (Lennox) media agar with tetracycline (10 μg/mL) overnight (Chang et al. 2017). A single colony was selected and inoculated in liquid LB media with tetracycline (10 μg/mL). *E. coli* was grown overnight at 37 °C with shaking (180 rpm). The following morning, cultures were centrifuged to achieve a ~2x concentration, and plasmid DNA was extracted using QIAprep Plasmid Spin mini-prep kits per the manufacturer's instructions (Qiagen). DNA concentrations in the plasmid extract (~50-100 ng/μL) were measured with a Nanodrop 1000 spectrophotometer.

### 4.2.5 Transformation assays

Frozen aliquots of *A. baylyi* were inoculated on LB media agar and incubated overnight at 30 °C. A single colony was selected from the agar plate, inoculated in liquid LB media, and incubated 30 °C with shaking (240 rpm) until it reached stationary phase (16-18 hours). The cultures were diluted 10x in LB media and incubated at 30 °C with shaking at 240 rpm. After two hours, they reached early exponential phase and were competent for transformation. At this point, a 480 µL aliquot of competent cells was combined with 20 µL of the experimental solutions (hydrolyzed urine or nuclease-free water spiked with plasmid DNA) in culture tubes. The mixture was incubated for 24 hours at 30 °C with shaking (240 rpm). Following incubation, the sample was serially diluted and plated on LB media agar and on selective LB media agar that contained either tetracycline (10 µg/mL) or ampicillin (100 µg/mL). After overnight growth at 30 $^{o}$C, colonies were counted on all plates. Transformation assay conditions, including the optimum plasmid concentration, the point in the *A. baylyi* growth curve that plasmids were added, and the incubation time for the plasmid and bacteria, were determined previously (Chang et al. 2017). The shaking speed was optimized in this study (Appendix B section B1). Transformation efficiencies (cfu/cfu) were calculated by dividing the number of colonies counted on LB media with antibiotics (cfu) by the number of colonies counted on nonselective LB media (cfu), accounting for dilutions. Using a culture-based control eliminated the effects of a viable but non-culturable state that may have been present during the experiment.

### 4.2.6 Gel electrophoresis

The conformation and integrity of plasmid DNA was evaluated using gel electrophoresis. Frozen 10 µL control samples (plasmid DNA in nuclease free water) and experimental samples were thawed, mixed with 6X blue loading dye (Promega), and loaded onto 0.5% agarose

(Biorad) gels diluted in 1X TAE buffer (Biorad). The Biorad PowerPac Basic gel electrophoresis system was run at 40V for 80-120 minutes. DNA bands were visualized with SYBR safe DNA gel staining (Life Technologies), and the molecular mass of the plasmid DNA was confirmed with GeneRuler 1 kb DNA ladders (ThermoFisher Scientific).

### 4.2.7 qPCR measurements

The concentrations of pWH1266 segments were measured throughout the experiments with qPCR. Three regions of the pWH1266 plasmid were quantified, namely the *tetA* gene (1191 bps), *blaTEM-1* gene (861 bps), and the origin of replication (*ori*) gene (901 bps). Primers were designed to cover the entire resistance gene sequences and the entire origin of replication sequence. Primers (5' to 3') for the *tetA* and *blaTEM-1* genes were obtained from Chang et al. (Table 4-1) (Chang et al. 2017). Primers (5' to 3') for the *ori* gene were designed using the NCBI Primer-Blast tool (Table 4-1). qPCR was conducted with a Mastercycler RealPlex 2 system (Eppendorf, Hamburg, Germany) with Fast EvaGreen qPCR Master Mix (Biotium). Standard curves were conducted in duplicate, with concentrations ranging from $10^3$ to $10^8$ gene copies/mL. The 10 µL standard reactions and sample reactions included 5 µL 2X Biotium Fast EvaGreen master mix, 0.05 µL of each forward and reverse 100 µM primers, 0.13 µL of 50 mg/mL bovine serum albumin, 3.77 µL nuclease free water, and 1 µL of DNA template (0.5- 5 ng). qPCR efficiencies were greater than 70%, and $R^2$ values were greater than 0.991.

**Table 4-1 Primers of pWH1266 used**

| Gene | Length of amplicon | Forward primer (5' to 3') | Reverse primer (5' to 3') |
|---|---|---|---|
| *tetA* | 1191 | CGTGTATGAAATCTAACAATGCGCT[a] | CCATTCAGGTCGAGGTGGC[a] |
| *blaTEM-1* | 861 | TTACCAATGCTTAATCAGTGAGGC[a] | ATGAGTATTCAACATTTCCGTGTCG[a] |
| *ori* | 901 | AGGCGGTAATACGGTTATCCAC[b] | GAGATAGGTGCCTCACTGATTAAG[b] |

[a]Chang et al. 2017
[b]Designed using NCBI Primer Blast

The sensitivity of the qPCR methods to detect small decreases in amplicon concentrations (*tetA*, *blaTEM-1*, *ori*) was tested by diluting the plasmid by 10%, 20% and 25% (i.e., 0.9x, 0.8x, and 0.75x the plasmid concentrations in the urine at t = 0) with nuclease free water (n = 5). The undiluted and diluted samples were then quantified with qPCR.

### 4.2.8 Data analyses

Statistical analyses were completed in GraphPad Prism software. Student t-tests were used to compare transformation efficiencies at different plasmid incubation times in hydrolyzed urine. Linear regressions were employed to determine if slopes of the experimental data deviated from zero. *P* values were evaluated at a 95% confidence level.

## 4.3 Results and Discussion

### 4.3.1 Plasmid DNA stability in hydrolyzed urine measured with transformation assays

The detection limits of the transformation efficiencies (*i.e.*, fraction of *A. baylyi* cells that could have been transformed) on the ampicillin and tetracycline selective media ranged from 1.4 x $10^{-8}$ – 5.0 x $10^{-7}$, and our initial transformation efficiencies were in the range of 6.3 x $10^{-7}$ – 1.2 x $10^{-5}$. We were therefore able to observe approximately 2-log of transformation efficiency loss in our assays. When incubated in hydrolyzed urine, the transformation efficiency of plasmid DNA did not decrease significantly after 10 hours (student t-test; tetracycline, $p = 0.1553$; ampicillin, $p = 0.5023$). After 24 hours of incubation, the plasmid transformation efficiency had decreased by approximately 99% (student t-test; tetracycline, $p = 0.0208$; ampicillin, $p = 0.0003$; Figure 4-1). This trend suggests a lag in plasmid DNA degradation or structure, followed by a

faster rate; consequently, the data did not follow first order kinetics and could not be characterized with first order rate constants.

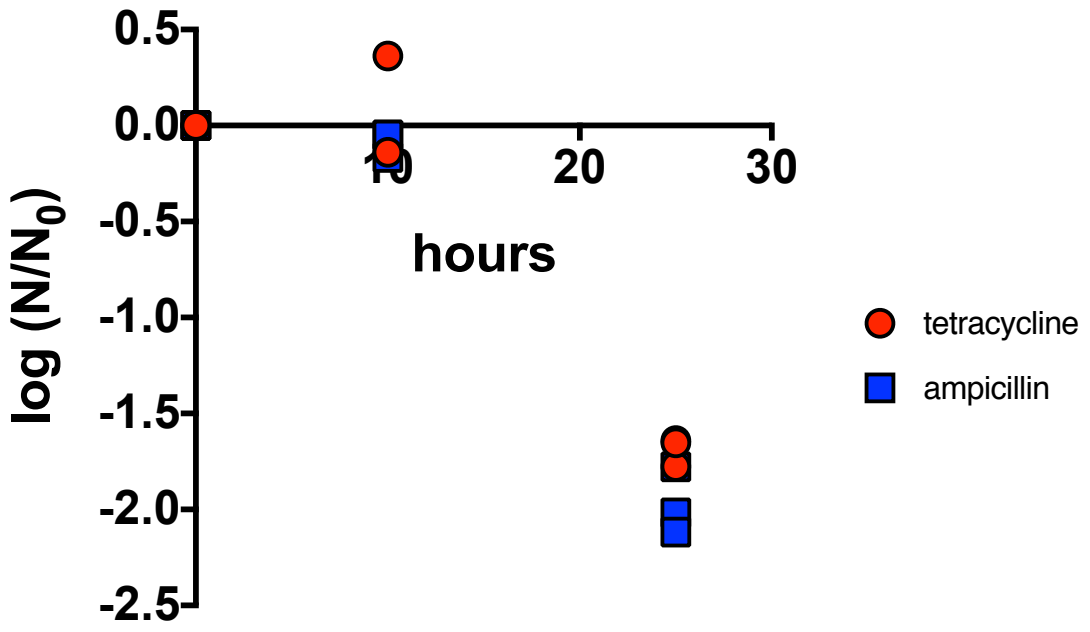


**Figure 4-1. Transformation efficiency relative to the initial efficiency for tetracycline (circle) resistance and ampicillin resistance (square) of the pWH1266 plasmid in hydrolyzed urine (n=3)**

### 4.3.2 Plasmid gene stability in hydrolyzed urine measured with qPCR

We first hypothesized that inactivation of the transformation efficiency was due to modifications in the resistance genes and that the damage was detectable with qPCR. After 24 hours in urine, the concentrations of tetracycline and ampicillin resistance genes on the plasmid as measured by qPCR, did not decrease statistically (Figure 4-2; student t-test: $p = 0.9741$ for *tetA,* tetracycline; $p = 0.9708$ for *blaTEM-1,* ampicillin; $p = 0.9807$). We therefore concluded that the transformation loss was not due to detectable damage on the antibiotic resistance genes. It should be noted that tetracycline and ampicillin resistance genes were not detected in the hydrolyzed urine samples before plasmid pWH1266 was added (data not shown).

We next hypothesized that inactivation of the plasmid transformation efficiency was due to damage incurred throughout the plasmid, that the damage was normally distributed across the plasmid, and that the damage was detectable with qPCR. The plasmid was approximately 8.9 kbps, and each of the three measured qPCR regions was approximately 1000 bp. Consequently, each qPCR assay could only detect approximately one-ninth of the overall plasmid damage at any given point in an experiment, given the damage was normally distributed across the plasmid. We next calculated the extent of damage incurred by each qPCR region after a 99% loss in plasmid transformation efficiency, given the damage was normally distributed across the entire plasmid and detectable with qPCR, based on the following relationship (Pecson et al. 2011):

$$log\frac{N}{N_0} = log\frac{n}{n_0} \times \frac{plasmid\ size}{amplicon\ size}$$

where $N/N_0$ is the concentration of the entire plasmid (8.9 kbps) and $n/n_0$ is the concentration of the measured regions of the plasmid. Based on this relationship, a 99% loss of transformation efficiency (i.e. decrease in total plasmid concentration) would correspond to a 46% decrease in the tetracycline resistance gene concentration, a 36% decrease in the ampicillin resistance gene concentration, and a 37% decrease in the *ori* gene concentration.

To assess if we could detect these concentration differences in our three qPCR assays, we determined the sensitivity of each qPCR assay to small decreases in the amplicon concentrations. Based on student t-test results of 5 replicates, qPCR analyses could detect statistical differences with a 25% decrease of the *tetA* gene concentration (student t-test, $p = 0.0247$), a 20% decrease of the *blaTEM-1* gene concentration (student t-test, $p = 0.0342$), and a 25% decrease of the *ori* gene concentration (student t-test, $p = 0.0158$). This suggested that following incubation in

hydrolyzed urine for 24 hours, the tetracycline resistance gene concentration, the ampicillin resistance gene concentration, and the *ori* gene concentration had not decreased by more than 25% and 20%, and 25%, respectively. Furthermore, the sensitivity results suggest that if the damage incurred in the plasmid was normally distributed and detectable by qPCR enzymes, the qPCR assays should have detected concentration decreases in the three regions when 99% of transformation efficiency was lost.

Taken together, these results suggest that either the inactivating modifications in the plasmid DNA are not normally distributed across the entire plasmid sequence and are outside the *tet*, *blaTEM-1*, and *ori* genes, or that the polymerase enzyme used in qPCR does not detect the DNA modifications that inactivate the plasmid.

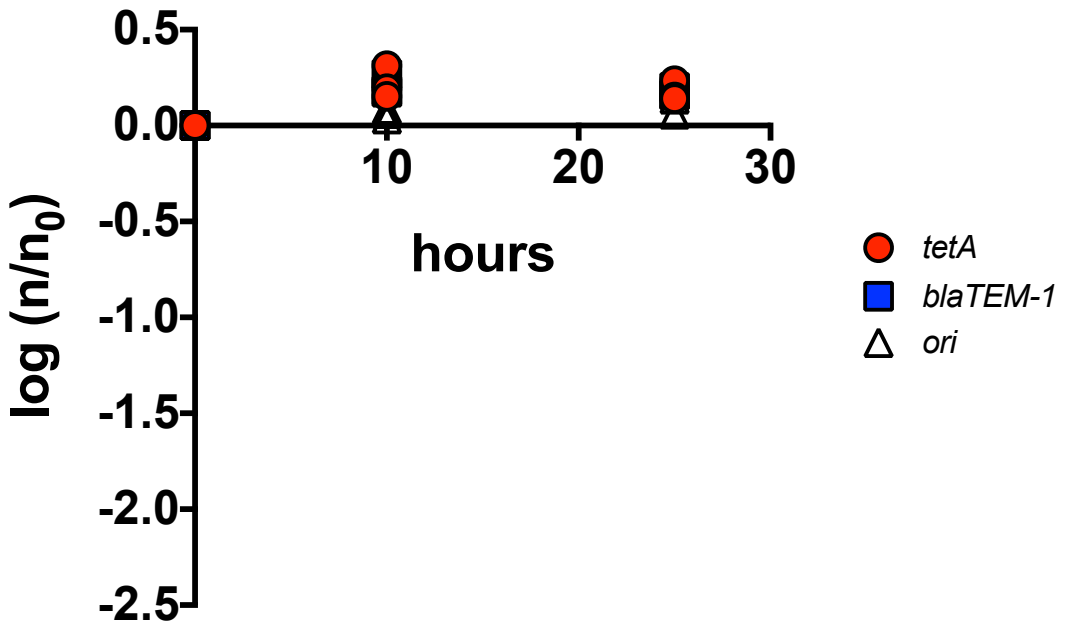


**Figure 4-2. Normalized concentration of tetracycline resistance gene copies (*tetA*), ampilcilin resistance gene copies (*blaTEM -1*), and the origin of replication gene copies (*ori*) on the pWH1266 plasmid in hydrolyzed urine at different experimental time points. Triplicates experiments were conducted.**

Previous research has demonstrated that the rate of extracellular DNA degradation depends on the environmental conditions. Plasmid DNA in activated sludge degraded within 6 hours of incubation, based on a PCR assay of a 1042 bp DNA region. The authors attributed the

rapid loss of PCR signal to both endo- and exonuclease activity in wastewater (Worthey et al. 1999). In groundwater, extracellular DNA can bind to aquifer material, making it resistant to DNases. As a result, DNA in groundwater environments tends to be stable and retain its ability to be transformed by bacteria (Romanowski et al. 1993). Likewise, the integrity and transformation ability of plasmid DNA added to soil microcosms can be retained for long periods of time due to adsorption to soil particles. For example, a DNA segment that was ~2 kbps was detected with PCR and gel electrophoresis after 40 days in soil (Widmer et al. 1996) and could be transformed up to 15 days in soil (Gallori et al. 1994). In another study, Southern hybridization was employed to determine plasmid conformation after incubation in soil. In that case, plasmid DNA, which is naturally supercoiled, had relaxed into its circular or linear form after only 1 hour in soil. After 2 to 5 days, the full length DNA molecule could no longer be detected; the specific time point at which it degraded depended on the soil type (Romanowski et al. 1992). To our knowledge, the fate of DNA in urine has not previously been explored.

Our transformation and qPCR data suggest that the reactions that caused a decrease in plasmid transformation efficiency either occurred outside of the three measured regions or that the polymerase enzyme used in qPCR does not detect the DNA modifications that inactivate the plasmid. In a previous study, a 900 bp region of the BK polyomavirus dsDNA genome (5.1 kbps) remained intact in hydrolyzed urine for more than three weeks despite the rapid loss of virus infectivity within 11 hours (Goetsch et al. 2018). In that case, the viral dsDNA was initially encapsidated, but the protein capsid eventually disassembled. The viral DNA was stable despite the fact that the capsid was no longer protecting the genome. Instead of modifications to the dsDNA, the inactivation mechanism was attributed to protein capsid modifications. Here, we were dealing only with dsDNA, so inactivation must be due to plasmid DNA modifications. The

loss of antibiotic resistance transfer ability, as measured by plasmid transformation, did not correlate with degradation of the corresponding antibiotic resistance gene, as measured by qPCR. We therefore hypothesized that DNA conformation changes were contributing to the loss of transformation in hydrolyzed urine.

### 4.3.3 Plasmid DNA conformation in hydrolyzed urine measured with gel electrophoresis

We next evaluated the conformation and integrity of plasmid DNA in hydrolyzed urine by gel electrophoresis over the same timescale studied in the transformation experiments. Gels conducted with the plasmids in nuclease free water controls demonstrated that the supercoiled conformation exhibited an apparent size of 5 kbps (Figure 4-3). The reason that the 8.9 kbps plasmid appears at 5 kbps is because supercoiled DNA appears smaller than linear DNA on gels. This same supercoiled plasmid band is visible in the hydrolyzed urine samples immediately following the spike addition of the plasmid DNA (0 hour samples).

Following 10 hours of incubation in the hydrolyzed urine, the 5 kbps band lightened, and a band representing an apparent size of ~8.9 kbps appeared. This 8.9 kbps band corresponds to the plasmid in a linear form. Following 24 hours of incubation in hydrolyzed urine, the supercoiled plasmid band disappeared, and the band at 8.9 kbps was the only band remaining (Figure 4-3). Based on these results, the linearization of the plasmid DNA occurred over the same timeframe that the plasmid transformation efficiency was reduced 99%. Previous research has demonstrated that linearized plasmids have lowered transformation efficiencies. For instance *Bacillus subtilus* was not transformed with either linearized or nicked plasmid DNA (Contente & Dubnau 1979). Likewise, Chang et al. observed a loss in transformation efficiency when plasmid DNA was cut either inside or outside of the *tetA* gene region with restriction enzymes (Chang et al. 2017).

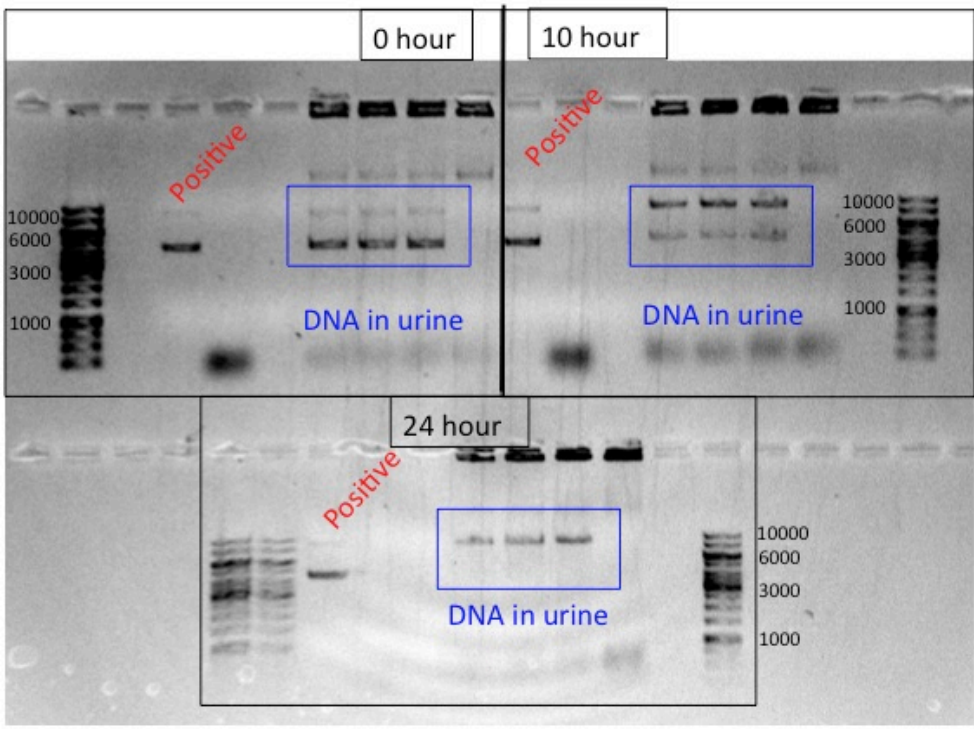


**Figure 4-3. Plasmid DNA conformation changes over time in unfiltered, hydrolyzed urine. Gel electrophoresis shows plasmid pWH1266 supercoiled in positive controls time over time and no plasmid in hydrolyzed urine in the negative controls (lane to the left of the plasmid in urine samples). pWH1266 is supercoiled at time 0 hour in urine, but it begins to linearize after 10 hours in urine. As transformation frequency goes down, the plasmid linearizes in unfiltered urine.**

DNA damage was not detected in the three regions measured with qPCR, but the gel results demonstrate that the plasmid was cut. Together, these results suggest that linearization was inhibiting plasmid transformation, but that the cuts were occurring outside of the three measured amplicon regions. We next sought to determine the attributes of hydrolyzed urine that caused the DNA linearization and the loss in transformation efficiency.

### 4.3.4 Impact of filtration and pasteurization of hydrolyzed urine on plasmid DNA fate

In our prior work on the fate of urinary tract virus polyomavirus in hydrolyzed urine, virus inactivation was prevented when the hydrolyzed urine was filtered through a 0.22 μm filter (Goetsch et al. 2018). In that study, we concluded that the virus inactivation was due to microbial activity. To test if a similar phenomenon was taking place with extracellular plasmid DNA, we spiked the plasmid into hydrolyzed urine that was pre-filtered through a 0.22 μm membrane filter.

The transformation efficiencies of the unfiltered urine were below the assay detection limits within 24 hours, in agreement with the previous experiments with unfiltered urine (Figure 4-1 and Figure 4-4). In the filtered urine (0.22 μm), we observed a ~1-log statistically significant decrease in transformation efficiency after 24 hours (student t test; $p = 0.0001$ (tetracycline), $p = 0.0268$ (ampicillin), Figure 4-4). After 48 hours, we observed a ~2-log decrease in transformation efficiencies (student t test; $p = 0.0001$ (tetracycline), $p = 0.0067$ (ampicillin), Figure 4-4). These data suggest that particles smaller than 0.22 μm pores are playing a role in the loss of plasmid transformation efficiency when incubated in hydrolyzed urine.

Next, transformation efficiency was evaluated in ultrafiltered (100 kDa cut-off) hydrolyzed urine. In contrast to the membrane filtered samples, there was no significant decrease in transformation efficiency over the entire timescale of the experiment, based on student T-test statistical comparisons between time 0 and 24 hours ($p = 0.1281$ (tetracycline), $p = 0.0875$ (ampicillin), Figure 4-4) and between 0 and 48 hours ($p = 0.7528$ (tetracycline), $p = 0.1698$ (ampicillin), Figure 4-4). These results suggest that the material responsible for loss of transformation in hydrolyzed urine is larger than 100 kDa and smaller than 0.22 μm. This is in contrast to RNA, which undergoes base-catalyzed transesterification reactions that are driven by soluble species in hydrolyzed urine, such as $NH_3$ and $OH^-$ (Decrey et al. 2014).

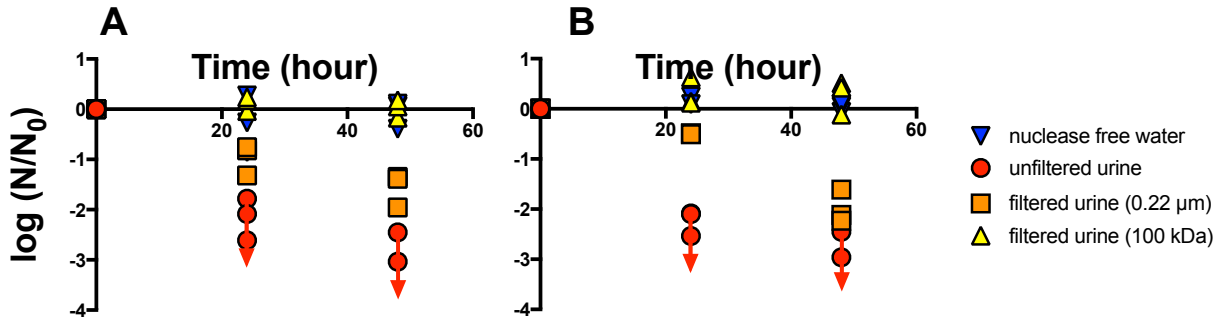


**Figure 4-4. Transformation efficiency for pWH1266 in *A. baylyi* conferring A) tetracycline resistance and (B) ampicillin resistance. The number of colonies on the selective agar plates (i.e. transformants) was normalized by the total number of viable cells culturable on nonselective LB media (transformation efficiency). Transformation efficiency over time (N) is**

**normalized to the transformation efficiency at the beginning of the experiment ($N_0$). Experiments were conducted in nuclease free water control, unfiltered urine, hydrolyzed urine filtered through 0.22 μm pore filters, and hydrolyzed urine filtered through 100 kDa pore filters. Transformation efficiencies at levels below assay detection limits for unfiltered urine are depicted with red arrows.**

In soil, plasmid DNA degradation and the loss of transformation ability has been attributed to microorganisms (Romanowski et al. 1991). Bacteria, for example, can metabolize DNA for microbial growth (Finkel & Kolter 2001) and produce enzymes that disrupt DNA (Priest 1977; Flenker et al. 2017). Here, removing material greater than 0.22 μm did have a significant impact on the inactivation of the plasmid over time. Although most bacteria are larger than 0.22 μm, some are small enough to pass through 0.22 μm pores (Wang et al. 2007). Extracellular nucleases are naturally excreted from bacteria and can exist after the bacteria are removed (Flenker et al. 2017). Nuclease enzymes can range in size (~20 kDa up to 400 kDa) (Xu & Schildkraut 1991; Boyer 1971; Arber & Linn 1969), structure, and function (Yang 2011). They are also heat sensitive, and lose their ability to cut nucleic acids at elevated temperatures.

To assess the potential role of small bacteria or extracellular enzymes in the hydrolyzed urine, we heated the filtered (0.22 μm) urine to 75 °C for 10 minutes and evaluated the impact this had on the inactivation of plasmid DNA. This temperature treatment should inactivate any remaining bacteria, and nuclease activity (e.g., BamHI, HindIII, EcoRI, PstI, DNase I, etc.) is irreversibly inhibited when the enzymes are heated above 60 °C (Pohl et al. 1982).

Heating and then cooling the filtered hydrolyzed urine prior to adding the plasmids had a significant impact on the plasmid transformation. Specifically, no significant decrease of transformation efficiencies was detected after incubation in the heated and cooled urine (Figure 4-5) for 24 hours. Without heating, the transformation efficiencies were below the detection limit after 24 hours. Due to the observed impacts of heating and filtering the hydrolyzed urine, we conclude that the inactivation of the plasmid DNA in hydrolyzed urine is microbially driven. The processes could be due to either intact bacterial cells that pass through 0.22 μm pores (Wang et al.

2007), or due to extracellular nucleases that are greater than 100 kDa. Extracellular enzymatic activity has been hypothesized for impacting plasmid DNA transformations elsewhere (Romanowski et al. 1991; Chang et al. 2017).

We do note differences in kinetics of loss of transformation in filtered (0.22 µm) urine samples (Figure 4-4 and Figure 4-5). Transformation efficiency was below the detection limit after 24 hours in one sample (Figure 4-5) but was still detectable after 24 hours in urine samples used in previous experiments (Figure 4-4). We observed similar variability in the inactivation kinetics of polyomavirus in different urine samples (Goetsch et al. 2018) and hypothesized that it was due to differences in the microbial communities (Lahr et al. 2016).

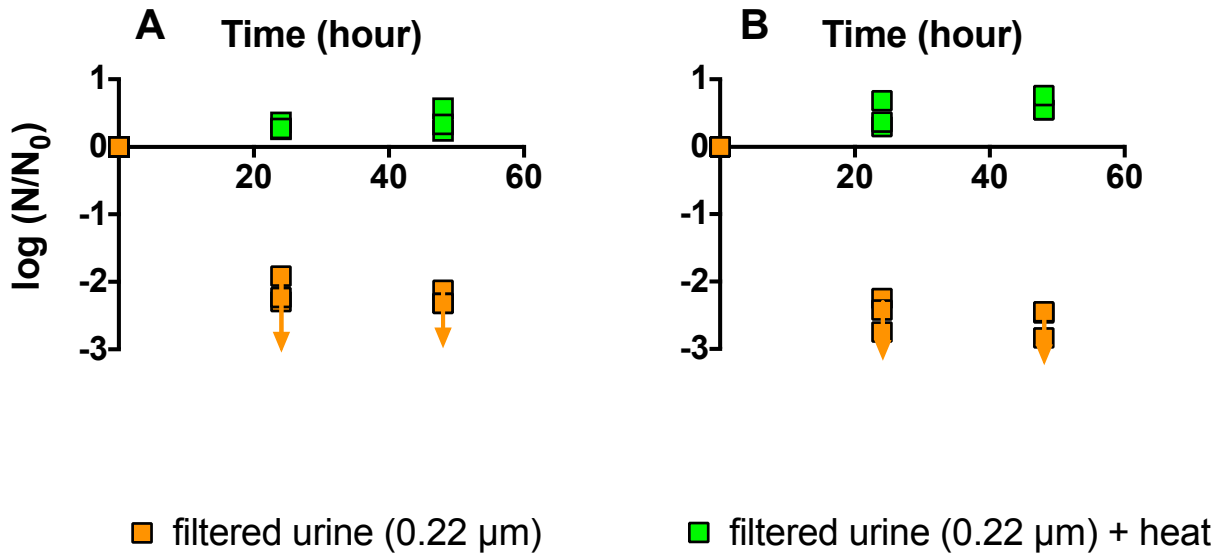


**Figure 4-5. Transformation efficiency for pWH1266 in filtered urine (0.22 µm) with and without heat treatment. Transformants were counted on tetracycline (10 ug/mL) (A) and ampicillin (100 ug/mL) (B) plates and normalized to total viable cell counts on LB media. Triplicate experiments are shown (n=3). Transformation efficiency below detection for unfiltered urine is depicted as orange arrows.**

### 4.3.5 Plasmid DNA conformation, integrity, and stability in filtered and pasteurized urine

We next sought to assess plasmid conformation, integrity, and gene concentration after incubation in the filtered urine and the filtered urine that was also heated. Both tetracycline and ampicillin resistance genes concentrations decrease over time in filtered urine, but the gene

concentrations remained stable in urine that was filtered and heated (Figure 4-7). Gel electrophoresis of samples in the filtered urine show smearing of the plasmid band following 24 and 48 hours of incubation (Figure 4-6). Furthermore, the supercoiled plasmid band at 5 kbps disappeared after 24 hours of incubation in hydrolyzed urine. In the heated samples, the supercoiled plasmid band was stable following 48 hours of incubation and smearing was not observed following either 24 hours or 48 hours of incubation. These results suggest the plasmid is fragmenting over time, as noted through a decrease in signal in the gel and a decrease in gene copy concentrations through qPCR. The smearing observed in the gel suggests that the plasmid DNA is cut in several locations on the plasmid; if the DNA were cut in only one or a few locations, one would observe clear bands with specific sizes. Plasmids were stable in urine that had been both filtered and heated. These results further demonstrate that heat sensitive microbial material is responsible for plasmid inactivation.

It is worth noting that the qPCR and transformation results in the second round of experiments (Figure 4-5 and Figure 4-7) differed from the first round of transformation in the unfiltered samples (Figure 4-1 and Figure 4-2). Specifically, in the first experiment, the gene copy concentrations in unfiltered urine did not decrease over 24 hours but the transformation efficiency decreased by 99% (In Figure 4-2). In the second round of experiments, the gene copy concentrations did decrease significantly after 48 hours of incubation in the hydrolyzed urine that had been filtered (0.22 μm). We propose that this is due to the greater loss in transformation efficiency observed in the second round of experiments, where the assay detection limits were reached after 24 hours.

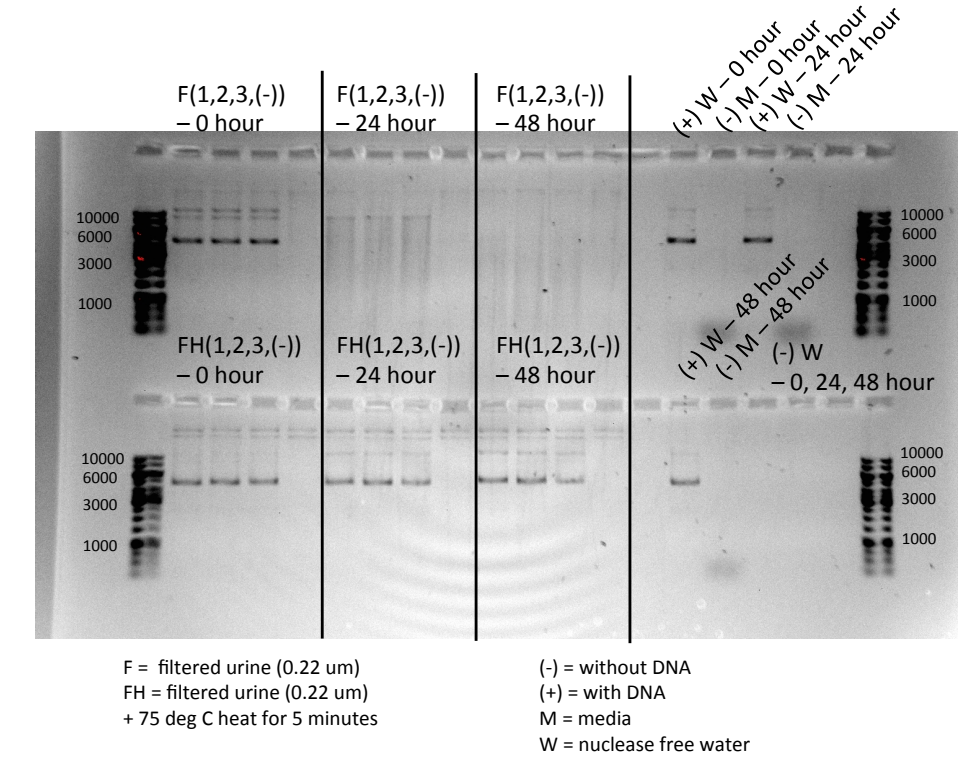


**Figure 4-6. Gel electrophoresis of filtered and heat denatured samples with pWH1266. The plasmid remains supercoiled over time in filtered and heated urine (FH, bottom row), as the band remains ~5000 bp for 48 hours. After 24 hours in filtered urine (F, top row) the bands start to smear, while the darkest band remains at ~9800 bp, the size of the plasmid.**

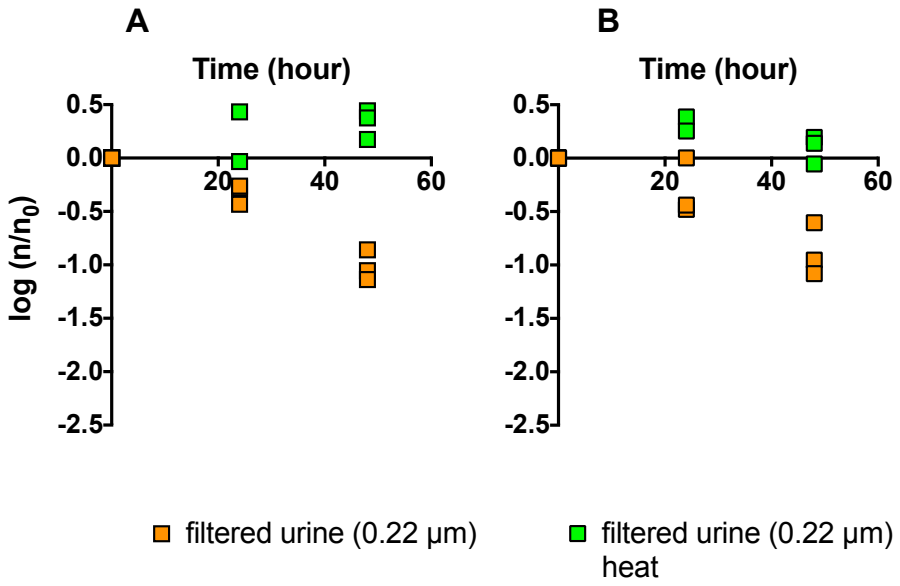


**Figure 4-7. Normalized gene copy levels for the tetracycline (*tetA*) and ampicillin (*blaTEM-1*) genes of pWH1266 in filtered (0.22 µm) urine with (green squares) and without heat treatment (orange squares) Gene copy levels are normalized to the initial gene copy concentrations at time 0. Triplicate experiments are shown (n=3).**

### 4.3.6 Summary and conclusions

We have evaluated the fate of plasmid DNA in stored hydrolyzed urine, as urine storage for long periods of time is a simple way to convert urine into fertilizer (WHO 2006). The resulting high ammonia and high pH environment in stored urine is known to have a biocidal impact on some bacteria (Chandran et al. 2009; Warren 1962; Schonning & Stenstrom 2004), reducing the risk of these bacteria to the applied environment. However, whether this biocidal impact applies to plasmid DNA had not been evaluated prior to this study. Based on the above results, if extracellular antibiotic resistance genes are released in hydrolyzed urine, they will likely lose their ability to transform new bacterial species. We also determined that the loss of transformation was microbially driven.

In light of these results, it is important to note the limitations of the study. We employed the model *A. baylyi* plasmid system to track the loss of natural transformation efficiency of plasmid DNA. It is possible that other extracellular DNA may retain its transformation ability and that other bacteria may have different tolerances for the modified plasmids. In this work, we assumed that the polymerase enzyme employed in qPCR detected all important DNA modifications at the primer-binding site and within the amplicon. This study focused on the horizontal gene transfer mechanism of transformation, yet transduction via bacteriophages may also occur in hydrolyzed urine, and future work is required to understand how microbial activity impacts transduction. While we only evaluated storage of urine as a treatment, pasteurization through heating is another common method used to create fertilizer (WHO 2006), and further research is needed to understand how heating impacts the ability of extracellular DNA in hydrolyzed urine to transform bacteria.

Future research should seek to identify the specific organisms and enzymes that degrade nucleic acids in hydrolyzed urine and other environments. Our previous work suggests that bacterial communities shift as urine hydrolyzes and is subsequently stored (Lahr et al. 2016). The bacterial communities in source-separated urine samples from different sources are quite diverse, but tend to converge after several weeks of storage. Hydrolyzed urine can be considered an extreme environment for bacteria, given its high pH, ammonia concentration, and salinity. Extremophiles in other environments are the source of unique enzymes, although most of the detected enzymes are proteases, dehydrogenases, and cellulases and do not necessarily degrade DNA (DasSarma & DasSarma 2015; Van den Burg 2003). Our model system of plasmid DNA in hydrolyzed urine suggests that heat sensitive, large enzymes (>100 kDa), or small bacteria are responsible for inactivating plasmids, and that inactivation occurs within the same timeframe as plasmid linearization based on gel electrophoresis results. Linearizing plasmid DNA reduced transformation to competent bacteria, reducing the spread of antibiotic resistance from plasmid DNA.

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# Chapter 5.
# Toward a virus exposure assessment from urine-diverting and conventional toilet flushing

## 5.1 Introduction

Viruses can be transmitted in a number of ways, including direct contact of an infected person, direct ingestion of contaminated food, inhalation of aerosols, or touching contaminated surfaces such as door handles, toilet flush handles, telephones, etc. (Gallimore et al. 2008). Toilet flushing is suggested to be the most common process for generating infectious aerosols and droplets that can be harmful to human health (Darlow and Bale 1959). Several studies have focused on the potential spread of aerosol contamination of surfaces after flushing toilets from enteric viruses and bacteria. Spiked bacteriophage MS2 and *E. coli* adsorbed to the surface of the toilet bowl and remained infectious in the toilet bowl after subsequent flushing (Gerba, Wallis, and Melnick 1975). The same phenomenon was observed with other organisms like *Serratia marcesens* (Barker and Jones 2005). Droplets produced by flushing can harbor MS2 and *E. coli* as detected by presoaked gauze stretched over the toilet seat (Gerba, Wallis, and Melnick 1975). *S. marcescens* was also detected on agar-filled settle plates arranged on the floor outside different types of toilets (cistern-fed, gravity-flow, and mains-fed pressure-valve) indicating flushing of different toilets produce bacteria filled droplets (Jessen 1955). These studies confirm that microorganisms persist on the toilet bowl surface and in the bowl water and can be disseminated into the air by further flushes.

Most toilet flushing virus exposure studies focus on potential risk from enteric viruses that are excreted in feces; however, a number of viruses can infect the urinary system and are excreted in urine. Polyomaviruses, for example, infect the urinary tract (Monini et al. 1995) and can be excreted in urine (Urbano et al. 2016; Antonsson et al. 2010). This non-enveloped, double-stranded DNA (dsDNA) virus readily infects a large percentage of the public asymptomatically (Dorries 1998), but can cause severe diseases in immune-compromised individuals (Sílvia Bofill-Mas, Pina, and Girones 2000). Even for individuals who do not exhibit symptoms, polyomaviruses BK and JC can be present in urine at high gene copy levels (100-$10^4$ BK gene copies per mL of urine; $10^4$-$10^7$ JC gene copies per mL of urine) (Urbano et al. 2016). It is not fully understood how these polyomaviruses are transmitted. Due to the potential role that urine exposure could play in viral transmission (Silvia Bofill-Mas 2017), polyomavirus exposure from toilets should be better understood.

Although usually treated as a waste, urine can be separated from the rest of the waste stream and converted to fertilizers. Indeed, the majority of nitrogen and phosphorus found in municipal wastewater comes from urine (Larsen and Gujer 2001; Otterpohl 2002; Maurer, Pronk, and Larsen 2006; Lienert, Bürki, and Escher 2007; Meinzinger and Oldenburg 2009). Recovering nitrogen and phosphorus from urine can offset some of the intensive energy used in synthetic fertilizer production processes (Maurer, Schwegler, and Larsen 2003) and also reduce the required nutrient removal at wastewater treatment plants (Wilsenach and van Loosdrecht 2006; Jimenez et al. 2015). If not removed from municipal wastewaters, nutrients can end up in waterways where they can cause harmful algal blooms and eutrophication (Smith, Tilman, and Nekola 1998). Separating urine from the wastewater streams and using it as fertilizer introduces a number of risks, however, including risks associated with the pathogens and pharmaceuticals in

the source-separated urine. Thus far, studies evaluating risks associated with source-separated urine have primarily focused on risks encountered downstream of urine collection (e.g., urine treatment and food fertilized with urine) and mitigating these risks through treatment processes (Höglund, Stenström, and Ashbolt 2002; Decrey et al. 2011; Bischel et al. 2015). However, we have not found published studies on risks associated with the collection of urine, such as the use of source-separating toilets.

Urine can be collected from source-separating toilets that have different plumbing than traditional toilets. For example, the Wostman EcoFlush and WB Dubbletten are urine-diverting toilets that can connect to existing sewer plumbing but also collect urine. These toilets have a separator in the main bowl of the toilet that collects urine in the front portion and collects feces and toilet paper in the back portion. The front bowl is plumbed to collect urine, while the back bowl connects to the sewer like conventional toilets. Furthermore, the flushes of these toilets involve different volumes of water; in fact, some involve no flushing at all following a urinating event. Due to the different designs and flushing characteristics, the exposure of toilet users to viruses while flushing may be different between conventional toilets and source-separating toilets. Given the importance that flushing plays in the generation of aerosols, this is an important consideration when evaluating the risks associated with urine diversion.

In this study, we examine the release of viruses from urine-diverting toilets and conventional toilets when flushed. We employed single-stranded RNA (ssRNA) and dsDNA surrogate viruses to represent enteric and urinary viruses and measured the amount of infectious particles in droplets that are released with flushing using settling plates. To better understand how urinary viruses are transmitted via toilet flushing, we validated a swabbing protocol that has been used elsewhere to measure endogenous polyomavirus in university bathroom surfaces

(Julian et al. 2011). We ultimately used this experimental data, along with information in the literature to estimate urinary virus exposures from toilet flushing.

## 5.2 Materials and Methods

### 5.2.1 Toilets

A conventional flush toilet and a source-separating urine-diverting toilet were used in these experiments. The conventional flush toilet (Kohler model 4330-0) was installed in 2016 and has both a low flush (4.83 L per flush) and high flush (6.1 L per flush) option. The urine-diverting toilet (Wostman Ecoflush) was installed in 2016 and has a 2.5 L ("large") flush option. While this toilet is not designed to need a flush with a urinating event, the facility it is currently installed in requires toilet paper to be flushed rather than disposed in garbage cans in stalls. Thus, every urinating event results in a flush. While most of the water goes down the back of the toilet, we determined that 380 mL of flush water enters the small bowl and is thus collected with the urine after each urinating event. The tested toilets were located in two different female restrooms in a university building. Sodium thiosulfate (Fisher Scientific) (10,000 mg/L) was used to quench residual chloramine in the toilet bowls before virus addition. Total combined chlorine readings (Hach) were measured before and after quenching to ensure chloramine concentrations were below the detection limit and thus not inactivating the added virus.

### 5.2.2 Virus surrogates and their hosts

Bacteriophages MS2 and T3 were used in flushing experiments to represent nonenveloped, ssRNA and dsDNA viruses present in urine and feces. MS2 is an *E.coli* nonenveloped ssRNA bacteriophage (3.6 kbps, 25 nm) and is often used as a surrogate for ssRNA enteric viruses (e.g., poliovirus, norovirus). T3 is an *E.coli* nonenveloped dsDNA

bacteriophage (38.2 kbps, 50 by 20 nm). In this case we are using this dsDNA bacteriophage as a surrogate for enteric and urinary tract viruses that contain dsDNA (e.g., adenovirus, polyomavirus). MS2 and T3 were propagated in their *E.coli* hosts (ATCC 15597 and 11303). They were extracted using a chloroform extraction method (USEPA 2001) and purified with an Econo fast protein liquid chromatography system (Bio-Rad, USA), with a HiPrep Sephracyl S-400 column (GE, USA). The purified virus fraction was concentrated using a 100 kDa Amicon ultracentrifugal filter and was filter sterilized with 0.22 μm polyethersulfone (PES) membrane filters (Millipore, USA). The final MS2 and T3 stocks ($10^{11}$ plaque forming unit (pfu) /mL and $10^{10}$ pfu/mL) were stored in phosphate buffer (5 mM $NaH_2PO_4$ and 10 mM NaCl, pH 7.5) at 4 °C.

BK polyomavirus was used to validate a swabbing protocol published previously for detecting bacteriophage MS2 and MS2 RNA on polyvinyl chloride (PVC) plastic and stainless steel surfaces (Julian et al. 2011). The culturable BK polyomavirus Dunlop variant is a genetic variant to endogenous polyomavirus that can be propagated at a high titer. It was propagated and purified using previously published methods (Broekema and Imperiale 2012; Jiang et al. 2009). Briefly, polyomavirus was propagated in 293TT cells. After cells were grown to 70% confluence, they were infected with BKPyV crude lysate at a multiplicity of infection (MOI) of 0.1 infectious unit per cell, and incubated at 37 °C for 10 days. Virus lysates were purified over density CsCl gradient centrifugation, and the collected virus fraction was dialyzed overnight in buffer (10 mM HEPES, 1 mM $CaCl_2$ , 1 mM $MgCl_2$, 5 mM KCl, pH 7.9).

### 5.2.3 Virus exposure from toilet flushing experiment

Bacteriophages MS2 and T3 were added to toilet bowl reservoirs to quantify the viruses that are released in droplets by flushing. Viruses were diluted in phosphate buffer (5 mM

$NaH_2PO_4$ and 10 mM NaCl, pH 7.5), resulting in a final concentration of $10^9$ pfu/mL (MS2) and $10^8$ pfu/mL (T3). Bacteriophages MS2 ($10^{10}$ pfu) and T3 ($10^9$ pfu) were added to each toilet at levels similar to what can be shed in infectious diarrhea (*e.g.*, $10^{10} – 10^{11}$ infectious enteric viruses (norovirus, rotavirus, and adenovirus) per gram of feces) (C. Haas, Rose, and Gerba 2014). This virus solution was added to the liquid containing part of the toilet bowl to mimic the typical use for a conventional flush toilet and as a worst-case scenario of use of the urine diverting toilets (Figure C-1 and Figure C-2). After approximately 1 minute, the toilet was flushed (6.1 L conventional toilet flush and 2.5 L urine-diverting toilet flush).

Samples of the bowl water were taken before adding virus to the toilet, immediately after adding virus to the toilet, one minute after flushing, and five minutes after flushing. Petri dishes with 10 mL of phosphate buffer (5 mM $NaH_2PO_4$ and 10 mM NaCl, pH 7.5) were placed around the toilet in six different locations (on the floor: left, front, right, front left, and front right; and back of the toilet). A schematic of the location of the petri dishes is provided in Figure C-1 and Figure C-2. The dishes on the floor were located 20-30 cm from the base of the toilet. The dishes on the backside of the toilet were located at the same elevation of the toilet seat. For the urine-diverting toilet, the dishes were taped on top of the back of the seat. For the conventional toilet, the dishes were 3-6 cm from the edge of the toilet seat. Two sets of petri dishes for each location were placed to capture droplets that spend different amounts of time in the air. The first set of petri dishes were covered after 1 minute post flushing; the second set of petri dishes were covered after 5 minutes post flushing. At the conclusion of the experiment, liquid was collected from the petri dishes and pipetted into 15 mL falcon tubes. The tubes were placed on ice, and plaque assays were completed within 30 minutes. Two sets of petri dishes with 10 mL of phosphate buffer were also placed on the floor of the adjacent stall (55-60 cm from the base of

the flushed toilet) and on the sink surfaces in the bathroom as negative controls and covered at the same time intervals as the experimental dishes.

### 5.2.4 Virus plaque assay

Bacteriophages were enumerated by the double-layer agar plaque assay (USEPA 2001). In brief, 100 µL of the recovered flushing experiment solution was combined with 100 µl of an overnight culture of the bacteriophage's respective host *E.coli* and 5 mL of soft agar media. Plaques of MS2 and T3 were enumerated after overnight incubation at 37 °C.

### 5.2.5 Virus swabbing recovery experiments

Virus swabbing experiments were based on a method developed previously for recovering MS2 on PVC and stainless steel surfaces (Julian et al. 2011). To first validate the swabbing method for our purposes, purified BK polyomavirus Dunlop strain (BKPyV) was added to surfaces and then recovered. In these experiments, two ~10 cm x ~10 cm squares were outlined with tape on stainless steel and black epoxy coated silica lab bench surfaces to recover added BKPyV (positive) and nuclease free water (negative). The surfaces were cleaned with ethanol, 10% bleach, and MilliQ water prior to adding virus solution and nuclease free water. The virus was diluted in nuclease free water to a final concentration of $10^{8.2}$ gene copies/mL. 0.7 mL of virus solution and 0.7 mL of nuclease free water was added to each square. The virus solutions and nuclease free water dried for several hours, until all visible droplets had disappeared. The surface was swabbed with sterile polyester-tipped swabs (BD Company) that had been wetted in 1.5 mL of phosphate buffer solution (pH 7.4) (Gibco). Phosphate buffer solution was selected for recovering the virus based on successful virus recoveries reported elsewhere (Julian et al. 2011; Foulongne et al. 2011). Constant and consistent pressure was applied to the surface, sweeping back and forth, up and down, and diagonal across the surface for

20-30 seconds total. The swabs were then placed in a 15 mL falcon tube with 1.5 mL of phosphate buffer solution (pH 7.4) and capped. The 15 mL tubes were then vortexed for 1 minute. Swabs were removed, and 300 µL aliquots were used for viral DNA extraction.

### 5.2.6 Viral DNA extraction

Viral DNA extraction was completed using Maxwell total viral nucleic acid extraction kits (Promega) with a Maxwell 16 instrument (Promega). 100-300 µL of sample were used for extractions. Extracted samples were measured for polyomavirus using qPCR.

### 5.2.7 qPCR reactions

Polyomavirus was quantified using established qPCR reactions. Primers (5' to 3') specific for endogenous BKPyV and the Dunlop strain were selected (152 bp; forward, AAGGAAAGGCTGGATTCTG; reverse, TGTGATTGGGATTCAGTGCT) (Bennett et al. 2015). The 10 µL reaction mixtures included 5 µL 2X Biotium Fast EvaGreen master mix, 0.5 µM primers, 0.625 mg/mL bovine serum albumin (BSA), nuclease free water, and 1 µL of DNA template (0.5 ng to 5 ng). Temperature conditions consisted of initial denaturation at 95 °C for 2 minutes, 40 cycles of denaturation at 95 °C for 5 seconds, annealing at 56 °C for 5 seconds, and extension at 72°C for 25 seconds. Standard curves were prepared between $10^3$ and $10^8$ gene copies per mL. All efficiencies were greater than 93%, and $R^2$ values were greater than 0.994. The qPCR method quantification limit (MQL) was determined by measuring at least 7 replicates of diluted standards ($10^3$ – $10^1$ gene copies/mL) and determining the relative standard deviation. The lowest standard with a relative standard deviation below 25% was considered the method quantification limit.

### 5.2.8 Comparative exposure assessment parameters and estimation

An exposure assessment was conducted to estimate the exposure of enteric and urinary tract viruses from conventional flush toilets. Literature based parameters were used to estimate polyomavirus and norovirus exposures and were combined with the fraction recovered from the virus exposure experiment. First, the numbers of urination and defecation events occurring at a workplace were estimated. Based on four of five total flushes attributed to urination and one for defecation, assuming three out of those five total flushes occurring at work, and interpolating the 4:1 flush ratio, and we assume a 2:0.85 urination to defecation ratio per person (Wilsenach and van Loosdrecht 2004; Berndtsson 2006). Polyomavirus is a urinary tract virus that can be present in urine of healthy individuals between 100-$10^4$ gene copies per mL of urine (Urbano et al. 2016); we used $10^4$ gene copies per mL of urine in our calculations. Enteric viruses (norovirus, adenovirus, rotavirus) can be found in stool samples at $10^{10}$ infectious units per gram of stool (C. Haas, Rose, and Gerba 2014). Urinating and defecation volumes and masses were estimated using literature values. It was assumed that a person will urinate ~250 mL during each event (Rauch et al. 2003; Fewless, Sharvelle, and Roesner 2011; WHO 2006). For defecation events, 32 grams per capita per day was assumed (Rose et al. 2015).

Based on these assumptions, we calculated the expected amount of deposited viruses in terms of gene copies or infectious units per flushing event. We also use the virus fraction recovered from the virus exposure experiment to estimate exposure of urinary tract viruses from flushing and compare this to the polyomavirus swabbing results.

### 5.2.9 Data Analysis

Student t-tests were used to determine significance of plaques detected outside the toilet from the virus exposure experiment. Plaques detected outside the conventional and urine-

diverting toilets were compared; plaques detected at different locations around the toilets were compared; and plaques detected at different collection times were also compared. *P* values were evaluated at the 95% confidence interval.

### 5.3 Results and Discussion

#### 5.3.1 Virus droplets outside flushing toilet

Both bacteriophages MS2 and T3 were detected in petri dishes after flushing the conventional toilet (Figure 5-1). Specifically, an average of 5600 total MS2 pfu were detected outside the toilet, whereas $10^{10}$ pfu were added to the toilets. For T3, a total of 1700 pfu were detected on average in the plates outside the toilet, whereas $10^{9}$ pfu were added to the toilet bowls. The fraction of the viruses recovered outside of the toilet after flushing was calculated for MS2 ($5.6 \times 10^{-7}$) and T3 ($1.7 \times 10^{-6}$) (calculations in section C.1). Other studies using MS2 for similar flushing exposure assessments observed varying recoveries of virus-laden droplets (Gerba, Wallis, and Melnick 1975; Barker and Jones 2005). For example, Barker and Jones measured MS2 on semi-solid agar plates placed on the floor around the toilet, the toilet seat, and the cistern and a shelf at the back of the toilet. The average total number of plaques detected was 365 pfu when $10^{10}$ pfu were added to the toilets, which shows a recovery (fraction recovered was $3.7 \times 10^{-8}$) one to two orders of magnitude smaller than what we observed (Barker and Jones 2005). Differences in these outcomes could be attributed to methodological variations (liquid buffer instead of agar to capture virus), the number of plates used to measure droplets outside of the toilet, and the flushing velocity of the different types of toilets. Gerba et al. observed fractions closer to our observed values, but they measured MS2 from flushed droplets by applying gauze over the top of the toilet to capture a larger fraction of what could be dispersed

(Gerba, Wallis, and Melnick 1975). When $10^{10}$ MS2 pfu was added to the toilet and then flushed, ~$10^{3}$ pfu could be recovered in the gauze, a value similar to our MS2 results.

Few significant differences across locations around the conventional toilet were observed. Using student T-tests to compare counted plaques at different locations, the only statistical difference was detected between the front and back plates for T3 ($p$ = 0.0497) and between the front and right front plates for T3 ($p$ = 0.0427). All other locations detecting T3 and all of the locations outside the toilet detecting MS2 were statistically similar ($p$ values located in the Table C-1) indicating that viral deposition was generally equal around the toilet. No statistical difference was observed between plaques collected after 1 minute or 5 minutes post flushing which may be in part due to the method of where the plates were placed.

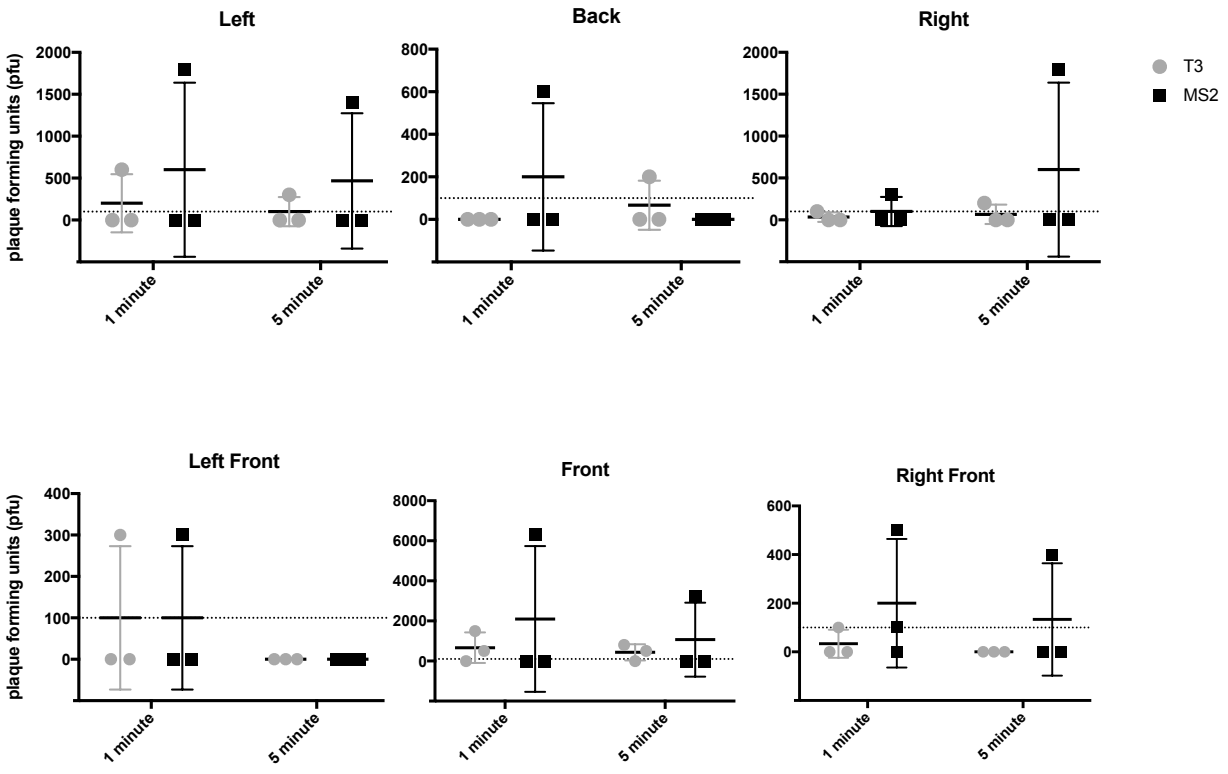


**Figure 5-1. Plaque forming units (pfu) collected from 10 mL of buffer in petri dishes collected 1 minute and 5 minutes around a conventional toilet after flushing. Gray circles indicate T3 pfu recovered; black squares indicate MS2 pfu.**

**Median values are shown and error bars report standard deviation for three replicate experiments. The limit of quantification (100 plaque forming units) is shown on each graph.**

Fewer virus plaques were detected around the urine-diverting toilet than the conventional flush toilet. In fact, plaques were detected in only one plate along the backside of the urine-diverting toilet, with 200 MS2 pfu detected five minutes post flushing when $10^{10}$ pfu were added to the toilets (Figure 5-2). Thus the average fraction of MS2 recovered from the three experiments was $6.7 \times 10^{-9}$ pfu/pfu (MS2). For all other conditions, no plaques were detected in all three replicate flushing experiments of the urine-diverting toilet (Figure 5-2). Because $10^{9}$ T3 pfu was added to the toilets, we estimate the fraction of recovered T3 to be $< 3.3 \times 10^{-8}$ pfu/pfu (section C.1). Using student T-tests comparing the sum of all plaques counted outside the conventional toilet and the urine-diverting toilet, there is a significantly higher plaque count outside the conventional toilet compared to the urine-diverting toilet ($p = 0.0073$ (T3) and $p = 0.0267$ (MS2)). Control samples, which consisted of petri dishes placed on the floor of the next stall over and on a neighboring sink, contained no culturable bacteriophages following the conventional and urine-diverting toilet flushing experiments (data not shown).

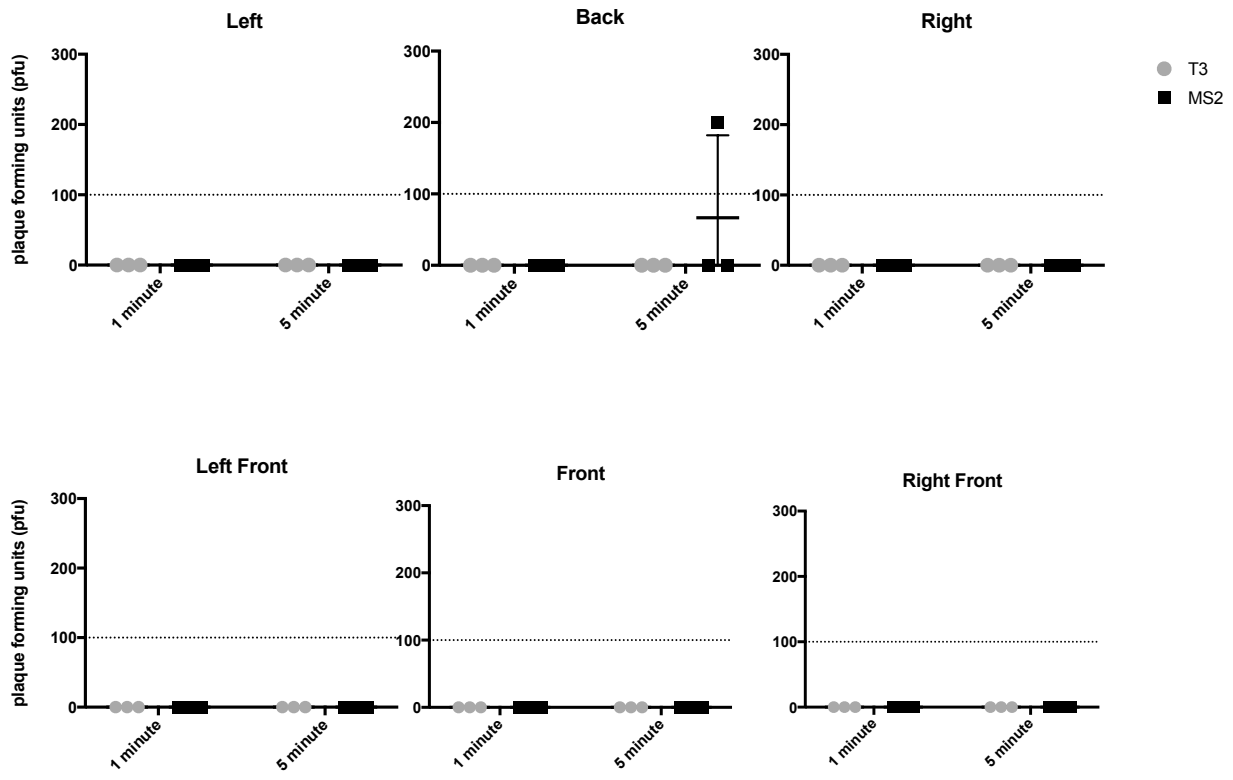


**Figure 5-2. Plaque forming units (pfu) collected from 10 mL of buffer in petri dishes collected 1 minute and 5 minutes around a urine diverting toilet after flushing. Gray circles indicate T3 pfu recovered; black squares indicate MS2 pfu. Median values are shown, and error bars report standard deviation for three replicate experiments. The limit of quantification (100 plaque forming units) is shown on each graph.**

Toilet flushing has been considered an important infection hazard since droplets and aerosols of infectious particles can land outside of the toilet (Gerba, Wallis, and Melnick 1975; Darlow and Bale 1959; Hutchinson 1956). While future work must investigate aerosolized viruses, our data demonstrates that the specifics of the flush will impact the amount of virus-laden droplets that are released from the toilet. Specifically, the urine diverting toilet, even when flushed, results in less virus-laden droplets; consequently, the exposure via contact with pathogenic viruses on surfaces is likely less. In this experiment, we added virus to the toilet bowl reservoirs. When urine-diverting toilets are used appropriately, the urine is released to the front of the toilet, where there is no reservoir, and drains before the toilet is flushed. Feces are deposited in the back of the toilet into the reservoir and are released down the drain when the

toilet is flushed. This experiment provides a worst-case scenario for determining viruses released from urine-diverting toilets, when both urine and feces are deposited in the back of the toilet. In other words, these results suggest that an added benefit to using source-separating technologies is a lower exposure to viruses from flushing. This is particularly important in areas of sensitive populations such as hospitals, where opportunistic urinary tract infections might be prevalent.

### 5.3.2 Comparative exposure assessment of enteric and urinary tract viruses in conventional toilets

As described above, people use toilets for urination more frequently than they use toilets for defecation. We therefore estimated the exposure loads released from a single toilet for a urinary tract virus versus a fecal-borne virus. We used literature values to calculate the expected exposure during each type of flushing event with the assumption of worst-case concentrations of viruses in urine and feces separately. Thus, for each urinating event of 250 mL volume of urine and $10^4$ gene copies of polyomavirus per mL of urine, we estimate 2.5 x $10^6$ gene copies per person per flush is entering the toilet (calculation in section C.2). We did the same calculation for enteric viruses and defecating events to estimate enteric viruses entering the toilet from one person (i.e., 0.85 flushes for defecation per day, multiplied by the $10^9$ infectious particles per gram of stool, multiplied by the 32 grams of stool per flushing event) and estimated 2.7 x $10^{10}$ infectious particles per flushing event (calculation in section C.2). These potential exposures were then multiplied by the fraction of exposures determined from the spiked bacteriophage experiment according to the genome type (T3 for dsDNA polyomavirus and MS2 for ssRNA enteric virus). Even if accounting for more frequent flushes for urinating events (we assumed two flushes per day in this calculation), we predict that enteric viruses could be found outside the conventional flush toilet 3 orders of magnitude higher than urinary viruses. Of course not every

fecal event results in levels near $10^9$ infectious units per gram of stool. When we assume a higher polyomavirus concentration of infected individuals being excreted ($10^{10}$ gene copies/mL of urine) (Randhawa et al. 2004), urinary viruses are found 3 orders of magnitude higher than enteric viruses. Thus, our assumptions in this calculation of worst case-scenarios, how often a diarrheal event may occur, and that gene copies are equivalent to infectious units are important when making predictions for urinary virus fate in bathrooms.

### 5.3.3 Polyomavirus swabbing of surfaces

We next sought to measure actual urinary viruses on bathroom surfaces. A viable method to recover polyomavirus was first verified by spiking a lab variant of BK polyomavirus (Dunlop) to two lab surfaces (stainless steel and epoxy-lined silica lab bench surfaces), air-drying for several hours, swabbing using PBS buffer, and analyzing by qPCR. Of the $10^8$ gene copies of polyomaviruses added to the surfaces, 47 ± 25% were recovered from the stainless steel surface and 51± 7.2% were recovered from the black epoxy coated silica surface. This particular swabbing method recovered 40% of infectious MS2 and 7% of MS2 RNA in a previous report (Julian et al. 2011). In that study, the swabbing material (cotton-tipped, polyester-tipped swabs, and antistatic cloth), different eluents (0.85% saline, one-quarter-strength Ringer's solution, and viral transport media), and different surfaces (PVC plastic and stainless steel) were tested for infectious MS2 and MS2 RNA recovery, and the polyester-tipped swabs with saline solution or Ringer's solution swabbing method had the highest recoveries from non-porous fomites (Julian et al. 2011).

Once we established that the swabbing method recovered BK polyomavirus variant, we employed it to monitor endogenous BK polyomaviruses on bathroom surfaces. Specifically, the floor, toilet seat, stall wall, and sink surfaces were swabbed once to measure polyomavirus in

three different university female bathrooms. One of the bathrooms swabbed was the same bathroom where the conventional toilet flushing experiment was conducted. Polyomavirus genes were below the qPCR MQL on the swabbed university bathroom surfaces (qPCR MQL $10^3$ gene copies/mL corresponded to <250 gene copies in the 100 $cm^2$ surface swabbed). It is worth noting that the surfaces were swabbed in bathrooms on a university campus in the summer when university occupancy is low. Consequently, the polyomavirus concentrations on surfaces may not be representative of what is present in a busy, public bathroom with traditional flush toilets. Our ongoing efforts include monitoring for polyomavirus at different times of the year and in different bathrooms with different types of flush toilets.

In an effort to predict the amount of polyomavirus that could be present on surfaces next to a conventional toilet, we assumed a concentration of BK polyomavirus found in urine of healthy individuals ($10^4$ gene copies/mL) and used fractions determined from the virus deposition data experiments with dsDNA T3. Based on these assumptions, we predict ~4 gene copies of virus to land within the area we measured outside the toilet per flush (calculations described in section C.2). Because we detected less than 250 gene copies on a 100 $cm^2$ swabbed surface in the swabbing experiment, we next determined the number of flushes that would need to occur to be able to detect more than 250 gene copies per 100 $cm^2$ swabbed surface. In this calculation we assumed 1 $m^2$ total surface area outside of the toilet within the toilet stall cubicle. Based on these assumptions, we calculate that nearly 6000 flushes are required to be able to detect BK polyomavirus above the qPCR MQL (calculations described in section C.3). This analysis was done using the dsDNA bacteriophage T3 fraction data to predict the fraction of the dsDNA polyomavirus that may be deposited outside the toilet. Of course, not all viruses of similar genome type behave the same way, and we have seen examples of polyomavirus

behaving differently from other dsDNA viruses (Goetsch et al. 2018). Therefore using the fraction of T3 to determine the expected amount of virus outside of the toilet may not apply to polyomaviruses. When we use the deposited fraction determined for MS2, we would predict ~1 gene copy landing outside the toilet per flush, resulting in ~18,000 flushes required to be able to detect polyomavirus outside the toilet. Because it is likely that bathroom surfaces will be cleaned more often than the time required for 6000 flushes to occur, we would hypothesize BK polyomavirus exposure from bathroom surfaces after flushing is low or that our limit of detection is too high to detect this potential exposure route. JC polyomavirus is another polyomavirus found in high concentrations in urine (Urbano et al. 2016) and should also be monitored on bathroom surfaces in future studies. More importantly, to truly evaluate the risk of polyomavirus, future research is required to determine the infectious dose of polyomavirus and if this dose is lower than our method detection limit.

### 5.3.4 Summary and Conclusions

Most research thus far on virus exposure from toilet flushing has focused on enteric virus fate. Given the viral presence in urine, the frequency of urinating events compared to fecal events, and the corresponding number of flushes for urine that could lead to virus-laden droplets, we need to have a better understanding of urinary virus fate after flushing. This work employs a swabbing method to quantify the urinary tract virus polyomavirus on bathroom surfaces. This work also compares the potential exposure of viruses after flushing urine-diverting toilets and conventional toilets. We find that the estimated numbers of ssRNA and dsDNA bacteriophages found outside of a conventional toilet are significantly higher than bacteriophages found outside the urine-diverting toilet, indicating an added benefit of using urine-diverting toilets. Further research is needed to fully determine polyomavirus exposures in bathrooms, the infectious state

of the virus to determine if toilet flushing could be a potential route of transmission, and the potential role aerosolized viruses could play in exposure.

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# Chapter 6.
# Conclusions and Engineering Significance

## 6.1 Overview

This work evaluated the fate of microbial contaminants as unprocessed urine is collected and converted to fertilizer. This research contributes to the broader goals of evaluating the risks of using source-separated urine for fertilizer production. Ultimately, the risks and benefits of source separation can be compared to risks and benefits of conventional toilet use and fertilizer production.

Chapters 3 and 4 of this dissertation focused on the fate of microbial contaminants in source-separated urine after collection and the exposures of these contaminants to people who handle fertilizer or who consume food fertilized with urine-derived fertilizers. We demonstrated that a common urinary tract virus is rapidly inactivated in hydrolyzed urine and that extracellular DNA carrying antibiotic resistance rapidly loses its ability to convey antibiotic resistance to environmental bacteria. In Chapter 5, we focused on another potential interaction between people and urine, namely when the public uses urine-diverting and conventional flush toilets. Exposures between urine-diverting toilets and conventional toilets were compared, and we demonstrated that using urine-diverting toilets leads to less virus-laden droplets being released from the toilet.

There are a number of aspects of this research that set it apart from other work that has been conducted on the exposures associated with the source separation of urine. First, it takes a conservative approach to understanding exposure by integrating both culture-based and molecular-based methods, whereas most research tends to use only qPCR or culturing. The

research employed real urine samples collected for fertilizer production instead of synthetic urine, and multiple urine samples were collected from hundreds of donors with functional urine-diverting toilets rather than a single collection from limited users. This strengthens the research, as the results are more likely representative of other urine samples collected with urine-diverting toilets.

### 6.2 Main findings and significance

One focus of this dissertation research was polyomavirus, a urinary tract virus found in high abundance in urine that has not yet been studied in source-separated urine. Polyomavirus genes were detected for long periods of time in hydrolyzed urine, yet infectivity assays indicated a loss in infectivity within several hours due to the protein capsid breaking apart. The results in this dissertation suggest that microbial agents are responsible for the polyomavirus inactivation. While the role of microbes in virus inactivation has been previously suggested (Decrey & Kohn 2017), the outcomes of this study confirm the importance of understanding factors that inactivate microbes. Perhaps most importantly, the polyomavirus results underscore the limits of using pathogen surrogates to predict the behavior of specific pathogens in the environment. In this case, the bacteriophage T3 surrogate behaved entirely differently than polyomavirus, despite having the same genome type. This study suggests that other characteristics beyond genome type are important to consider when choosing surrogates for environmentally relevant viruses. For polyomavirus, the disulfide bonds found in the polyomavirus capsid, which are generally thought to have a stabilizing effect in virus capsids (Walter & Deppert 1975), could have caused polyomaviruses to have a higher susceptibility to inactivation compared to the surrogate. A range of surrogates and pathogenic viruses with various genome types, genome sizes, envelope

presence/absence, and capsid features should be studied in the future to identify the characteristics that dominate virus fate.

This dissertation research suggests that dsDNA is extremely stable inside the bacteriophage T3 and polyomavirus capsids. Interestingly, naked plasmid DNA lost its ability to be transformed to competent bacteria in hydrolyzed urine within a day and was fragmented due to microbial activity. Beyond the insights to the mechanistic inactivation of extracellular DNA, this work demonstrates that encapsidated DNA is protected from the microbial activity that degrades extracellular DNA, even after the virus had been inactivated.

Both of these studies underscore the importance of method limitations when tracking microbial contaminants. qPCR is useful for measuring gene presence and concentrations in environmental samples, and is especially important when culture-based methods are not available (Allen et al. 2010). Nonetheless, qPCR assays repeatedly overestimated the presence of infectious viruses and transferable antibiotic resistance genes (Luby et al. 2016). In the polyomavirus study, large sections of the genome remained intact for several weeks after the infectivity assays demonstrated that the virus had lost its infectivity. Likewise, antibiotic resistance gene copies measured with qPCR did not correlate to the gene's ability to be picked up by other bacteria, as measured in transformation assays. It is therefore critical to use multiple methods to evaluate the potential risk of exposure or spread of microbial contaminants.

This work suggests that urine-diverting toilets may decrease the exposure of viruses that are released from flushing toilets, thus adding to the benefits of using urine-diverting toilets. This is particularly interesting for the case of urinary tract viruses, due to the fact that urination takes place more frequently in toilets than defecation. The urinary tract virus polyomavirus, for example, infects large percentages of the public asymptomatically, and its transmission is poorly

understood. No polyomaviruses were detected when bathroom surfaces were swabbed and analyzed by qPCR, but this may be due to the low volume of toilet users during the time of sample collection.

### 6.3 Recommendations for Future Research

The results from this dissertation identify a number of additional research questions and directions that should be explored in the future. First, this work focused primarily on urine storage as a means of creating fertilizer. The fate of microbial contaminants will need to be studied with other methods used to make fertilizer, such as acid (Hellström et al. 1999) or base (Randall et al. 2016; Dutta 2012) addition to prevent urea hydrolysis, struvite precipitation, reverse osmosis, distillation, pasteurization, etc. We can hypothesize from this work that in the case of high pH environments, it is likely that viruses will be inactivated and that the enzymes that are likely responsible for virus inactivation and DNA degradation may also be inactivated.

Future work should also explore the microbial processes that drive both polyomavirus inactivation and plasmid DNA transformation loss. Here, we hypothesized that the disulfide bonds present in the polyomavirus capsid were susceptible to microbial activity. Additional research is required to identify the microbial agents that drove capsid disassembly. This information will be useful for predicting the fate of other environmental viruses, especially those that share the disulfide bridge characteristic (e.g., HIV, hepatitis B, and hepatitis C) (Ivanov et al. 2007; Wynne et al. 1999; Blanchard et al. 2002). Enzymatic activity likely plays a role in the degradation of extracellular ARGs in many environments, and future research should identify the organisms and enzymes responsible in different environments, including hydrolyzed urine. Future work should determine how other plasmid DNA-bacteria models are impacted by microbial activity in urine and other environments (Lorenz & Wackernagel 1994).

In terms of the bathroom exposure study, further work is needed to determine if polyomaviruses are present on bathrooms surfaces, and the potential role of toilet flushing on polyomavirus transmission should also be studied in more detail. Whereas this study focused on viruses in droplets from flushing, future work should study the role of aerosols in polyomavirus transmission (Gerba et al. 1975; Jessen 1955; Atmar & Estes 2006; Teunis et al. 2008; Hung et al. 2004; Liu et al. 2004; Booth et al. 2005; Olsen et al. 2003). Finally, for a quantitative microbial risk assessment of polyomavirus and other viruses released from flushing, dose-response relationships must be established for each of the viruses of interest.

Overall, the results presented in this dissertation suggest low microbial risks associated with collecting and storing urine for fertilizer production. Consequently, the research strengthens the arguments for implementing urine diversion to recover nutrients and close the nutrient cycle.

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# Appendices

# Appendix A Supplementary Material for Chapter 3

# Fate of the Urinary Tract Virus BK Human Polyomavirus in Source-Separated Urine

**Table A-1. qPCR temperature conditions and duration**

| **Step** | **No. cycle** | **BKPyV (152bp)** | **BKPyV (900 bp)** |
|---|---|---|---|
| Initial denaturation | | 95° C for 2 minutes | 95° C for 2 minutes |
| Denaturation | 40 x | 95° C for 5 seconds | 95° C for 5 seconds |
| Annealing | | 56° C for 5 seconds | 52.2° C for 5 seconds |
| Extension | | 72° C for 25 seconds | 72° C for 1 minute |
| $R^2$ | | >0.996 | >0.999 |
| Efficiency | | 96-97% | 70% |

**Table A-2. Buffer composition used for polyomavirus inactivation.**

| **Buffer Name** | **pH*** | **$Na_2PO_4$ $2H_2O$** | **$Na_2CO_3$** | **NaCl** | **$NH_4Cl$** |
|---|---|---|---|---|---|
| PC7 | 7 | 60mM | 50mM | 1770mM | |
| PC9 | 9 | 60mM | 50mM | 1770mM | |
| AmPC7 | 7 | 60mM | 50mM | 1770mM | 147mM |
| AmPC9 | 9 | 60mM | 50mM | 1770mM | 147mM |

* adjusted with HCl/NaOH

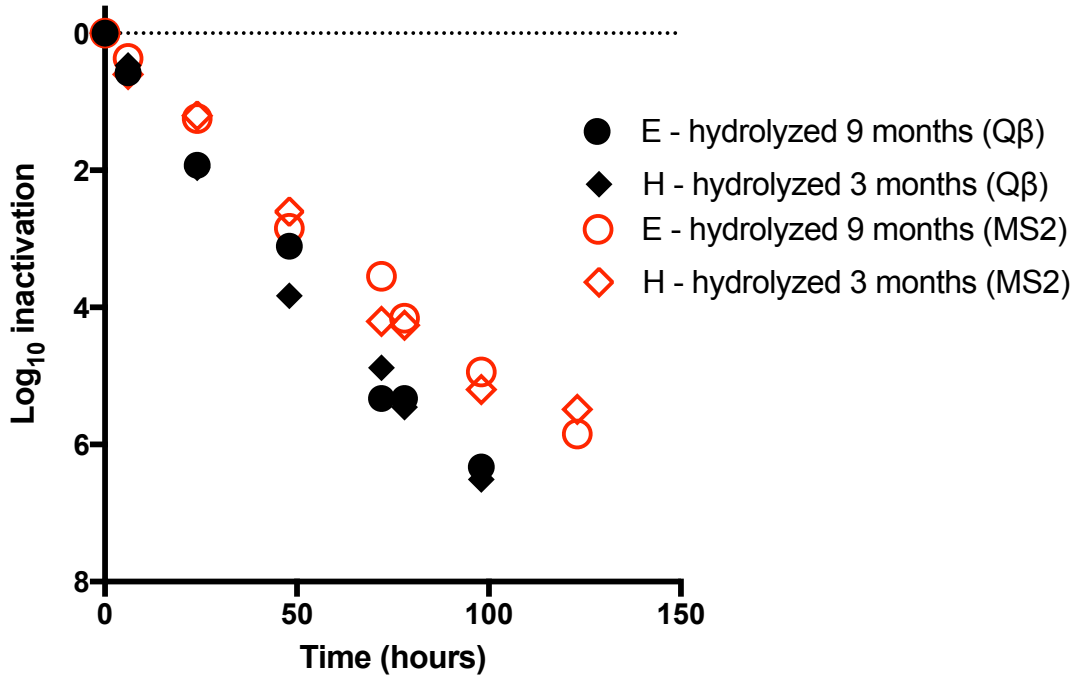


**Figure A-1. Inactivation of Qβ (black) and MS2 (red) in different ages of urines E (n=2) and H (n=2) over time. Virus was added to hydrolyzed urine at $10^8$ – $10^9$ pfu $mL^{-1}$, and infectious virus particles were measured over time**

# Appendix B Supplementary Material for Chapter 4

# A mechanistic study of DNA persistence, conformation changes, and transformation ability in source-separated urine

## Section B.1 Sequence of pWH1277.

pWH1277 (4578 bp) is inserted into pBR322 between the two PvuII restriction sites (CAG'CTC). PvuII is italicized and bolded.

***CAGCTG***CtGCtGtTGCTGCTGTTCAaGTGTCaATTGCTCTACTCTGTTGCTCGTCTCAATG
ATAGACATATCAAGCCTTTCAGCTTTTTTATTGCGCTTTTTGATTTCATCATTACGCTT
GACGATCTCCAGTTCCTCCCCCCTGCGCTTGGCGTGGGTGGCTTCAAGTCCCTCCTTG
AATGTCGGCTCGATGTCTAGTCCACGATCCTTATGACTGCGGTGATCAACTCTCACC
TCAAGCCCTGCTCGCTCTAGGTGGACGTTCGTCAGATCCGCCACTTTTTCCCTGATTT
TTTTCAGCGTTGAGTTCTGATCTAGCTCCCTGACTTTCTTCCCTAGCCCTTGCGGTGT
AAGGCTTCTTGTTGTCATTAGTATGTGGGCGTGATGGTTTCTTTCATCACTTCCCCCA
TGCACATGAGGGGCATGGATCGCTACGTCCACCGCCACTCCATAGGCTTTAACTAAG
GACTGGCACAACTCGGTAACAAGTGCCTGACGCTGTGTCTTATCCAGTTCATGCGGC
AATGCTATCTCTACTTCCTTTGCTAATCTTGCCTCCTGTTTGATGTCACCGTTCTTTTT
TAGTTCGGATTGCTCTACCCGATTCCATAGGGTTTGACGATCTAACATGTCAGGACT
TGCCCCAGTTGGGGCAAAAATTTGGGTGTATTCAATGCCTGTTTTTTTGGTGTAGTCC
TGCTCTTTTCCGTACGTATCACAGTACAATTTTTCGCCTGCTCGGTATGCTGCACATG
CCACGATTGAGCGACCATCTGACCTCGAAATGTTCTGCATTTCACAaTGGTaAATTGC
CATTTTCATCACCTG-TTTATTAACAGCCGCCCC-AAGTTTT-GGGC-
GGTTGGGGTGTCGGGG-TTTCCCTGA-CCGTACTTG-
CAAAATTTGGCGTAGCAAAAATTTTGCGTAAGTGCGCCCTTCGGGAACTCTGCGAGG
CTAAAAGCAAAAGCAAGAGCAATAAGGCTTTGACTTTGTTTTTTGATTTTGCTCGCT
GCGCTCGGATCTTGATGTAAGTCGAGTTTTTGAAGTAGAACACTTTTCACATTGATG
ATGGTTCATACTTCGGAAAATAGGTTGAGACACAGGCATTAAAAATGGTCAAAAAA
ACAGCTATGGAATTGGGTCAAATGCTCGATGAAGAAAAGGAAAAATTGGAACGTCA
GGAAAAGGCAAGAAAGGATCTAGCTGACGCTGTGGTGAAAGGTCGAGAGCAAAAA
CAGGCAAGGTCAGACGATGCAAAACGCAAGATCCTGATCGGGGCTTACTTACTGAA
GAAACATGATAATGACATCAAGAAACTTGTGGAACAAAACCCCGATTTTATTGGGT
ACATTAGAGAAAATGATAAGCACTTATTTTCATAGGCATTAGTAGTTATAAAAAACC
CTCATAAATCGAGGGTTTCTTTTTTATCTGCATTACTTTTTGAATCAATGTACTTTTTT

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CACCCTTATCTATAAACACCTCCAAACGTTGACTATCAAGGCTTTTGCGTTTTTCACG
ATATGCTTTTTGTCGTTCGGCATTGGTTTTTGGCGGCTCTTTTAGCATGTCCTGYGTTT
TTCTGATCGTTCGGGTCTTTCaTGTCTTTAACTCCCATATAGTATATCATAGTTACAGG
ATAACATTGTTTTTAGTTACAGGATAACTTTTT-ATAAAATTTATTTGT-
AAAAATTTATGCTATCCTGCAACTATTTTTAAATTACAGGATAACTATTTAACGTTAC
CCTGTAACTATAGTTATCCTGTAACGCTAAATTAATTACCCTGTAACATATATTTTGT
TATCCTGTAACAACTAAATATTGAAATAATACTACCAAAGTTACCCTGTAACGAAAA
TAACTAGGTTAAAAAATGATCGGATTTTAACATTTTGCGTTGTTCCAAAAGTTATCA
ACAGCCTAGAACGTCATAGGAAGCGATTACAGACACTTTATAGCTATCAGCATGGG
AACATAAGGGCAGGATGAAATATGGGTCTTAAAACGCAAATGGTGAGGTTTTAGAG
GTATTTTTTGAAGATGATTAAGGCGGTTTGTTTTTAAAATTTTTGGCGGCTCTCAGGC
TGCTTACATTTTAACCAGTTCAGTGAAAAGTTCTTTTTCAGCAAATTTCTGTTTAGCA
CCATAGCTAAAACTTGCGTGGAACATATTAAGAATTGACCGAAATGACACAATCTC
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TAAGTATACATTCATTTTAATAATTAATCTTTATTTAACAATGATTTATCTATATTCA
ATTGTTTAATTATTCTTACTAATATTATCTCTATATCAATATTTTTTATTTAAAAACAT
ATGTTTAGTAGTGCTTTTGATTAAAGTACCAGAGGGAGGGAGCAGAGCTGAATGGG
NAAATACTCACCCTAGAGCGATTCTTAAAAaTCA-
CCCTAAAGTATTCCCATTCGATGTACCGTCGG-
TCGGTCGCTTTCGCATCAGGGATGACATCACTGTATCAAGCTGCCACTGTTATGATT
ACGATTGATAGCACCGCCTGAACACGCTCATAACCGCCAATTAAATGACTACTCATT
GCGTCCGCTACTCTGTTCAGTTCCCCTAGTAATAGCGTTTTTCCGATGTGTGCTAGCG
TCACTGTACCTCATCACCCACACATGGACAGATTGAGTTACAGACATTGGCTAAATT
TTTGGGGTCTATGCTTGACAAAGCGAGTTAAAACCTTAGAATTTAAGACAGGTACAT
TAAGCCCCTGTGGTGAAATCATTAGCGGTCGTCAAACCTTAATGCTTTCGATTGCCG
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TAAATAATCCAAAGTACATGAGTAAGTCATGACCACTCCTGCGATGTGTGTAGCACT
CTGAGTATCCGTATTATATCAGTGATGTCATATACAACCACATAATGCGGATGTATC
ACTAACTCCCTAGTATCTTTCTGTCTGCCTGTGCGCCCCATCAGTCGATTATCAACAA
GTAAATCTGTCTTTTCTTCAATTAAATCATCAATTTCAATAGCTGCTATAGGGTTGCG
TTCTTCAAGATAATCATAGATATTTCTACGATCTTGGCGAGCTGTTTCAGTCCACTCA
ATCTTCATTCTTCGCTGCCAGGTTTCGCCAATGTTGCAGCACGTCTAGCGGC-
AACTCTGCCTTTACGTCCTCATGTGGAATCAACTTGCCTG-
CATTGGCTTCATTTAAGCCGA-TCTGTACCTGCTG-CTTG-
AACCATTTGTCATAGTCTGCGGCTTCCTGTTGCTTCTTAACAAAGTCACGCATGAAGT
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TCGGCTTAGGCTGTTCTTGGTCATAACGT-
GGTTTAAATGATGGCTGTCGCTCATTCTCAAGTAAAATTTTCCGTTT-ATTAACAG-
CCTGTTGCATCTGCTCATAGTAAGGCATCAACTGCGGTAAAATATCAGCAAGTGCCT
GTCTGTCTTTATCCAGGATACGGCTCAATTGCCCGATTTTTTTCAAGTCACGCTCATA
GGCTATTCTGTCGCTGATCTGCTCGTATTTTTGCTTTGAACACCTTATGTACTCTTGC

ATATCGTTACTTGCCCATGCCTGCGCCTGAAGACGTGTAAACATGGAAATGTCTGAA
GCAACATGGTCGCTGATATGGTCATACTCAGCATCTTTGACATAGCCGTATTTTGAT
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CTTTCTATCTGCTGTTGTTTTCGTTGCTGTTCCTGTTGCTCCTCTTTTATCTCTCTGTGA
TAAATGGAAAGGCTAGATTGATCATTATCCCAACGTGTGAGCATGTCCTCAAAGCCG
TAATAATCAGCACTAAACCAACCTG-
TTTTCACTTTCACTGGTGGCGGCAGTGGCTCTCC-
GATCTcCCTTAGCAGTGCTGTAGCTGCTCGT-
CCCTCTTTGATCACTTTATCAAGCTGCAAGCGGTCGATATTGTCTTGC-
AAAGCCTTGTAAAATCTATGCTCTGCTCGC-TTCTCATCC-GTTTTTACTTCAATC-
AAATACACATCTATAAGCGTTTGGCGGTGTTGTTGGTGGTCGTATAGTGCCTGTTGT
GCGGTGGCATAGACTGCGGGGGCTTGGTCTACGGCATCCTC***CAGCTG***

## Section B.2 Optimization of Transformation Assay Conditions.

The purpose of the optimization was to increase the transformation efficiency capabilities to account for the hydrolyzed urine chemical conditions impacting transformation efficiency. Optimized conditions from Chang et al. were used initially. The shaking speed parameter was changed to increase transformation efficiency. *Acinetobacter baylyi* was grown in LB broth at 30 deg C with shaking at 240 rpm. Growth curves were conducted to identify the new timing of log and stationary phases (Figure S1). Based on these results, plasmid DNA with urine solutions were added to bacteria two hours after a stationary cell culture was diluted in LB broth.

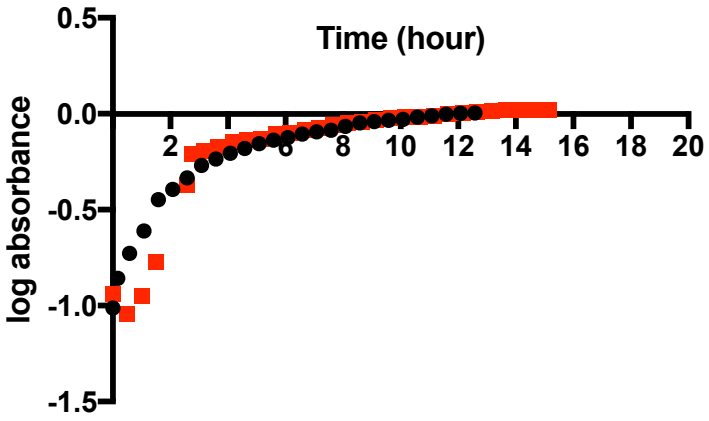


**Figure B-1. Growth curves of *Acinetobacter baylyi* (n=2)**

# Appendix C Supplementary Material for Chapter 5

# Towards a virus exposure assessment from urine-diverting and conventional toilets


**Figure C-1. Urine-diverting toilet with petri dish layout to collet virus-laden droplets. Two dishes for each location (starting at the back of the toilet and rotating clockwise: back, right, front right, front, front left, left) are used to collect samples 1 minute and 5 minutes after flushing. Experiments were completed in triplicate. Urine diverting toilets differ from conventional toilets with a separate front bowl and plumbing to collect urine. The mesh screen shown here was added to the toilet to prevent other items besides urine from entering the bowl and provide easier cleaning for when this occurs.**

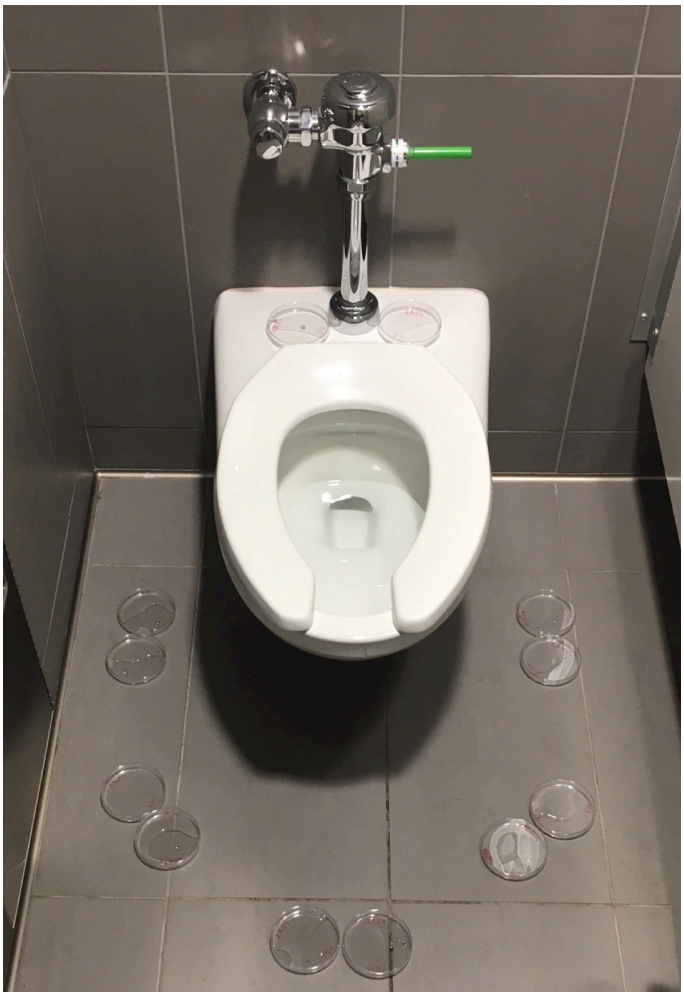

**Figure C-2. Conventional flush toilet with petri dish layout to collet virus-laden droplets. Two dishes for each location (starting at the back of the toilet and rotating clockwise, back, right, front right, front, front left, left) are used to collect samples 1 minute and 5 minutes after flushing. Experiments were completed in triplicate.**

**Table C-1. P values of student-t test comparing T3 and MS2 plaques counted at different locations around the toilet.**

T3

| | Left | Right | Front | Back | Left Front | Right Front |
|---|---|---|---|---|---|---|
| Left | | 0.3763 | 0.1421 | 0.3044 | 0.401 | 0.228 |
| Right | 0.3763 | | 0.0563 | 0.7342 | 1 | 0.401 |
| Front | 0.1421 | 0.0563 | | **0.0497** | 0.0588 | **0.0427** |
| Back | 0.3044 | 0.7342 | **0.0497** | | 0.7872 | 0.6643 |
| Left Front | 0.401 | 1 | 0.0588 | 0.7872 | | 0.5413 |
| Right Front | 0.228 | 0.401 | **0.0427** | 0.6643 | 0.5413 | |

MS2

| | Left | Right | Front | Back | Left Front | Right Front |
|---|---|---|---|---|---|---|
| Left | | 0.6926 | 0.3751 | 0.251 | 0.1913 | 0.3239 |
| Right | 0.6926 | | 0.2957 | 0.4397 | 0.3383 | 0.5651 |
| Front | 0.3751 | 0.2957 | | 0.2008 | 0.1859 | 0.2198 |
| Back | 0.251 | 0.4397 | 0.2008 | | 0.6642 | 0.6341 |
| Left Front | 0.1913 | 0.3383 | 0.1859 | 0.6642 | | 0.2909 |
| Right Front | 0.3239 | 0.5651 | 0.2198 | 0.6341 | 0.2909 | |

**Section C.1. Calculations of the fraction recovery for T3 after flushing conventional toilet**:

$$fraction\ recoverd\ (T3) = \frac{1700\ pfu}{10^9 pfu} = 1.7\times10^{-6}$$

Fraction recovery for MS2 after flushing conventional toilet:

$$fraction\ recoverd\ (MS2) = \frac{5600\ pfu}{10^{10} pfu} = 5.6\times10^{-7}$$

Fraction recovery for T3 after flushing urine-diverting toilet (100 pfu is the lowest amount to be detected, averaged over three replicates):

$$fraction\ recoverd\ (T3) < \frac{100/3\ pfu}{10^9 pfu} = 3.3\times10^{-8}$$

Fraction recovery for MS2 after flushing urine-diverting toilet:

$$fraction\ recoverd\ (MS2) = \frac{200/3\ pfu}{10^{10} pfu} = 6.7\times10^{-9}$$

## Section C.2. Calculations estimating urinary and enteric viruses outside the toilet

Polyomavirus gene copies added to toilet assuming 250 mL of urine per flushing event and $10^4$ gene copies per mL of urine:

$$\frac{BK\ PyV\ gene\ copies}{flush} = \frac{250\ mL\ urine}{flushing\ event} \times \frac{10^4 gene\ copies\ of\ BK\ PyV}{mL\ urine} = \frac{2.5\times10^6\ gene\ copies\ of\ BkPyV}{flush}$$

Estimated polyomavirus recovered outside of a conventional toilet:

$$\frac{BK\ PyV\ gene\ copies}{flush} \times fraction\ recovered\ (T3)$$

$$= \frac{2.5\times10^6\ gene\ copies\ of\ BkPyV}{flush} \times 1.7\times10^{-6} = \frac{4\ gene\ copies\ BK\ PyV\ outside\ toilet}{flush}$$

For multiple flushes for urinating events during the day assuming 2:0.85 ratio of urinating to defecating rate:

$$= \frac{2\ flushes\ for\ urinating}{per\ person} \times \frac{4\ gene\ copies\ BK\ PyV\ outside\ toilet}{flush}$$

$$= 8\ gene\ copies\ per\ person$$

Enteric virus added to toilet assuming 0.85 flushes for defecating per day, 32 grams of stool per flushing event, and $10^9$ infectious particles per gram of stool:

$$\frac{infectious\ particles}{flush} = \frac{32\ grams\ of\ stool}{flushing\ event} \times \frac{10^9 infectious\ particles}{gram\ of\ stool} \times \frac{0.85\ flushes\ for\ defecating\ at\ work}{flushes\ for\ defecating} =$$

$$\frac{2.7\times10^{10}\ infectious\ particles}{flush}$$

Estimated enteric virus recovered outside of conventional toilet:

$$\frac{infectious\ particles}{flush} \times fraction\ recovered\ (MS2)$$

$$= \frac{2.7\times10^{10}\ infectious\ particles}{flush} \times 5.6\times10^{-7}$$

$$= \frac{1.5\times10^{4}\ infectious\ particles\ outside\ toilet}{flush}$$

**Section C.3. Calculations of the number of flushes required to detect above 250 gene copies per 100 cm$^2$ swabbed surface.**

$$Number\ of\ flushes > \frac{\frac{250\ gc}{100cm^2} \times \left(\frac{100\ cm}{m}\right)^2 \times 1m^2}{\frac{concentration}{flush} \times fraction}$$

Using the fraction of dsDNA T3 bacteriophage recovered outside of the toilet (1.7 x $10^{-6}$)

$$Number\ of\ flushes > \frac{\frac{250\ gc}{100cm^2} \times \left(\frac{100\ cm}{m}\right)^2 \times 1m^2}{\frac{2.5\times10^6 gene\ copies\ per\ person}{flush} \times 1.7\times10^{-6} pfu/pfu} = 6000$$

Using the fraction of ssRNA MS2 bacteriophage recovered outside of the toilet (5.6 x $10^{-7}$)

$$Number\ of\ flushes > \frac{\frac{250\ gc}{100cm^2} \times \left(\frac{100\ cm}{m}\right)^2 \times 1m^2}{\frac{2.5\times10^6 gene\ copies\ per\ person}{flush} \times 5.6\times10^{-7} pfu/pfu} = 18000$$