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Pathogen Removal in Natural Wastewater Treatment and Resource Recovery Systems:

Solutions for Small Cities in an Urbanizing World

by

Matthew Eric Verbyla

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Environmental Engineering
Department of Civil and Environmental Engineering
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Keywords: water reuse, water reclamation, climate change, food security, sanitation, waste stabilization ponds, pathogens, viruses, quantitative microbial risk assessment, Sustainable Development Goals

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DEDICATION

I dedicate this dissertation to my parents, for their love and support; to my wife, Wendy, for her patience and encouragement; and in memory of my friend, Luis Alberto Gamboa, who enjoyed learning and made learning enjoyable.

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ABSTRACT

Sanitation, renewable energy, and food security are among the most pressing global development needs of the century, especially for small cities with rapid population growth. Currently, 53% of the world's population either lacks access to improved sanitation or discharges fecal waste to the environment without treatment. Furthermore, 80% of food consumed in developing regions is produced by 500 million small farms, and while many of them are still rain-fed, irrigated agriculture is increasing. The post-2015 Sustainable Development Goals, recently adopted by the United Nations, include targets to address the water-energy-food nexus. Wastewater reuse in agriculture can be an important solution for these goals, if it is done safely. Globally, 18 – 20 million hectares of agricultural land are irrigated with wastewater, but much is untreated, unregulated, or unsanctioned, causing concerns and uncertainty about health risks.

There is a need to better understand pathogen removal in natural and non-mechanized wastewater treatment systems, such as waste stabilization ponds (WSPs) and upflow anaerobic sludge blanket (UASB) reactors, which are commonly used in small cities and towns. Riverbank filtration (RBF) is also a natural technique used by farmers in developing countries to treat surface water polluted with untreated sewage, but pathogen removal in these systems has seldom been assessed in developing countries. The focus of this dissertation is on pathogen removal in natural and non-mechanized wastewater treatment and reuse systems, to evaluate the health implications of water reuse for irrigation, with the following three objectives: 1) assess the current understanding of virus removal in WSP systems through a systematic review of the literature; 2) measure the removal of viruses and their association with particles in systems with WSPs, UASB reactors, or both; and 3) assess the fate and transport of pathogens and fecal indicators in wastewater treatment systems with direct and indirect reuse for irrigation to estimate microbial risks.

To advance the understanding of virus removal in WSP systems, a comprehensive analysis of virus removal reported in the literature from 71 different WSP systems revealed only a weak to moderate correlation of virus removal with theoretical hydraulic retention time (HRT). For each $\log_{10}$ reduction of viruses a geometric mean of 14.5 days of retention was required, but the 95th percentile of the data analyzed was 54 days. Also, whereas

virus-particle association and subsequent sedimentation has been assumed to be an important removal mechanism for viruses in WSPs, the literature review revealed a lack of evidence to confirm the validity of this assumption.

The association of human adenovirus (AdV) with wastewater particles was assessed in five full-scale wastewater treatment systems in Bolivia, Brazil, and the United States (two with only WSPs, two with a UASB reactor and WSPs, and one with only UASB reactors). A mesocosm study was also conducted with WSP water from one of the full-scale systems, and some samples were also analyzed for pepper mild mottle virus (PMMoV), F+ coliphage, culturable enterovirus (EV), norovirus (NoV), and rotavirus (RV). Results indicate that WSPs and UASB reactors affect virus-particle associations in different ways, which may differ for different viruses. In UASB reactor effluent, PMMoV was more associated with particles <180 µm, showed no indication of settling in subsequent ponds, and appeared to degrade in pond sediments after 5 days. In contrast, AdV in UASB reactor effluent was associated with small and large particles, and in subsequent ponds, particle-associated AdV showed evidence of possible settling or more rapid decay at the water surface. AdV and culturable EV were also more volumetrically-concentrated in UASB reactor sludge than they were in untreated sewage, WSP water, UASB effluent, and WSP sediments, indicating that the reactors may cause these viruses to become entrapped and concentrated in granular sludge. Some viruses may be removed in the sludge, but others exit the reactors in solution and attached to particles. The resuspension of pellets from centrifuged UASB reactor sludge samples in an eluant buffer indicated reversible AdV association with granular sludge, but some associations with particles in solution may not be reversible.

The fate and transport of pathogens and fecal indicators was assessed in Bolivia for two WSP systems with direct reuse for irrigation, and one on-farm RBF system used to treat surface water polluted by untreated sewage. In the WSP systems, despite HRTs of 10 days, pathogen and fecal indicator removal was generally $\leq$1-$\log_{10}$, possibly due to overloading and short-circuiting from sludge accumulation. The RBF system provided removals on the order of 2-$\log_{10}$ for protozoan parasites, 3-$\log_{10}$ or more for viruses, and 4-$\log_{10}$ or more for bacteria. The use of RBF also reduced cumulative estimated health burdens associated with irrigated lettuce. Irrigation of lettuce with untreated river water caused an estimated disease burden that represents 37% of the existing burden from acute diarrhea in Bolivia; when RBF was used, this decreased to only 1.1%, which is not epidemiologically-significant, and complies with the World Health Organization guidelines. Ratios of concentrations of microorganisms in irrigation water to their respective concentrations in soil or crops were calculated, to assess transfer from irrigation water to soil or crops. These ratios (with units mL g^{-1}) were generally < 0.1 mL g^{-1} for coliphage, between 1 and 100 mL g^{-1} for

Giardia and *Cryptosporidium*, and generally between 100 and 1,000 mL g^{-1} for helminth eggs. Higher ratios could indicate more efficient transfer from water to soil or crops, longer persistence in soil or on crops, or slower leaching away from soil or crops.

The results from this research demonstrate that pathogen removal in full-scale natural wastewater treatment systems happens via complex mechanisms that vary with respect to pathogen type, treatment systems configuration, and other environmental and operational parameters. Future research and innovation efforts should focus on the use of a combination of natural and non-mechanized technologies, surface-flow systems (e.g., WSPs) and subsurface systems (e.g., RBF), applied at both semi-centralized (e.g., wastewater treatment plant) and decentralized levels (e.g., on farms), to evaluate how this affects the efficiency and resiliency of pathogen removal. Also, future research is needed to further elucidate reasons for the observed differences in virus-particle associations in natural wastewater treatment systems.

1 INTRODUCTION

1.1 Background

In September 2015, the United Sanitation adopted the post-2015 Sustainable Development Goals (SDGs), which include sanitation, renewable energy, and global food security among the most pressing sustainable development needs of the century (United Nations 2015a; b). The Millennium Development Goals (MDGs), which expired in 2015, included targets to half the proportion of the population experiencing extreme hunger (Target 1.C) and lacking basic sanitation (Target 7.C). While progress has been made for Targets 1.C and 7.C, both targets were missed, with one in four children still experiencing stunted growth, and 2.4 billion people in the world without access to improved sanitation (United Nations 2015b). Furthermore, improved sanitation was poorly defined by the MDG Joint Monitoring Program (JMP), as it did not take into account the safe reuse or return of collected waste to the environment. For example, simple access to a sewer connection was counted as improved sanitation by the JMP, however fewer than 35% of cities in developing countries actually treat their wastewater (UNEP and WHRC 2007), and 1.5 billion people worldwide discharge sewage to the environment without any form of treatment at all (Baum et al. 2013). This practice leads to a geographical translocation of health risks (often from wealthier urban neighborhoods to poorer slums located downstream and in flood plains), as well as the widespread *de facto* (indirect unplanned) reuse of untreated wastewater.

The post-2015 SDGs stress a more integrated approach to sustainable development, placing emphasis on health as well as the nexus of food, energy, and water systems (United Nations 2013). There is now a large body of accumulating evidence about the interconnectivity of food security and nutrition with sanitation, hygiene and health. Food insecurity and malnutrition manipulate the human gut microbiome, which is thought to exacerbate health risks caused by enteric pathogens (Kau et al. 2011). Diseases associated with poor sanitation currently account for 280,000 diarrhea deaths that occur every year in low- and middle-income countries (Prüss-Ustün et al. 2014). In addition, nearly 13% of all people in developing regions of the world are undernourished and each year, more than 90 million children are underweight (United Nations 2015b). From an economic standpoint, reducing malnutrition

among children (by enhancing food security and improving sanitation and hygiene) is considered to be the most cost-effective development intervention, yielding nearly $45 for each dollar invested (United Nations 2013).

Energy was not addressed by the MDGs, but is central to the post-2015 SDGs, which have a specific target to increase renewable energy. Global food security is strongly influenced by the management of water, energy, and nutrients. For example, the dependency of fertilizer prices on the cost of fossil fuels was partially blamed for past food crises (Esmaeili and Shokoohi 2011); also, climate change and recent increases in water demand for biofuel production have contributed to the exacerbation of global water scarcity, causing food price spikes (Rosegrant et al. 2009). Wastewater treatment technologies that integrate a waste-to-energy approach can have synergy with multiple SDGs. For example, anaerobic wastewater treatment technologies can potentially produce more than 0.5 kWh of net energy gains per cubic meter of wastewater treated (McCarty et al. 2011), which supports SDG targets 7.2 and 6.2. Waste-to-food systems such as sanitation and wastewater treatment technologies that enable the safe reuse of treated water for irrigation or stabilized biosolids for soil amendment can contribute simultaneously to SDG targets 6.2, 6.6, and 2.4, and help manage nutrient cycles. The phosphorus in human waste alone comprises 22% of global phosphorus demands (Mihelcic et al. 2011), and the nutrients in domestic wastewater account for 10 to 15% of fertilizer imports for countries with food deficits and net food imports (see Section 2.2.2 of this dissertation). Access to clean water and better management of nutrients (i.e. managing the nitrogen cycle) have also been identified as two of the fourteen grand engineering challenges of this century (National Academy of Engineering 2008).

Small cities and towns can be defined as urban centers with fewer than 500,000 residents (United Nations 2011). These small cities and towns tend to have poorer access to water and sanitation relative to larger urban centers, and fall into what has been described as an infrastructure management gap: neither the community-based management models successfully used in rural areas, nor the centralized utility models that have been effective in larger cities, work well for small cities and towns (World Bank 2008). Because of the lack of access to highly-skilled operators, the use of non-mechanized and natural wastewater treatment systems is very common in small cities and towns, especially in developing countries (Mara 2003). For the purposes of this dissertation, non-mechanized wastewater treatment systems are defined as systems that do not require large inputs of external energy and materials, and which generally do not have mechanized equipment or moving parts (except for wastewater conveyance). Natural wastewater treatment systems would include a subset of non-mechanized systems that are constructed using mostly natural materials (e.g., soil, gravel, sand, clay). Examples of natural systems include waste

stabilization ponds, constructed wetlands, overland flow systems, rock filters, or reed beds. Examples of non-mechanized systems that are not considered natural systems include anaerobic reactors, such as Imhoff tanks, anaerobic filters, upflow anaerobic sludge blanket reactors, and expanded or fluidized and granular bed reactors. Natural wastewater systems have been used globally for centuries for wastewater reuse. They also have more preferable social, environmental, and economic sustainability indicators than mechanical treatment systems (Muga and Mihelcic 2008), especially when used for integrated resource recovery (Cornejo et al. 2013).

Operation and maintenance of wastewater treatment systems is central to their success. In low-resource regions and developing country settings, many natural wastewater treatment systems are overloaded and under-maintained. For example, in a recent study of wastewater management in 105 Bolivian cities and towns (89% of which had populations of 50,000 or less), the Bolivian Ministry of Water and the Environment found that 37% of natural (non-mechanized) wastewater treatment plants studied were not operating at all, and less than 5% were achieving acceptable treatment efficiency (Ministerio de Medio Ambiente y Agua 2013). Operation and maintenance was identified as the principal problem for 63% of these malfunctioning treatment plants. Furthermore, despite the inadequate treatment, farmers were still reusing nearly half of the volume of this wastewater to irrigate more than 5,700 hectares. Worldwide, at least 4.3 billion cubic meters of treated municipal wastewater and 8.3 billion cubic meters of untreated municipal wastewater are used to irrigate more than 6 million hectares (FAO 2015), though others have estimated that untreated or polluted water is used to irrigate as many as 18 million hectares (Drechsel et al. 2010), or 20 million hectares (Raschid-Sally and Jayakody 2008). Globally, there is a widespread need for wastewater management systems that provide efficient and resilient pathogen removal to enable the safe reuse of water in agriculture, and that can be easily operated and maintained.

Wastewater treatment ponds (lagoons), or waste stabilization ponds (herein referred to as WSPs), are shallow engineered basins enclosed by earthen berms, and are designed to stabilize and treat wastewater via natural processes involving sunlight, algae, and bacteria. Of the wastewater treatment facilities in the United States that are required to comply with the Clean Water Act, WSPs are the most commonly-used technology, not necessarily in terms of flow rate, but in terms of the total number of facilities, according to the Clean Watershed Needs Surveys of 2000 and 2008 (US EPA 2000, 2008a, 2011). WSPs are also the most common technology used to treat wastewater in New Zealand, Mexico, Brazil, Bolivia, and the Dominican Republic (Mara 2003; Ministerio de Medio Ambiente y Agua 2013; Noyola et al. 2012). There are approximately 8,000 WSP systems in the United States (US EPA 2000,

2008a) and 2,500 in France (Mara and Pearson 1998) (Figure 1). Because they are relatively inexpensive and simple to construct, operate and maintain, they are particularly common in small cities and towns, especially when there is ample available land space. The use of chlorine, peracetic acid, and UV germicidal lamps for disinfection of treated wastewater may be common in larger cities and industrialized regions, however these technologies may not be accessible, affordable, or appropriate for small cities and towns, especially those located in low-income regions. Many WSP systems throughout the world rely solely on natural mechanisms for pathogen removal, such as sedimentation, predation, starvation, pH extremes, and sunlight-mediated mechanisms.

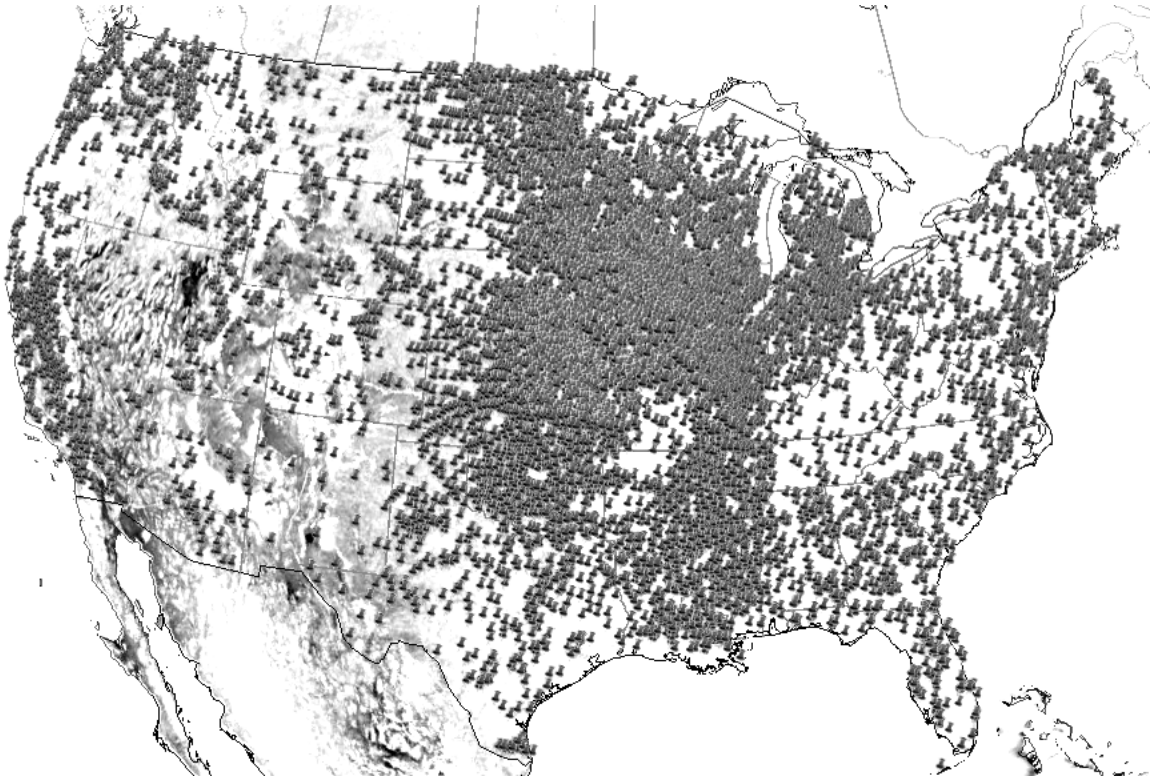

Figure 1. Location of waste stabilization pond (lagoon) systems in the United States, as reported in the Environmental Protection Agency's Clean Watersheds Needs Survey Reports to Congress from 2000 and 2008 (US EPA 2000, 2008a).

Connecting wastewater treatment with resource recovery, environmental sustainability, and food security development goals may be an effective sustainable wastewater management strategy for small cities and towns, especially in developing countries. The livelihoods of residents in these regions are highly dependent on food production and ecosystem management services, with farming and fishing being two of the most important activities (FAO 2011).

1.2 Research Gaps

The natural mechanisms responsible for pathogen removal and inactivation in WSPs are extremely complex and differ by pathogen type. For example, helminth eggs and protozoan parasites are mostly removed via sedimentation, while bacteria and viruses are removed by sunlight disinfection, predation, and inactivation due to environmental factors. In particular, the efficiency of virus removal in WSPs and the relative importance of different mechanisms is still not very well understood. This prevents the optimization of virus removal in WSP systems through the modification of operational or design parameters. Recent laboratory-scale studies have demonstrated the complexity of sunlight-mediated mechanisms, specifically how inactivation rates can differ for different types of viruses and can also be affected by a virus' associations with particles or their proximity to natural organic matter (Bosshard et al. 2013; Kohn and Nelson 2007; Love et al. 2010; Mattle et al. 2015; Romero et al. 2011, 2013; Rosado-Lausell et al. 2013; Templeton et al. 2008). Despite this, the study of virus-particle associations in WSPs has been extremely limited (Ohgaki et al. 1986; Sobsey and Cooper 1973). Also, during the past 30 years, high-rate anaerobic technologies, such as the upflow anaerobic sludge blanket (UASB) reactor, have become more common for the treatment of domestic wastewater in countries with warm climates (Lettinga 2010). While energy from biogas can be easily recovered from these anaerobic reactors, post-treatment is required for additional pathogen removal. WSPs are often used for the post-treatment of domestic wastewater from UASB reactors. A 4-$\log_{10}$ reduction in the concentration of *Escherichia coli* was reported for shallow WSPs treating the effluent of a UASB reactor (von Sperling and Mascarenhas 2005), however the removal of viruses in WSPs treating effluent from UASB reactors has not been studied. Research on this topic is needed, especially since UASB reactors transform the microbial community and the nature of organic matter and particles in the wastewater, potentially affecting the way viruses associate with particles.

Centralized or semi-centralized wastewater treatment systems provide one barrier to the transmission of pathogens between feces and irrigated food products. Understanding pathogen removal in centralized or semi-centralized wastewater management systems is only one part of developing policies to protect public health during water reuse activities. As highlighted above, there is a large percentage of people in the world using sewerage systems with no wastewater treatment or with wastewater treatment systems that are overloaded, under-maintained, failing, or abandoned. Given the high demand for irrigated agriculture, there is a need to evaluate the effect of overloading on pathogen removal in natural wastewater treatment systems, and also provide intermediary or

alternative solutions for safe water reuse, such as on-farm water treatment methods. Riverbank filtration (RBF) is commonly used in developing countries to treat irrigation water from polluted rivers, but its effectiveness at removing pathogens from river water that is highly contaminated by untreated sewage (as is the case in many developing countries) has not been well-studied. On-farm solutions such as RBF can be used in combination with centralized wastewater treatment systems or as intermediary solutions, while centralized wastewater treatment systems are being constructed or rehabilitated. The World Health Organization (WHO 2006) recommends the use of quantitative microbial risk assessment (QMRA) to estimate health risks associated with wastewater irrigation. QMRA has been used to guide water policy in the Netherlands and Australia (Bichai and Smeets 2013; Mara 2013), and to estimate illnesses from drinking water (Macler and Regli 1992), water reuse (US EPA 2012a), and recreational bathing (US EPA 2010a) in the United States. However, the use of QMRA in developing countries is more challenging, particularly because data are often scarce (Howard et al. 2006). The following limitations are associated with previous uses of QMRA to evaluate the risk of wastewater irrigation: a lack of data needed to estimate exposure (Hamilton et al. 2006a; Hamilton et al. 2007; Pavione et al. 2013); the oversimplification of dose-response models (Schmidt et al. 2013; Teunis and Havelaar 2000); and the failure to incorporate uncertainty associated with model parameters (Donald et al. 2011).

1.3 Research Objectives

The overall goal of this dissertation is to evaluate the health implications of wastewater reuse for irrigation in small cities by studying pathogen removal in natural and non-mechanized wastewater management systems. The following are the specific research objectives:

1. Assess the current understanding of virus removal in WSP systems through a systematic literature review
2. Measure the removal of viruses and their association with particles in WSPs, UASB reactors, and systems with UASB reactors and WSPs
3. Evaluate direct and indirect wastewater reuse for irrigation a) by comparing the fate and transport of pathogens and fecal indicators in WSPs and in on-farm RBF systems treating sewage-impacted river water, and b) by estimating health burdens from consuming wastewater-irrigated lettuce using a QMRA model that incorporates parameter uncertainty

1.4 Dissertation Synopsis

This dissertation is organized into six chapters. The present chapter provides background and a brief overview of the research needs. Chapter 2 includes a policy analysis about wastewater management for small cities and a review of emerging challenges for safe resource recovery in natural wastewater treatment systems. Chapter 3 includes a critical and systematic review of virus removal in WSP systems, addressing objective 1. Chapter 4 includes results from studies of virus removal and particle associations in one pilot-scale and four full-scale wastewater treatment systems and in a mesocosm using WSP water from one of the systems. Chapter 5 includes an investigation of the fate and transport of pathogens and fecal indicators in two WSP systems with direct water reuse for irrigation, and one site where sewage-contaminated river water is used for irrigation, with or without treatment from an on-farm RBF system. A QMRA model is used to assess microbial risk and the health burden associated with consumption of irrigated lettuce. Overall conclusions and recommendations for future research are presented in Chapter 6.

2 EMERGING CHALLENGES FOR INTEGRATED WASTEWATER RESOURCE RECOVERY IN SMALL CITIES

2.1 Integrating Protection of Health and the Environment with Resource Recovery and Food Security[1]

2.1.1 Introduction

More than half of the urban population in developing countries lives in cities with less than 500,000 inhabitants, which are generally more connected with agricultural production zones than they are with other urban areas (United Nations 2011). These small cities are expected to double both in number and in population by 2025 (WaterAid/BPD 2010). Residents of these areas have some of the worst access to water, sanitation, and hygiene services, and their economic prosperity and health is dependent on food security and ecosystem management (FAO 2011). Water and sanitation solutions for these areas are complex; for example, dry sanitation technologies such as composting latrines have been promoted and provide an appropriate solution for some areas, but will not work in others for social and cultural reasons (Nawab et al. 2006).

Between 1990 and 2000, 248 million urban dwellers in Asia, Africa, and Latin America made new connections to sewer systems, representing a third of the total urban population gaining access to improved sanitation during that time (WHO-UNICEF 2000). Growing populations in small cities of developing countries will likely continue to connect to sewer systems; however, without adequate treatment, the discharges from those systems will degrade surface waters in areas that already have poor water quality, leading to increased health risks and decreased economic opportunities for those who depend on water quality for their livelihood (Fry et al. 2008).

In larger industrialized cities, wastewater is often treated via mechanized processes that require energy inputs, only a fraction of which can be recovered by harvesting biogas from the anaerobic digestion of sludge. While anaerobic wastewater treatment processes can potentially increase overall energy recovery yields (McCarty et al. 2011), wastewater reuse also provides significant energy and carbon emission savings by offsetting energy needed

[1] Reprinted with permission from: Verbyla, M.E., Oakley, S.M., Mihelcic, J.R. (2013) "Wastewater Infrastructure for Small Cities in an Urbanizing World: Integrating Protection of Human Health and the Environment with Resource Recovery and Food Security," *Environmental Science and Technology*, Volume 47, Number 8, pages 3598 – 3605. Copyright 2013 American Chemical Society. Permission is included in Appendix F.

for manufacturing and distributing fertilizers and supplying irrigation water (Mo and Zhang 2012). Wastewater reuse is also one solution to provide global food security amidst the looming phosphorus crisis (Cordell et al. 2011).

In developing countries, community-managed infrastructure often fails prematurely due to maintenance deficiencies that result from decreasing financial durability and community activity as the system ages (Schweitzer and Mihelcic 2012). Small cities in developing countries tend to fall into what has been termed an infrastructure management gap (World Bank 2008)—they are large and compact enough to have centralized utilities (such as sewer collection systems), but they are too small to have the resources within the community to manage highly-mechanized infrastructure. Wastewater treatment systems in developing countries are often overloaded or abandoned altogether due to energy demands, lack of skilled operators, and inadequate sludge management (Oakley 2005a). In fact, it's estimated that 1.5 billion people use sewerage facilities with no treatment (Baum et al. 2013) and global estimates of the total area irrigated with untreated wastewater range from 3 million (Drechsel et al. 2006) to 20 million hectares (Raschid-Sally and Jayakody 2008). This places farmers, vendors, and consumers at a greater risk for excreta-related diseases, which account for one-tenth of the global disease burden (Mara et al. 2010b).

When determining the most appropriate way to sustainably manage wastewater in small cities of developing countries, policy-makers, development agencies, and local stakeholder groups face a continuum of alternatives and tradeoffs. Wastewater contains water, nutrients, and energy resources, which can each be recovered for reuse. Alternatively, treated effluents can be discharged, if nutrients are removed to limits that protect receiving waters from nutrient imbalances. Yet, the tradeoff for this may be the need for additional complexity, as well as energy and chemical inputs for retrofitting or expanding treatment plants to include tertiary nutrient removal, and for operation, maintenance, laboratory monitoring, and evaluation tasks (US EPA 2008b). However, if treated wastewater is used to irrigate crops, nutrient limits do not need to be monitored, and farmers may even be able to expand operations, increase productivity, or offset costs associated with commercial fertilizers. The public health impact of wastewater irrigation, if pathogens are not removed or inactivated, is yet another tradeoff. The removal of helminth eggs is especially critical if wastewater is to be reused for irrigation in developing communities, where incidence rates of helminthiasis are high.

Given the need to make informed decisions about wastewater management in urbanizing developing countries amidst this continuum of alternatives and tradeoffs, priorities should be determined regarding the recovery of water, nutrient, and energy resources. Furthermore, it is important to evaluate resource recovery in systems with

appropriate technologies that fill the infrastructure management gap in small developing country cities, in a way that links sanitation goals with urban water management and food production. Can water, nutrients, and energy all be recovered, while meeting appropriate health targets and protecting the environment?

Accordingly, the purpose of this study is to evaluate the resource recovery potential of wastewater from two treatment systems serving small (and growing) cities in the Yungas region of Bolivia: one consisting of three stabilization ponds in series (three-pond system), and the other consisting of an upflow anaerobic sludge blanket (UASB) reactor followed by two polishing ponds in series (UASB-pond system). Specifically, the two systems are compared with respect to their removal of conventional wastewater quality parameters, nutrients, pathogens (helminth eggs, *Giardia* cysts, *Cryptosporidium* oocysts), and bacterial pathogen indicators, using the 2006 World Health Organization (WHO) guidelines (WHO 2006) as a benchmark. Nitrogen and phosphorus concentrations in the effluent are measured, to determine their potential fertilizer value for agricultural reuse. Assessing the wastewater reuse potential in geographical settings like this is particularly important since a recent study of the watersheds in this agriculturally-productive region concluded that they exhibit water stress due to climate change (Fry et al. 2012).

Stabilization ponds have been used globally to treat wastewater for over 100 years, and are especially common in developing country cities of all sizes. The UASB reactor is an advanced anaerobic wastewater treatment technology that allows for the recovery of biogas. Originally developed to treat industrial wastewater, these reactors were successfully demonstrated at a pilot scale for the treatment of domestic wastewater in the 1980s, and thousands of full-scale systems have since been implemented worldwide, especially in Latin America and South Asia (Lettinga 2010).

2.1.2 Materials and Methods

The two systems in this study are located within 20 kilometers from each other, at elevations of 400 and 460 meters above sea level, in a tropical region with an average annual ambient temperature of approximately 25°C (Instituto Nacional de Estadistica 2011). The three-pond system started out serving a population of approximately 420 people in 2006, and by 2012, the population connected to the system had grown to 780 people. Twenty-four hour flow measurements taken in this system during the month of June between 2007 and 2012 resulted in average daily flowrates that ranged from 52 m^3/day (in 2007) to 121 m^3/day (in 2012). The average per capita flow in this

community was 120 ±32 liters/person/day. The UASB-pond system started out serving a population of approximately 780 people in 2006, but has since increased coverage to now serve approximately 1,310 people. Twenty-four hour flow measurements taken during the month of June between 2007 and 2012 show that the average daily flowrate in this system has ranged from 43 m^3/day (in 2007) to 124 m^3/day (in 2012). The average per capita flow between 2007 and 2012 in this system is only 63 ±21 liters/person/day. The apparent difference in the observed per capita flow rates may be explain by the fact that water supply in the UASB-pond community is metered (customers pay a rate that is based on consumption), whereas customers in the three-pond community pay a flat monthly rate regardless of use.

Figure 2 depicts a schematic of both systems, showing the sampling locations. All wastewater samples were collected in June (during the dry season). Grab samples were collected from 2007 to 2011 during the hours of peak daily flow (between 7:00 a.m. and 10:00 a.m.) and analyzed for thermotolerant coliforms, in accordance with either the most probable number method or the membrane filtration method (APHA et al. 2012). The maximum holding time for grab samples was 24 hours. Composite samples were generally collected in one or more 10-liter plastic containers in the field at hourly intervals over the course of 24 hours, such that the volume collected each hour was proportional to the flow rate measured during that hour. Composite samples were analyzed annually from 2007 to 2011 for five-day biochemical oxygen demand (BOD_5), total suspended solids (TSS), total nitrogen (TN), and total phosphorus (TP) in accordance with *Standard Methods* (APHA et al. 2012). Composite samples were also analyzed for helminth eggs (in 2011 and 2012) and for *Giardia* and *Cryptosporidium* (in 2012). The sample volumes for physical-chemical parameters were 2.0 liters each; sample volumes for helminth egg analysis ranged from 2.0 liters to 32.1 liters; and sample volumes for *Giardia* and *Cryptosporidium* analysis were 4.0 liters each. The maximum holding time for composite samples was 48 hours. All samples were either stored on ice in a cooler, or in a refrigerator, until the time of analysis in the laboratory.

Helminth egg samples that were larger than 4.0 liters in volume were concentrated in the field as follows: samples were transferred to buckets and placed on a flat surface for at least four hours to allow eggs to settle, after which time the supernatant was decanted using a siphon and the remaining sediments were transferred into pre-rinsed 2-liter plastic bottles and transported within 48 hours on ice to the Centro de Aguas y Saneamiento Ambiental (CASA) laboratory (Cochabamba) for further processing and analysis. Samples for conventional water quality parameters and for *Giardia* and *Cryptosporidium* analysis were not concentrated in the field, but were transferred

directly into pre-rinsed 2-liter plastic bottles for delivery to the laboratory. Sludge core samples were also collected from the first pond in the three-pond system (in 2011 and 2012) and from the UASB reactor (in 2012), and were transferred directly into opaque plastic containers, placed on ice and transported within 48 hours to the laboratory.

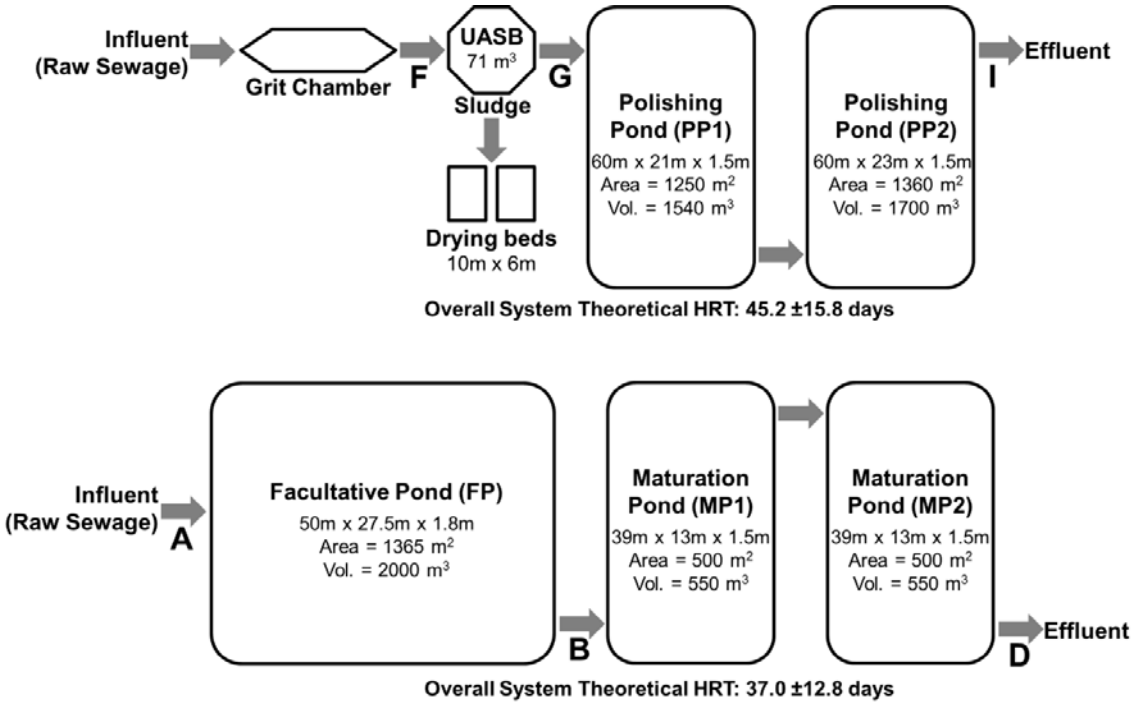

Figure 2. Schematic diagrams of the upflow anaerobic sludge blanket (UASB) reactor and pond (UASB–pond) system (top); and the waste stabilization pond (three-pond) system (bottom).

In the laboratory, helminth egg samples were further concentrated using CASA's standard protocol, which has been described in detail elsewhere (Verbyla 2012). Briefly, eggs were isolated from samples using sedimentation, flotation, and two-phase separation, as described by the Mexican Test Method for the Determination of Helminth Eggs in Water Samples (Secretaria de Comercio y Fomento Industrial 1999), with the exception that magnesium sulfate was used for flotation instead of zinc sulfate. Representative portions of the concentrated samples were transferred to 0.1-mm-deep Neubauer counting slides. Concentrated samples were gently mixed by turning the centrifuge tube over several times before removing the aliquot with a micropipette for observation under the microscope. Observations were made (2-4 repetitions) on an area of 18 mm2 of the slides under a microscope (Eclipse E600, Nikon) at either 400x or 1000x magnification, where helminth eggs were identified and enumerated. The average count was used to calculate the concentration of helminth eggs in the original sample. Minimum levels

of detection varied depending on the volume of sample collected, and the final concentrated volume of each sample (which ranged from 1.1 – 5.0 ml per sample).

The number of viable helminth eggs was judged by adding 0.1 ml of 0.4% Trypan Blue solution to a portion of the concentrated sample containing eggs. Trypan Blue, which stains non-viable cells, leaving living cells unstained, may over-predict the number of non-viable eggs, since the amount of stained eggs increases with respect to time (WHO 2004). To minimize these effects, samples were read within five minutes of adding the stain. Eggs that were unstained within the first five minutes were judged as viable. It is also important to note that some of the eggs may have been inactivated or destroyed by the ether used in the final step of the concentration process (Nelson and Darby 2001).

Samples were analyzed for *Giardia* and *Cryptosporidium* in general accordance with EPA Method 1623 (US EPA 2005). Briefly, samples were concentrated as described for the helminth egg analyses, with the exception that the flotation steps were omitted. *Giardia* cysts and *Cryptosporidium* oocysts were isolated from the concentrated samples using immunomagnetic separation with Dynabeads (Life Technologies). The entire volume of each concentrated sample (two repetitions per sample) was added to slide wells. Slide wells were prepared for differential interference contrast microscopic evaluation by adding mounting medium, a fluorescein-labeled monoclonal antibody reagent, and DAPI stain, yielding two final volumes of 100 µl in each well (100 µl per repetition). The number of cysts and oocysts were counted under the microscope, and the average count from the two repetitions is reported with the standard deviation.

2.1.3 Results

In order to compensate for the different per capita water usage rates in the two communities, a mass balance was performed on each system, and the removal of water quality parameters are compared as the percent of mass that was removed between the influent and effluent points (Figure 3). The observed removal of BOD_5, TSS, TN and TP was similar in both systems. Removal of BOD_5 was 91.2 ±5.4% for the three-pond system and 86.9 ±9.8% for the UASB-pond system, which is near the higher range of expected removal for systems using these technologies (Verbyla 2012). Removal of TSS was 84.8 ±8.9% for the three-pond system and 91.3 ±3.8% for UASB-pond system. The removal of TN and TP in the three-pond system was only 43.2 ±30.5% and 40.7 ±32.4%, respectively; and in the UASB-pond system, only 28.7 ±33.8% and 22.8 ±27.1%, respectively.

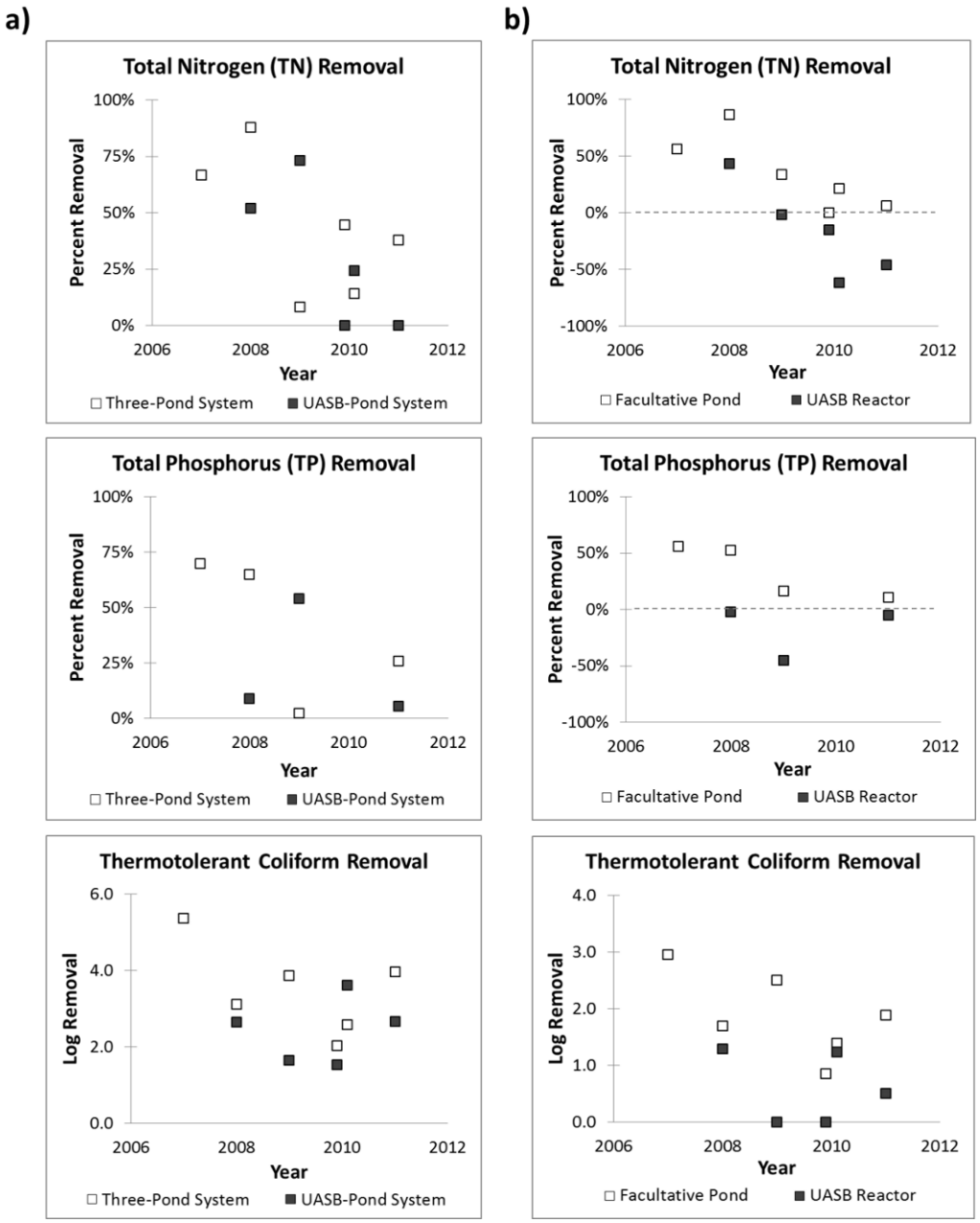

Figure 3. Observed mass balance removal of total nitrogen, total phosphorus, and thermotolerant coliforms (a) in the three-pond system (□) and in the UASB−pond system (■); also, (b) in the facultative pond from the three-pond system (□) and in the UASB reactor (■).

The removal of pathogens and pathogen indicators varied in both systems. The three-pond system provided a 3.4-$\log_{10}$ reduction of thermotolerant coliforms on average (n=6), which may be higher than the 2.3-$\log_{10}$ reduction (n=5) that was provided in the UASB-pond system (p=0.1261). The concentrations of parasites detected in samples from both systems are shown in Figure 4. In the three-pond system, *Giardia* cysts were detected at a concentration of 159.5 ±2.8 cysts/L at the influent and 22.8 ±3.9 cysts/L at the effluent. *Cryptosporidium* oocysts were detected at

6.0 ±1.4 oocysts/L at the influent and 4.0 ±1.4 oocysts/L at the effluent. The UASB-pond system appeared to provide similar removal: *Giardia* cysts were detected at 242.3 ±42.8 and 73.3 ±13.8 cysts/L at the influent and effluent, respectively. *Cryptosporidium* oocysts were detected at 9.5 ±6.4 and 6.5 ±1.4 at the influent and effluent, respectively.

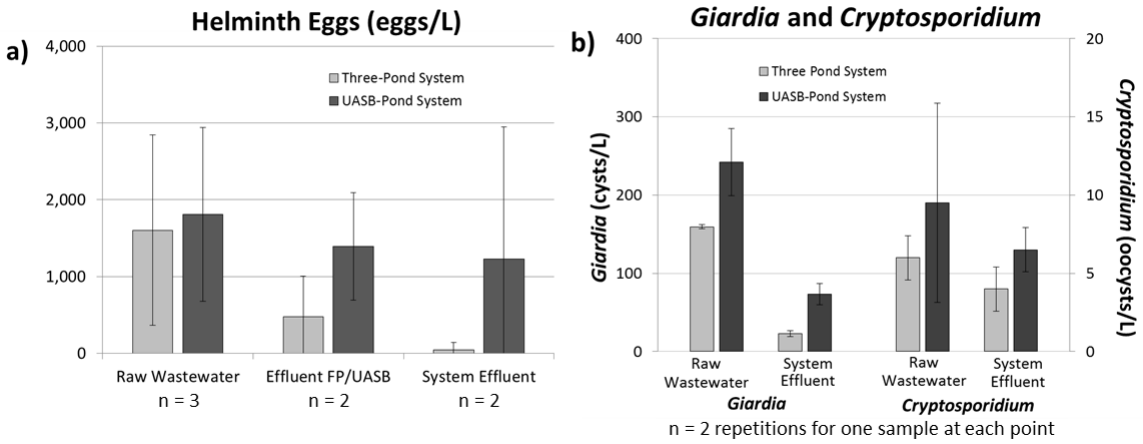

Figure 4. Observed removal of (a) helminth eggs, and (b) *Giardia* cysts, *Cryptosporidium* oocysts in both systems. Bar graph represents average concentration; all "non-detect" samples were replaced with a value equal to half of the limit of detection. Whiskers represent standard deviations.

Helminth eggs were detected in the untreated sewage of the three-pond system (1,605 ±1,242 eggs/L, n=3) and the UASB-pond system (average concentration of 1,809 ±1,129 eggs/L, n=3). The most common species detected in the untreated sewage samples were *Taenia* (78.9%), *Ascaris* (19.1%), *Trichuris* (1.7%), and Hookworm (0.3%). The only species detected in the treated effluent of the three-pond system was *Taenia*, but both *Taenia* and *Ascaris* eggs were detected in one sample from the effluent of the UASB-pond system. While *Taenia* is not one of the four helminth genera referred to in the WHO Guidelines for wastewater reuse (Mara 2007), *Taenia* eggs have been detected in the effluents of a waste stabilization pond system in Tunisia with a theoretical HRT of 20 days (Ben Ayed et al. 2009), and in 6-10 month-old composted feces from toilets in Panama (Mehl et al. 2011). The ingestion of eggs from the swine species (*Taenia solium*) can cause cysticercosis in humans, which is an emerging, neglected disease (Flisser 2011; Sorvillo et al. 2011) and the leading cause of acquired epilepsy in the world (García et al. 2003).

The three-pond system provided an average overall removal of 97% of all species of soil-transmitted helminth eggs (including *Taenia*), and *Taenia* was the only species detected in the system effluent. Conversely, a large number of helminth eggs (including both *Taenia* and *Ascaris* species) was detected in one of the two

composite samples collected at the effluent of the UASB-pond system. Sludge samples from the UASB reactor also had a lower average concentration of helminth eggs (236 ±225 eggs/g TS) than sediment samples collected near the inlet of the facultative pond in the three-pond system (841 ±606 eggs/g TS) (p=0.0951).

Parasites pose a risk to human health only if they are viable and infective to humans at the time of exposure. Therefore, it is important to understand not only how many parasites are present, but also what percentage is still viable. In this study, one out of six helminth eggs isolated from untreated sewage samples entering the three-pond system, and one out of eight helminth eggs from untreated sewage samples entering the UASB-pond system were judged to be viable. And while none of the eggs isolated from pond effluents in either system (n=18 eggs) were judged to be viable, one-third of the eggs isolated from the UASB reactor effluent samples (n=9 eggs) were judged to be viable. Sludge samples also showed greater egg viability: one-third of eggs isolated from UASB reactor sludge (n=15 eggs), and 9 of 24 eggs from the facultative pond sludge were judged to be viable.

2.1.4 Discussion

There is not much difference between the two wastewater treatment systems in terms of their ability to remove BOD_5 and TSS. While the average removal of BOD_5 was higher in the facultative pond (of the three-pond system) (81 ±8.6%) than it was for the UASB reactor (48 ±39%), the average removal of TSS was similar in both technologies (74 ±19% for the facultative pond and 77 ±18% for the UASB reactor). Based on the standard deviations, the facultative pond in this setting has provided a more consistent removal of BOD_5 than the UASB reactor over the lifespan of the project. While this apparent difference in consistency may be a result of the different per capita flow rates (and wastewater strengths) between the two communities, it ultimately does not affect the performance of the entire systems. Removal of BOD_5 and TSS was quite good in both systems, ranging from 85-91%.

Both systems rely on treatment mechanisms that produce methane and carbon dioxide emissions, but only the UASB reactor is designed for the collection of biogas, which contains 56 – 77% methane (Muga et al. 2009b). On average, 22 kg of COD was removed from the wastewater in the reactor per day. Theoretically, assuming that one-fifth of the COD removed from the wastewater is converted to biomass (Mara 2003), and that the remaining fraction is converted to biogas, in one day, the UASB reactor would produce biogas with 10 – 13 kg CH_4, and an equivalent harvestable energy content of 500 – 650 MJ. Measurements from the UASB reactor's biogas chamber in

2009 (Muga et al. 2009a) supports this result and suggests that the harvestable energy content of this reactor may even be slightly higher.

If the energy footprint of wastewater management is a primary concern, wastewater reuse may provide a greater energy savings than biogas recovery alone. For example, one recent study found that water reuse had a higher potential for offsetting carbon footprint and embodied energy than on-site energy generation and nutrient recycling via land application of biosolids (Mo and Zhang 2012). Eutrophication potential is also an important factor for wastewater management in developing countries, and in this study, wastewater reuse would decrease eutrophication potential by discharging high nutrient loads directly to land. The UASB-pond system discharges high concentrations of total nitrogen (51.8 ±28.1 mg N/L) and phosphorus (9.4 ±4.4 mg P/L) to a receiving water body that is a tributary of the Beni River, which is part of the Amazon basin and a local source for fishing. The concentrations discharged by the three-pond system are slightly less (34.7 ±14.1 mg N/L and 6.4 ±2.2 mg P/L), but are still high. Limited nutrient removal is not uncommon in UASB reactors (Chong et al. 2012) and stabilization ponds. For example, in a study of 178 different ponds, Racault et al. (Racault et al. 1995) reported removals of only 72% and 68% for total Kjeldahl nitrogen (TKN) and total phosphorous (TP), respectively, with 22 mg/L TKN and 8.5 mg/L TP detected on average in the system effluents. In order for a compliance-based approach to effectively manage the discharge of nutrients to receiving waters, communities in developing countries with these existing technologies would have to retrofit their systems to add additional nutrient removal components, which would require additional financial, complexity, energy, and material inputs. However, if effluents are applied to land, reclaimed nutrients can offset farmer's costs for fertilizer or may even augment production capacity. In fact, the phosphorus in human waste alone accounts for one-fifth of the global demand (Mihelcic et al. 2011).

Despite the observed difference in effluent concentrations, the mass discharge rate of nutrients per capita in the effluent of both systems is not significantly different, and was found to be 3.06 ±1.39 g/capita/day (total nitrogen) and 0.50 ±0.22 g/capita/day (total phosphorus). Based on published nutrient application rates and crop yields for wheat, maize, and rice in Latin America (FAO 2006), the volume of treated wastewater discharged from these systems over a period of six months is estimated to contain the same mass of phosphorus as commercial fertilizer that is used to support the production of 9.8 ±3.9 kg of wheat, 18.9 ±7.5 kg of maize, or 14.5 ±5.8 kg of rice per person that discharges wastewater to the treatment system. This represents approximately 100 – 400 MJ of food energy (Juliano 1993), which is equivalent to 10 – 75 days' worth of the recommended daily food energy

intake for one human (Purves and Sadava 2004). While agricultural yield will certainly vary based on a variety of regional and site-specific factors, the calculation shows the potential for integrating urban water infrastructure with food security efforts. From a regional management standpoint, it makes little sense to expend additional resources to remove nutrients from wastewater so that it may be discharged without impairing receiving waters.

If pathogens are present in the system effluents, the advantages of nutrient, water, and biogas recovery may be countered by public health risks. Assessing the potential health risks from helminth eggs is particularly important in these settings due to high incidence rates and because of the fact that the infectious dose is low. The infectious dose for helminths can be as low as one egg, and the eggs of some species, such as *Ascaris lumbricoides*, can survive for months or even years in soil, sediments, or sludge (see review in Verbyla 2012). In addition, helminthiasis can impact the nutritional status of children: Ascariasis alone may put as many as 1.5 million children under the age of 15 at risk for permanent growth retardation (de Silva et al. 1997).

The 2006 WHO Guidelines for wastewater reuse (WHO 2006), which are designed for a tolerable additional disease burden of 10^{-6} DALY per person per year, recommend that wastewater used for the irrigation of any crop have an average concentration of less than one helminth egg per liter. In this study, *Ascaris*, *Trichuris*, and Hookworm eggs were not detected in the effluent of the three-pond system, but *Ascaris* eggs were detected in the effluents of the UASB-pond system. *Taenia* eggs, which have a lower density than *Ascaris*, *Trichuris*, or Hookworm eggs (Feachem et al. 1983), were detected in the effluents of both systems. There was a 97% overall reduction of helminth eggs in the three-pond system (70% of which happened in the first pond), compared with only 23% overall reduction of helminth eggs in the UASB reactor. Thus, the UASB-pond system appears to be less effective at removing helminth eggs than the three-pond system, and reuse of the UASB-pond system effluent for irrigation may present higher health risks. Though several recent publications suggest that 10^{-4} or 10^{-5} DALYs per person per year may be a more appropriate health target for some regions (Mara and Sleigh 2010b; WHO 2007), and because the WHO recommendation for helminth eggs is based on a limited number of epidemiological studies, it has been suggested that wastewater with average concentrations of greater than one helminth egg per liter may still be suitable for reuse (Ensink and van der Hoek 2009). When treatment provides limited removal of pathogens, the 2006 WHO Guidelines suggest that risks for consumers can be further reduced by implementing non-treatment public health interventions in fields and on farms. For example, produce can be washed, rinsed or peeled prior to consumption (Amoah et al. 2007) irrigation can also be ceased several weeks prior to harvesting (Keraita et al.

2007a), or drip irrigation systems can be used to minimize contamination of edible portions of the crop (Keraita et al. 2007b). However, these interventions generally do not provide additional protection for farmers or field workers.

The results from the Trypan blue staining indicate that helminth eggs detected in the untreated sewage from both systems, in the effluent of the UASB reactor, and in the sludge from both systems were more likely to be viable than eggs detected in the pond effluents from either system. Sedimentation is one of the main mechanisms responsible for the reduction of parasites in stabilization ponds (Mara 2003), and larger parasites, such as helminth eggs, may be removed in UASB reactors by getting captured in the sludge bed (Chong et al. 2012). Therefore, many of the parasites that are removed from wastewater will become concentrated in the sludge. Non-viable eggs have a lower density than viable eggs, and therefore they may settle at a slower rate or become re-suspended during periods of peak flow and advance towards the pond outlet. Similar results were found in a study in Mexico, where sludge samples located near the pond outlet had a higher percentage of non-viable eggs than samples located near the pond inlet (Nelson et al. 2004). In the present study, sludge samples collected near the inlet of the first pond in the three-pond system had a higher concentration of helminth eggs than sludge samples from the UASB reactor, and sludge samples from both systems had similar percentages of viable eggs. However, stabilization ponds produce less sludge per kilogram of organic material removed than UASB reactors (Mara 2003), and the sludge from ponds only needs to be removed once every few years. Because operators of UASB reactors must remove sludge every 2 – 4 weeks, they have the potential to be exposed to sludge more frequently than operators of stabilization pond systems. Therefore, health risks associated with managing biosolids may be greater for a UASB reactor system than they are for a stabilization pond system.

The two systems in this study used technologies that are common in small cities of developing countries and can potentially fill part of the infrastructure management gap. The UASB reactor in this study produces 500 – 650 MJ of biogas energy which can be recovered daily. Both systems discharge effluents with the same mass of nutrients as that used in commercial fertilizers to produce food with an energy content equivalent to 10 – 75 days' worth of the recommended daily food energy intake for one human. However, the concentration of pathogens was reduced to a lesser extent in the UASB-pond system than in the three-pond system, and helminth eggs detected in the effluent of the UASB reactor were more likely to be viable than those detected in stabilization pond effluents. While wastewater management systems in large cities and in developed countries may be designed for the removal of nutrients and discharge into receiving waters, the technologies used in developing countries do not provide

sufficient nutrient removal to do this without impairing receiving waters. The reuse of wastewater nutrients for agriculture can offset eutrophication potential and create synergy between sanitation and food security development goals. Thus, wastewater management in small cities of developing countries should use technologies that remove pathogens so that water and nutrients can be safely reclaimed. Direct energy recovery from biogas may be a priority that should be subservient to water and nutrient recovery in these settings.

2.2 Emerging Challenges for Pathogen Control and Resource Recovery in Natural Wastewater Systems[2]

The purpose of this section is to address the emerging challenges associated with pathogen control and resource recovery in natural wastewater systems. Perspectives from engineering and the social sciences are used along with case examples from Bolivia to demonstrate how wastewater management and pathogen control objectives must be contextualized. Because natural methods of wastewater management rely on the integration of environmental, engineered, economic, and social systems, appropriate pathogen control objectives cannot be based on a single approach, and must reflect the broader context of health, integrated water management, and cultural perceptions of health and risk, which requires participation from stakeholders in multiple sectors. For the purposes of this discussion, natural wastewater treatment systems are divided into the following categories: 1) aquatic systems (e.g. WSPs, free water constructed wetlands, and marshes); and 2) subsurface or soil-based systems (e.g. rock filters, reed beds, leach fields, and subsurface flow wetlands). The former group consists of systems where wastewater is exposed to sunlight; for the systems in the latter group, wastewater is primarily filtered through soil, gravel, plant roots, and other subsurface substrates. This can have important implications on both pathogen removal efficiency and cultural acceptance of the technology.

2.2.1 Challenges with Pathogen Control in Natural Systems

2.2.1.1 Mechanisms, Rates, and Indicators of Pathogen Removal in Natural Systems

Wastewater pathogens comprise a diverse group of disease-causing organisms, including viruses (non-living infectious agents that utilize host cells to replicate), bacteria (single-celled prokaryotic microorganisms), helminth eggs (microscopic infective agents that develop into parasitic worms), and protozoa (single-celled

[2] This section contains text extracts, a figure, and a table that are reprinted with permission from: Matthew E. Verbyla, Maryann R. Cairns, Paola A. Gonzalez, Linda M. Whiteford, James R. Mihelcic, "Emerging challenges for pathogen control and resource recovery in natural wastewater treatment systems," *WIREs Water* 2015, Volume 2, Number 6, pages 701 – 714. doi: 10.1002/wat2.1101. Copyright 2015 Wiley Periodicals, Inc. Permission is included in Appendix F.

heterotrophic and mostly eukaryotic parasites). These different types of pathogens are removed via distinct mechanisms at a variety of rates in natural wastewater treatment systems (Table 1). The removal efficiency of pathogens in natural wastewater systems can be significantly affected by environmental factors such as temperature, sunlight, wind, and rainfall. Table 1 highlights the importance of the different mechanisms and the general range of efficiencies that may be achieved in aquatic and subsurface natural wastewater treatment systems. Large variations in performance should be expected in natural systems.

It is currently impractical to monitor the concentrations of all human pathogens in wastewater on a routine basis. The common approach thus far has been to use surrogate organisms as a proxy to estimate the removal of actual pathogens. Coliform bacteria, thermotolerant coliform bacteria, *Escherichia coli*, fecal streptococci, and enterococci are the most common fecal bacterial indicator groups used in practice today. However, they do not necessarily indicate the presence of pathogens and are not necessarily appropriate indicators for pathogen removal in aquatic or subsurface natural wastewater treatment systems (García et al. 2010; Maynard et al. 1999). One reason for this is that fecal indicator bacteria are often present in wastewater at much higher concentrations than human pathogens, and the inactivation of these organisms in natural systems can be more efficient at higher densities than it is at lower densities. Another reason is that *E. coli*, fecal streptococci, and enterococci are generally specific to the intestines of warm-blooded animals, but some coliforms are free-living in the environment, and there is evidence that *E. coli* can replicate in the environment (Ishii et al. 2006). Since most natural wastewater systems have long retention times and provide habitat for a variety of wildlife (including birds, bats, turtles, and small mammals), fecal indicator bacteria from non-human origin (which do not necessarily indicate a threat to human health) may be added to the wastewater throughout the system. Microbial source tracking (Harwood et al. 2009) is one technique that can help distinguish between animal and human fecal contamination in natural wastewater systems, and thus can provide a better understanding about actual microbial risks to humans.

New technology developments are revolutionizing the field of microbiology, and will soon enable the widespread use of alternative indications of pathogen removal in wastewater systems. Methods for monitoring pathogens in natural systems should be contingent upon the type of system used (aquatic vs. subsurface), and also on the end use of the treated wastewater (e.g. discharge to water bodies, reuse in agriculture, reuse in aquaculture). In aquatic natural wastewater systems, helminth eggs are removed mainly via sedimentation (Table 1).

Table 1. Mechanisms and general removal efficiencies for different types of pathogens in natural wastewater treatment systems.

Type of System	Microorganism Group	Removal Mechanisms	General Removal Efficiency	References
Aquatic Natural Systems (Ponds, Lagoons, Surface Flow Wetlands)	Bacteria	• Sunlight disinfection: directly (UV absorption by nucleic acids) and indirectly (sunlight absorption by photosensitizers, producing reactive species) • Predation and natural mortality • Sedimentation (after sorption to larger particles) • Environmental factors (temperature, pH, NH3)	Medians [interquartile range] of log_{10} removal of coliform bacteria in stabilization ponds, based on data from 186 different ponds, the majority with retention times <20 days (von Sperling 2005): Primary facultative ponds: 1.8 [1.4 – 2.3] Secondary facultative ponds: 1.0 [0.7 – 1.5] Maturation ponds: 1.2 [0.5 – 1.7]	(Crites et al. 2014; von Sperling 1999, 2002, 2005)
	Viruses, bacteriophage	• Sunlight disinfection: directly (UV absorption by nucleic acids) and indirectly (sunlight absorption by photosensitizers, producing reactive species) • Predation and natural mortality • Sedimentation (after sorption to larger particles) • Environmental factors (temperature, pH, NH_3)	Ratio of retention time per each log_{10} reduction in virus/bacteriophage concentrations: Geometric mean: 14.5 days (95^{th} percentile: 56 days) Bacteriophage PRD-1 and coliphage were found to survive longer in surface flow wetland sediments than they did in the water column	(Adhikari et al. 2013; Karim et al. 2004; Sinton et al. 2002; Chapter 3 of this dissertation)
	Protozoan Parasites (mainly *Giardia* and *Cryptosporidium*)	• Environmental factors (temperature, pH, NH_3) • Sunlight disinfection • Predation and natural mortality • Sedimentation	Removals reported in the literature vary greatly (from <1-log_{10} reduction in pond with 76 days retention to 1- to 2-log_{10} reduction in pond with 6 days retention). Discrepancies in the literature with regard to rate of decay in pond/wetland sediments vs. near the water surface, and with regard to the importance of sedimentation vs. other factors.	(Karim et al. 2004; Reinoso and Bécares 2008; Reinoso et al. 2008a; b, 2011)
	Helminth eggs	• Primarily sedimentation	Observed retention time [range] for helminth egg removal in ponds: 1-log_{10} removal: 3 [2 – 12] days of retention 2-log_{10} removal: 9 [5 – 23] days of retention 3-log_{10} removal: 18 [7 – 33] days of retention	(Ayres et al. 1992; Saqqar and Pescod 1992)
Subsurface Natural Systems (Subsurface Wetlands, Rock Filters, Reed Beds)	Bacteria, viruses, bacteriophage	• Adsorption to soil and wetland substratum • Environmental factors (temperature, pH, NH_3) • Predation and natural mortality • Sedimentation	Thermotolerant coliform removal in subsurface wetlands typically ranges between 1.25- and 2.5-log_{10} units; efficiency of removal by adsorption is not necessarily related to hydraulic retention time. Bacteriophage removal in subsurface wetlands is generally between 0.5- and 2-log_{10} units; virus survival may be prolonged in sediments	(Adhikari et al. 2013; García et al. 2010; Gerba et al. 1999; Jackson and Jackson 2008; Karim et al. 2004; Reinoso et al. 2008b; Stevik et al. 2004; Vidales et al. 2003; Vidales-Contreras et al. 2012; Vymazal 2005)
	Parasites (protozoan parasites and helminth eggs)	• Sedimentation • Physical filtration • Predation (especially protozoa) • Natural mortality	Horizontal subsurface wetlands generally provide between 0.4- and 3-log_{10} removal of protozoans Helminth egg removal is likely related to wetland or rock filter length; a concentration of <1 egg L^{-1} in 50 m reed bed, with 6 days retention.	(Bastos et al. 2010; García et al. 2010; Gerba et al. 1999; Karim et al. 2004)

The mechanisms responsible for the removal of protozoan parasites, viruses and bacteria are more complex, and depend on interactions between sunlight, biological, and environmental factors. It has been proposed that natural wastewater treatment systems that combine aquatic units with subsurface or soil-based units may provide more efficient overall removal of all pathogen types (Gerba et al. 1999).

2.2.1.2 Disease Transmission versus Pathogen Control

There are fundamental differences between controlling pathogens in a wastewater system and preventing the transmission of diseases; the ultimate goal for wastewater management should be to protect the environment and prevent disease in ways that are appropriate for the cultural and geographical setting. Disease prevalence and exposure to pathogens can vary both seasonally and regionally, and can also be disproportionately classified within a community depending on gender, class, and other forms of social stratification (Brown et al. 2011). Wastewater management can be thought of in terms of three integrated components: collection, treatment, and reuse or disposition. Wastewater collection helps the population avoid direct contact with raw sewage. Wastewater treatment helps avoid populations downstream from having direct contact with contaminants in the wastewater. Finally, the method of final disposition or reuse of wastewater will dictate who is exposed to contaminants, as well as how much they are exposed, how often they are exposed, and to which types of diseases they will be the most vulnerable. The effectiveness of wastewater collection and treatment, as well as the methods of final disposition or reuse, all have important implications for the distribution and severity of risks presented by wastewater management. This can be illustrated by examining the diversity of transmission routes for four different neglected tropical diseases caused by wastewater-transmitted helminths. Pathogen removal goals in wastewater management systems should be contingent upon the method of final disposition or reuse of wastewater (discharge to water bodies, application to soil, reuse in aquaculture) and should consider the diseases that are of concern for the different populations that may be exposed. Pathogen removal goals that are appropriate for one region may not necessarily be appropriate for another.

2.2.1.3 World Health Organization Guidelines for Wastewater Reuse

The risk of disease transmission resulting from exposure to pathogens must be low enough that it is safe and acceptable to society, without investing unnecessary financial, material, or energy resources. The determination of what level of risk is acceptable, i.e. “safe enough” for all members of society, is a task that should be defined

through participative action by community members and stakeholders. Simply recommending a maximum tolerable concentration of a single indicator organism, such as thermotolerant coliforms, for all different types of natural wastewater systems and all scenarios of wastewater discharge or reuse, may be an oversimplification of this task.

In 2006, the World Health Organization (WHO) published guidelines for wastewater reuse in agriculture (WHO 2006), based on the use of quantitative microbial risk assessment (QMRA). They suggest that pathogen concentrations in treated wastewater should be low enough to ensure that no more than 10^{-6} years of a person's life (on average) are lost due to poor health, disability or early death resulting from wastewater reuse (it was later suggested that 10^{-4} may be a more appropriate goal in communities where the existing disease burden is already high (Mara et al. 2010a)). While this is an adequate framework, there is still a great deal of uncertainty associated with risk estimates provided by QMRA models. In addition, the cultural perception and understanding of risk from wastewater reuse or discharge to the environment may be different from the actual risk, and the perceived or actual health risks may be disproportionately distributed amongst members of a society. In order to ensure effective system placement and management, it is important to understand that not only the average risk of disease transmission, but also the distribution of this risk and the distribution of cultural perceptions about this risk. This requires collaborative efforts from professionals with a broad range of disciplines, including public health, engineering, microbiology, and the social sciences.

2.2.2 Natural Wastewater Systems and Resource Recovery in Different Contexts

2.2.2.1 Industrialized Regions

The infrastructure responsible for water management, food production, and energy is often taken for granted in industrialized countries. Because the advanced technologies and engineering associated with this infrastructure are often not visible to end users, and its functionality depends on consistent monitoring, upkeep, maintenance, and cultural acceptance. In the United States, where approximately half of all wastewater treatment facilities used lagoons as part of their treatment process as of the year 2000 (US EPA 2011), it appears that natural wastewater treatment systems are gradually being replaced by more mechanized technologies that can reduce the concentrations of suspended solids, nutrients (especially ammonia), and fecal indicator bacteria to levels that meet increasingly strict permit requirements for discharge to water bodies (Gaston 2014; Morgan 2015; Sharp 2015). The reuse of wastewater for land application can eliminate the need to meet stringent nutrient limits. However, in some

locations, there are long distances between the source of treated wastewater and areas of agricultural water demand, and urban residential irrigation demands also tend to be very dispersed (Leverenz et al. 2011). During the past few decades, there has also been a shift in the philosophy of domestic wastewater treatment, which emphasizes energy recovery (McCarty et al. 2011); and it has been recognized that the recovery of energy from wastewater can potentially offset the direct operational energy costs associated with treatment (Mo and Zhang 2012).

There are examples of successful wastewater resource recovery in industrialized countries—but there have also been failures that are due in part to a lack of consideration for the systems' impact on societal structures (Guest et al. 2009). Public perceptions about the microbial quality of reclaimed wastewater can affect people's decisions and behavior (Wells et al. 2014). While this may be true globally, the few studies in the literature are from industrialized countries. For example, the majority of students at one university in the United States showed concern about the safety of using reclaimed water, even without specific knowledge about the details of water reuse practices in their universities (Vedachalam and Mancl 2010). Another study in Australia found that subjective norms (peer pressure), emotion ("ick" factor), and equity (fairness) all significantly and directly affect people's decisions and behaviors related to wastewater reuse (Nancarrow et al. 2008).

2.2.2.2 International Development

The majority of wastewater systems in less economically-developed countries have only partial treatment or no treatment at all (Baum et al. 2013). For the communities in these settings that do have wastewater treatment, and in countries with emerging economies that are increasing wastewater treatment coverage rates, natural systems are common (Mara 2003). For example, stabilization ponds and constructed wetlands are the most common technologies used for wastewater treatment in Bolivia (Ministerio de Medio Ambiente y Agua 2013) and they are also the most common systems used in the Dominican Republic, Mexico and Brazil (Noyola et al. 2012).

In the context of international development, the recovery of resources via water reuse could potentially help meet several goals simultaneously (e.g. energy demand, environmental sustainability (Cornejo et al. 2013), hunger eradication (e.g., Section 2.1 of this dissertation)). To demonstrate this, information about nutrient imports, total nutrient demand, urban sewer coverage, and agricultural water withdrawal was gathered for 52 net food-importing developing countries and 49 low-income countries with food deficits (as defined by the United Nations) (FAO 2013, 2015). Using estimates for the mass of nutrients in human waste (Strauss et al. 2003), and data about urban sewer

coverage (FAO 2015), it is clear that wastewater generated in these resource-challenged countries contains a considerable percentage of the nutrients and water that are respectively imported (as fertilizers) and withdrawn (as freshwater) for agricultural activities (Figure 5). On average, in these countries with food deficits and net food imports, the estimated amount of nitrogen in wastewater is between 10 and 15% of the total mass of nitrogen imported, and the amount of water the can be reclaimed accounts for ~5% of the water withdrawn for agriculture. However, in countries like Bolivia, where natural systems comprise the majority of all wastewater treatment plants (Ministerio de Medio Ambiente y Agua 2013), wastewater reuse can potentially offset the freshwater extracted and nutrients imported for agricultural production. Water also has an economic value—in the southern periurban district of Cochabamba, Bolivia, some residents pay up to US$5.00 per cubic meter of freshwater, and others use wastewater from a stabilization pond system to irrigate crops (authors' own experience). In summary, if implemented appropriately, natural wastewater systems with agricultural water reuse can assist countries that are seeking to improve economic, food production, and resource outcomes.

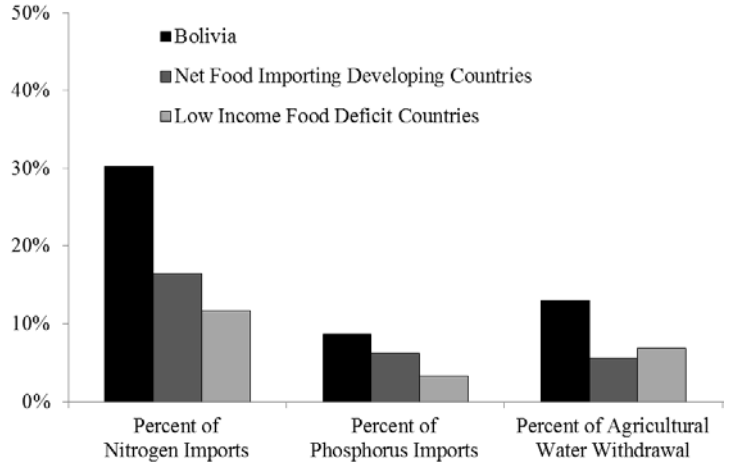

Figure 5. Ratio of the estimated quantity of nutrients and water in existing sewer systems compared to overall nutrient imports and agricultural water withdrawal in food-importing developing countries (dark gray) and low-income countries with food deficits (light gray), with Bolivia (black) as a case study.

The use of advanced anaerobic reactors (e.g. upflow anaerobic sludge blanket reactors) is rapidly being adopted globally, especially in tropical countries (Lettinga 2010). For reasons that are uncertain, in Bolivia, we have observed that systems with anaerobic reactors are being selected over (and sometimes replacing) natural wastewater systems, even in locations where agricultural wastewater reuse is common and large areas of flat land are available for the implementation of natural systems. The advanced anaerobic reactors may be viewed by some stakeholders as more "modern" technologies (Gamboa 2010), while natural systems may be considered by some to be "outdated"

(Ryan Shachoy and Moore 2013). Advanced anaerobic reactor technologies can improve energy recovery from the wastewater treatment process. However, some systems with advanced anaerobic reactors may be inherently less efficient at eliminating pathogens (Oliveira and von Sperling 2011). It is almost always necessary to use some sort of post-treatment after advanced anaerobic reactors to achieve additional pathogen removal. This post-treatment can be (and often is) provided by natural wastewater treatment systems. In communities with appropriate resources and technical support, the use of advanced anaerobic reactors in conjunction with natural wastewater treatment technologies can provide important benefits over natural treatment systems by themselves, by saving land space and allowing for the recovery of biogas. However, if biogas is not reused or flared, the methane emissions from advanced anaerobic reactors can contribute even greater greenhouse gas emissions according to a recent study (Bruun et al. 2014).

2.2.3 Addressing Cultural Perceptions of Pathogens and Risk

The efficacy of natural wastewater systems at removing pathogens is contingent on individuals' and communities' ability to keep these systems operating, the amount of capacity building that governing bodies provide to local communities who may manage these systems, and the extent to which individual behaviors and understandings related to health risk are in line with the technological abilities. Risk, therefore, can be as inherently cultural as it is biological. Individual choices about hand washing, irrigation practices, sanitation, water reuse, and the use of chemical disinfection methods will thus be as integral as the effectiveness of technology in reducing risk, and furthermore, these behaviors can be managed. In addition, governmental and non-governmental organizations can teach stakeholders new behaviors via informational campaigns and outreach materials, increase local interest in wastewater technologies, and encourage adoption of new practices through economic or social motivations. However, unless there is widespread adoption of these new techniques, in conjunction with attention to wider issues of sustainability (Shomar and Dare 2014), the efficacy of a wastewater management system that employs resource recovery on reducing health risks may be widely variable.

Public perceptions about the safety of effluent from a natural wastewater systems are multifaceted, and may vary widely between stakeholder groups (Wells et al. 2014). Furthermore, the benefits and risks resulting from reusing water from these systems can often be influenced by factors such as gender, class, and social status (see Chapter 5 of this dissertation). Marginalized and vulnerable groups in a society may feel they cannot trust authorities

and may also lack information about their organizations or affiliations (Sano 2000), which can undermine their willingness to adopt practices and policies implemented by agencies and governments. It is necessary to understand how infrastructure relates to human health and well-being, and especially how wastewater treatment infrastructure impacts communities' health when it is managed unsuccessfully. As water shortages and the contamination of water bodies continues, these relationships will become more relevant (Libralato et al. 2012).

2.2.4 Conclusion

The efficacy of wastewater management using natural methods relies on the integration of environmental, engineered, economic, and social systems. Because of this, the most effective form of pathogen control and monitoring cannot be dictated by a single approach (e.g. a single limit for the maximum tolerable concentration of faecal indicator bacteria). Natural systems that employ a combination of aquatic and subsurface units may provide better overall removal of different types of pathogens, but the overall context of the system and its final method of wastewater disposition are important, as certain diseases are of greater concern in some regions than they are in others. As such, wastewater system technology choice, methods of final disposition or reuse of treated wastewater, and resulting pathogen removal objectives have to be contextualized technically and socio-politically for each particular application. Because of this, partnerships across sectors for science, technology and innovation are an essential part of the solution to challenges related to pathogen control in natural wastewater treatment and resource recovery systems, and the management of these resources at the nexus of water, energy, and food security. In order to develop sustainable approaches to wastewater management that have synergy with renewable energy, food security, and public health protection goals, the following partnerships are crucial: 1) academic partnerships between experts from the natural sciences, engineering, health, and the social sciences to develop new solutions to wastewater management; and 2) broad participation between professional and community stakeholders from the water, sanitation, health, agriculture, and energy sectors. While fostering these relationships and partnerships will take time, energy and funding, the reality of water pollution and the need to safely recover resources from wastewater will make these efforts imperative.

3 A REVIEW OF VIRUS REMOVAL IN WASTE STABILIZATION POND SYSTEMS[3]

3.1 Introduction

Waste stabilization ponds (WSPs), also known as lagoons, are one of the oldest and most prevalent types of systems used to treat domestic wastewater in the world. More than half of the wastewater treatment facilities in the United States and in New Zealand utilize ponds (US EPA 2011; Mara 2003). They are also the most common technology used to treat domestic wastewater in Mexico, the Dominican Republic, and Brazil (Noyola et al. 2012). In France, there are approximately 2,500 WSP systems (Mara and Pearson 1998), and in Colombia, there are approximately 100 WSP systems (Miguel Peña Varón, personal communication). The low cost and simplicity of their construction, operation, and maintenance has caused them to be considered one of the most important wastewater treatment technologies, especially for small cities and towns, and in particular when the effluent is land-applied (Mara 2003; Oakley 2005b; Peña Varon et al. 2000). In fact, the land application of WSP system effluent can reduce the eutrophication potential, embodied energy, and carbon footprint of wastewater management over the life cycle (Cornejo et al. 2013); but pathogen removal for safe nutrient recovery from these systems may be an increasingly important priority for some small cities and towns (see Chapter 2). The removal of fecal indicator bacteria in WSP systems has been well-documented (von Sperling 2005), but it is not a good indicator for virus removal (Maynard et al. 1999).

Viruses are intracellular parasites with a genome contained inside a protein capsid. They can be divided into groups according to their genome type: double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), double-stranded RNA (dsRNA), and single-stranded RNA (ssRNA) (Flint et al. 2009). Of more than 100 known species of viruses that are excreted in human waste (Bosch 1998), some are particularly resistant to wastewater treatment. Symonds et al. (2009) detected adenoviruses, enteroviruses, noroviruses, and picobirnaviruses in treated wastewater from 12 different cities throughout the United States. Though they are not necessarily specific markers of human waste (Harwood et al. 2013), bacteriophages (viruses that infect bacteria) have been used as surrogates to

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study enteric virus removal in WSP systems (Castillo and Trumper 1991; Omura et al. 1985). Pepper mild mottle virus, a plant pathogen of dietary origin, has been recently proposed as a surrogate for enteric viruses (Rosario et al. 2009), but its application to the study of WSPs has been limited to one publication (Chapter 4 of this dissertation).

The efficiency of virus removal in WSPs with respect to theoretical hydraulic retention time (HRT) has been widely debated in the literature, with different authors often reaching contradictory conclusions over the past 40 years (Figure 6). In one early review, virus removal in WSPs was described as being "erratic" (Berg 1973). In another report (Feachem et al. 1983), it was concluded that WSP systems with temperatures exceeding 25°C were capable of reducing enteric virus levels by 1- to 2-$\log_{10}$ units for every five days of retention, implying that 30-day pond systems would achieve at least 6-$\log_{10}$ virus reduction. This report also partially attributed virus removal in WSPs to their adsorption to solid particles and subsequent settling. Shuval et al. (1986; 1990) reported that virus removal in a 20-day pond system with temperatures above 20°C would be as high as 2- to 4-$\log_{10}$ units, a statement which was more recently cited by Drechsel et al. (2010). Maynard et al. (1999) indicated that virus removal rates in tertiary (maturation) ponds appeared erratic, and that overall removal in pond systems was generally <1 $\log_{10}$-unit.

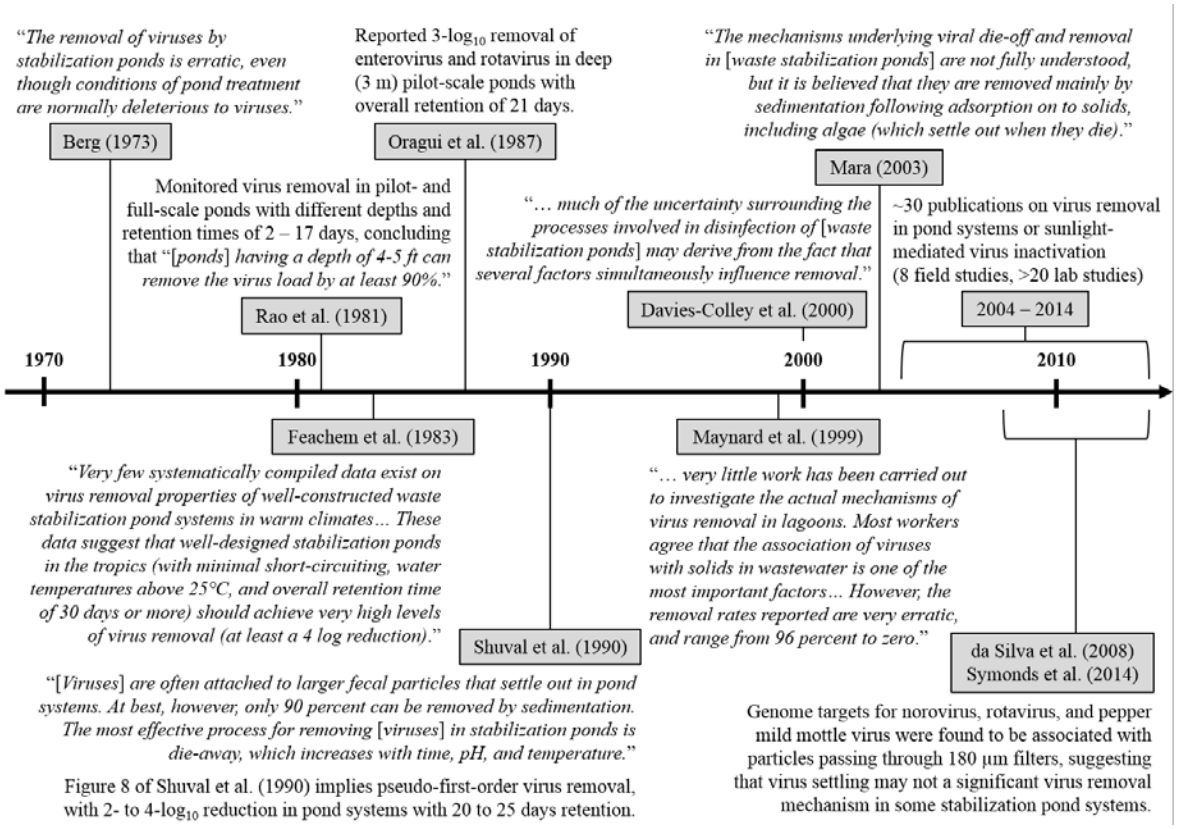


Figure 6. Chronological evolution of conclusions made about virus removal in waste stabilization ponds.

Previous reviews of pathogen removal in WSPs (Davies-Colley et al. 2000) and of the overall performance of maturation ponds (Maynard et al. 1999) have acknowledged that the mechanisms responsible for the removal of viruses in WSP systems are complex and rely upon interactions between physical, chemical, and biological characteristics of the pond water. However, as virus removal was not the primary focus of these previous review articles, the authors did not analyze results from a large number of field studies.

Given the widespread global use of WSP systems and their potential for integration with wastewater resource recovery, the purpose of this chapter is to critically review the major findings related to virus removal in WSP systems, and to statistically analyze results reported in the literature from field studies on virus removal in WSP systems. The literature describing phage removal in WSP systems is also reviewed, and statistical analyses are provided separately for phages and enteric viruses. It is important to note that authors from many of the studies included in this review have used bacteriophages as surrogates for enteric viruses in WSP systems. In particular, coliphages and phages infecting *Bacteroides fragilis* have been proposed as surrogates for studying enteric viruses in water, due to the fact that they have fundamental similarities with enteric viruses (e.g. structure, morphology, size), they are often found in greater concentrations than enteric viruses in water environments, and they mainly replicate inside the human gastrointestinal system (Grabow 2001). Nevertheless, the use of phages as surrogates for enteric viruses in WSP systems also has some important limitations. Until recently, methods used to measure concentrations of phages in water samples were not internationally-standardized; different authors have used a variety of different host cells and methods. Another limitation is that unlike enteric viruses, coliphages can be excreted by animals; and with the exception of F+ RNA coliphages, most coliphages have low specificity for human feces (Harwood et al. 2013). Somatic coliphages in particular represent a wide range of phages with different properties (Grabow 2001). While the primary focus of this review is on human enteric viruses and their surrogates, it should be noted that some enteric viruses may be capable of cross-infection between animals and humans (e.g. hepatitis E (Meng 2011)). RNA viruses in particular have a high potential for genetic mutations (Rosenberg 2014), and some have been known to jump between bird, human and other mammal hosts, creating emerging zoonotic viral diseases. It may be important to consider the influence of such zoonotic viruses in WSP systems; however, no information was found in the literature about this phenomenon as it relates to WSPs.

The following pond types are considered in this review: anaerobic, facultative, maturation, polishing, and surface-aerated ponds. Polishing ponds are defined here as ponds treating the effluent of any other type of

wastewater treatment system. The chapter is organized as follows: in Section 3.2, the mechanisms responsible for virus removal in WSPs are reviewed, with a particular focus on contributions that have been made over the past fifteen years. Section 3.3 provides an overview of some techniques that are used for additional disinfection of the effluent of some WSP systems. Section 3.4 includes a review of the interactions between viruses and particles in WSPs, and the potential impact that these interactions may have on virus removal. The influence of pond hydraulics on virus removal in WSPs is briefly discussed in Section 3.5. Section 3.6 includes a comprehensive review and statistical analysis of virus or bacteriophage removal reported from field studies of pilot- and full-scale WSP systems, pulling information from 48 different publications. Finally, Section 3.7 concludes by assessing the implications of the current state of knowledge, and identifying future needs for research and technology development.

3.2 Mechanisms for Virus Removal in Waste Stabilization Ponds

In many WSP systems, virus removal is achieved solely by natural mechanisms such as sedimentation (following the association of viruses with larger particles), predation by organisms of higher trophic levels, and sunlight-mediated inactivation mechanisms (Mara 2003). High temperatures and pH extremes alone can also inactivate viruses by denaturing their proteins and nucleic acids, however the effect of temperature and pH in WSP systems is more important for its interaction with other environmental factors which affect the efficiency of sunlight disinfection (Romero et al. 2011). Fecal coliforms (such as *E. coli*) are poor indicators for the removal of enteric viruses in WSPs (Maynard et al. 1999).

3.2.1 Adsorption to Solids and Sedimentation

Viruses are too small to settle by themselves in WSPs, but they may be removed via sedimentation if they associate with larger, settleable solids. Section 3.4 contains a more detailed review of the mechanisms responsible for virus-particle interactions. In older reports, virus adsorption to solids and removal by sedimentation was thought to be an important virus removal mechanism in WSPs (Feachem et al. 1983; Shuval 1990), however there is limited evidence that this is actually true. In early studies, the reversible adsorption of coliphage to algae in WSP water was reported under aerated conditions (with desorption under anaerobic conditions) (Ohgaki et al. 1986); poliovirus type 1 exhibited reversible adsorption to suspended WSP solids, conforming to a Freundlich isotherm model (Sobsey and

Cooper 1973). However, in these studies, high-speed centrifugation was used to separate particle-associated viruses; this is not necessarily an indication that the viruses would settle by gravity in a full-scale WSP system. Lijklema et al. (1986) found that bacteriophages in combined sewer overflow detention ponds were less likely to settle with particles than fecal indicator bacteria. In laboratory experiments with filtered (2.7 μm pore size) and unfiltered untreated sewage, facultative pond water, and maturation pond water, Frederick and Lloyd (1995) reported no significant removal of *Serratia marcescens* phage that could be attributed to adsorption to solids and sedimentation. Vorkas and Lloyd (2000) found that the phages of *S. marcescens*, *Erwinia ananas*, and *Erwinia amylovora* survived for a few days longer in filtered WSP water than they did in unfiltered WSP water. They suggested this difference might be due to phage adsorption to solids and subsequent sedimentation; however, they did not confirm the presence of the phages in the reactor sediment. Furthermore, their reactors were exposed to sunlight, so the shorter survival of viruses in unfiltered samples may have been due to the presence of algae which caused a rise in the pH and dissolved oxygen levels, potentially resulting in more efficient sunlight-mediated inactivation. In two recent studies, it was demonstrated that only a very small percentage of human norovirus (genotypes I and II) and human rotavirus (group A) in WSP samples were retained on 180-μm filters, implying that sedimentation of these viruses is not likely a dominant mechanism for their removal in WSP systems (da Silva et al. 2008; also Section 4.2 of this dissertation).

3.2.2 Predation by Organisms of Higher Trophic Levels

Virus predation in WSPs has not been extensively studied, and is poorly understood. The mechanisms driving virus internalization by higher trophic-level organisms (and the resulting protective or detrimental impact on virus viability) are likely complex. In one eutrophic freshwater reservoir in Japan, it was found that heterotrophic nanoflagellates consumed virus-like particles, decreasing their abundance in water (Manage et al. 2002), but in another eutrophic lake in France, less than 1% of virus-like particles were reportedly grazed by heterotrophic nanoflagellates (Bettarel et al. 2005). In one coastal system, viruses accounted for < 5% of all heterotrophic nanoflagellate predation (bacteria are the preferred target) (Miki and Jacquet 2008). The internalization of enteric viruses by free-living protozoa has been documented both *in vitro* (Danes and Cerva 1981) and *in vivo* (Danes and Cerva 1984). This internalization may actually protect viruses from inactivation, acting as vectors for their transmission and dispersal through water sources (Scheid and Schwarzenberger 2012). Alternatively, if these larger

organisms die or lose their motility, they may settle to the bottom of a pond, carrying the internalized viruses with them. A recent field study demonstrated that human adenovirus can be taken up and internalized by ciliates in wastewater, where it can persist for up to 35 days (Battistini et al. 2013); however, the authors were not able to determine the infectivity of the internalized viruses. Human adenovirus genome targets were also detected on 180-μm filters in samples from a maturation pond where water mites were also trapped in large quantities on the filters (see Section 4.3). More research is needed to better understand the effect of virus predation in WSPs, in particular to understand if the association of enteric viruses with higher trophic-level organisms in WSPs plays a significant role in virus removal or assists with virus sedimentation.

3.2.3 Sunlight-Mediated Inactivation

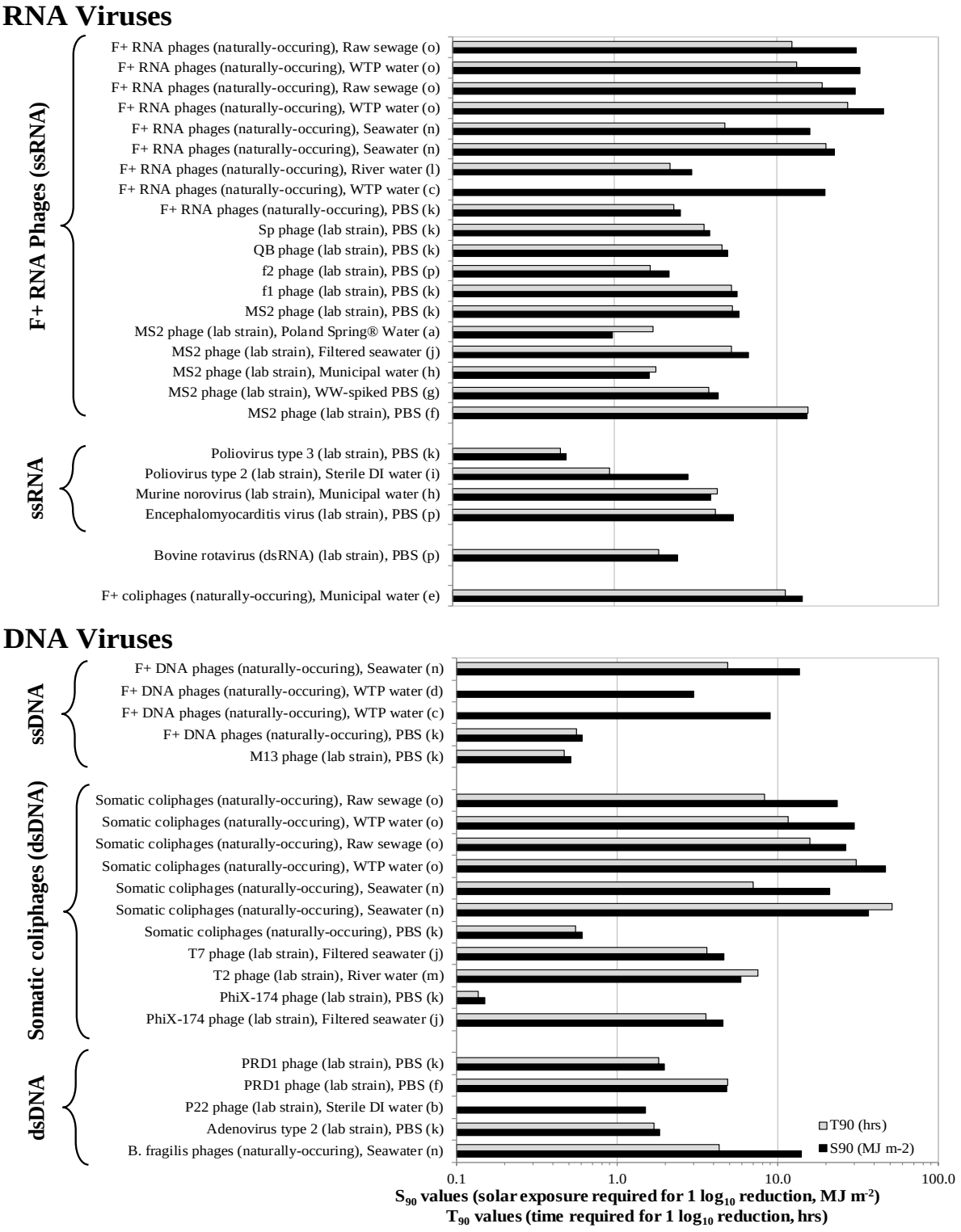
There are three different sunlight-mediated mechanisms that can contribute to virus inactivation in WSPs: direct inactivation, indirect endogenous inactivation, and indirect exogenous inactivation (Jagger 1985). The direct mechanism involves the absorption of photons by the virus, resulting in damage to the genome or capsid proteins. The indirect mechanisms involve the reaction of photons with sensitizer molecules, resulting in the formation of reactive intermediates or radicals that damage the virus. The mechanism is endogenous when the sensitizer molecules are part of the virus itself, and exogenous when the sensitizers are other molecules in the water column. The strength of radiation, the optical and physicochemical characteristics of pond water, and the properties of the particular virus species in question are all factors that influence the efficiency of sunlight-mediated inactivation mechanisms. Seasonal fluctuations in solar radiation may have important implications for WSP systems located in high-latitude regions. Radiation with wavelengths in the UVC (100-280 nm) and UVB (280-315 nm) ranges are the most efficient for direct inactivation, while wavelengths in the UVA (315-400 nm) and visible (400-500 nm) ranges can also contribute significantly to the indirect mechanism (Jagger 1985). Practically all of the UVC radiation produced by the sun is absorbed by the Earth's atmosphere, and only a small percentage of UVB radiation reaches the Earth's surface.

In a recent study of solar water disinfection, McGuigan et al. (2012) reviewed the literature on virus inactivation resulting from sunlight exposure, but did not report or calculate the corresponding S_{90} values (fluence required to achieve 90% inactivation). The S_{90} metric, which is analogous to the Ct (concentration × time) metric used for chemical disinfectants (Davies et al. 2009), provides a more useful comparison of the relative vulnerability

to sunlight-mediated inactivation for different types of viruses, since solar intensity can vary from region to region. Therefore, in order to make such a comparison, the S_{90} values for DNA and RNA viruses were collected from the studies referenced by McGuigan et al. (2012) (Dejung et al. 2007; Fisher et al. 2012; Harding and Schwab 2012; Heaselgrave et al. 2006; Rijal and Fujioka 2003; Safapour and Metcalf 1999; Sinton et al. 2002; Wegelin et al. 1994), and from several other studies of virus survival in water, wastewater or WSP water after sunlight exposure (Carey Walker et al. 2004; Davies et al. 2009; Davies-Colley et al. 1997, 2000; Fisher et al. 2011; Kapuscinski and Mitchell 1983; Love et al. 2010; Sinton et al. 1999). If S_{90} values were not provided by the authors, they were calculated (see Appendix A).

The resulting S_{90} values vary considerably with respect to virus type (even within viruses of the same family), water type, and experimental conditions (Figure 7). Laboratory strains of dsDNA and ssRNA viruses may not respond the same to sunlight as viruses in the environment; the S_{90} values for laboratory virus strains are significantly lower than the S_{90} values of their wild-strain counterparts ($p < 0.01$). The rate of sunlight-mediated virus inactivation in untreated sewage and WSP water also appears to be lower than the corresponding rate in phosphate-buffered saline (PBS). For example, the S_{90} value for wild-strain F+ RNA phage in PBS (based on data reported by Love et al. (2010)) is lower than the S_{90} values reported by Davies-Colley et al. (1997) and Sinton et al. (2002) for the same type of phage in untreated sewage and WSP water. This may be due to the fact that sunlight is much more rapidly attenuated in untreated sewage and WSP water than it is in PBS. Sunlight radiation decreases by an order of magnitude in the first 35 cm of a WSP, while the UV portion is attenuated within the first 10 cm (Davies-Colley et al. 2005a).

Data from most of the studies summarized in Figure 7 are for coliphage or animal viruses; the experiments done with human enteric viruses are all laboratory strains. Because of the limitations associated with the use of laboratory strains and enteric virus surrogates, inactivation rates for wild strains of actual enteric viruses may be different. Nevertheless, the results summarized in Figure 7 provide a general idea about the order of magnitude efficiency of solar disinfection. For example, most S_{90} values ranged between 1 and 10 MJ m^{-2}. For the most resistant groups of viruses (ssRNA and dsDNA), the time required for 90% inactivation (T_{90}) approach 50 hours, which is close to the mean HRT of some WSPs.



References

[a] (Carey Walker et al. 2004)
[b] (Davies et al. 2009)
[c] (Davies-Colley et al. 1997)
[d] (Davies-Colley et al. 2000)
[e] (Dejung et al. 2007)
[f] (Fisher et al. 2011)
[g] (Fisher et al. 2012)
[h] (Harding & Schwab 2012)
[i] (Heaselgrave et al. 2006)
[j] (Kapuscinski and Mitchell 1983)
[k] (Love et al. 2010)
[l] (Rijal and Fujioka 2004)
[m] (Safapour & Metcalf 1999)
[n] (Sinton et al. 1999)
[o] (Sinton et al. 2002)
[p] (Wegelin et al. 1994)

Figure 7. Summary of S_{90} and T_{90} values from solar disinfection studies of RNA and DNA viruses.

3.2.3.1 Direct Sunlight Inactivation

The most effective wavelength for direct light-mediated virus inactivation mechanism is ~260 nm (Jagger 1985). However, sunlight emitted at this wavelength does not reach the Earth's surface. Light in the UVB and UVA range can also directly inactivate viruses, but efficiency decreases with increasing wavelengths (Lytle and Sagripanti 2005). Due to the rapid attenuation of UV radiation in WSP water, direct sunlight inactivation likely only occurs at the pond surface.

Virus genome type influences the efficiency of direct sunlight-mediated inactivation. One of the most common virus genome deformations that results from the absorbance of photons is the formation of pyrimidine dimers (especially thymine dimers), which can prevent replication (Jagger 1985). Since RNA does not contain thymine, RNA viruses are typically more resistant to direct light-mediated inactivation than DNA viruses (Lytle and Sagripanti 2005). This has been demonstrated in laboratory experiments. For example, Davies-Colley et al. (1999) found that F+ RNA phage solar inactivation kinetics were strongly dependent on the concentration of dissolved oxygen and the presence of light-absorbing constituents in the water (inactivation likely dominated by indirect mechanisms), while the kinetics of F+ DNA phage inactivation were independent of these variables (inactivation likely dominated by the direct mechanism).

Genome size may also affect the vulnerability of viruses to direct sunlight-mediated inactivation. Lytle and Sagripanti (2005) reported that viruses with larger genomes are more susceptible to UV damage than viruses with smaller genomes. In fact, when they normalized virus inactivation rates at UV wavelengths of 254 nm (UV_{254}) to the size of the virus genomes, they found that the size-normalized sensitivities to UV_{254} were within the same order of magnitude for viruses with the same genome types (ssDNA, dsDNA, ssRNA, or dsRNA). After normalizing for genome size, dsDNA viruses are the most resistant of all to direct sunlight inactivation, followed by dsRNA viruses, ssRNA viruses, and ssDNA viruses, in that order (Lytle and Sagripanti 2005). Romero et al. (2011) also found evidence that genome size affects the vulnerability of RNA viruses to direct sunlight inactivation. They reported that porcine rotavirus was inactivated more quickly than MS2 coliphage under full sunlight, while the opposite was true when the UVB portion of the sunlight was screened. While this implies that porcine rotavirus (dsRNA) is more vulnerable to direct sunlight inactivation than MS2 coliphage (ssRNA), the size of the rotavirus genome is nearly five times the size of the MS2 genome (Michen and Graule 2010).

3.2.3.2 Indirect Sunlight Inactivation

Indirect sunlight inactivation of viruses in WSPs results from the absorbance of photons by sensitizer molecules that are endogenous (part of the virion) or exogenous (in solution) (Jagger 1985). The subsequent energy transfer to dissolved oxygen forms oxidants that can damage nucleic acids or proteins (Bosshard et al. 2013). Endogenous virus photosensitizers may include amino acid chromophores in the proteins of virus capsids (e.g. histidine, tyrosine, phenylalanine, cysteine, tryptophan) (Davies 2003; Kohn and Nelson 2007). Tryptophan exhibits the highest absorbance of solar UV, however it is not present in high quantities in virus proteins, therefore, endogenous sunlight-mediated inactivation of viruses is essentially negligible (Mattle et al. 2015). Common exogenous photosensitizers in WSPs include dissolved natural organic matter (NOM) and algal particulates (Davies-Colley et al. 1999; Kohn and Nelson 2007). Pseudo-first-order kinetic models are generally used to model overall solar virus inactivation with respect to time, but the rate of exogenous sunlight-mediated virus inactivation follows second-order kinetics (Kohn and Nelson 2007; Mattle et al. 2015). The concentration of dissolved oxygen and the extent of light penetration in WSP water are important factors for indirect sunlight inactivation mechanisms. Sunlight may penetrate deeper into maturation ponds than it will in more turbid facultative and anaerobic ponds. The concentration of dissolved oxygen is also highest in maturation ponds, followed by facultative ponds, then anaerobic ponds.

The rate of indirect exogenous inactivation in WSP water has been particularly well-characterized for MS2 coliphage (ssRNA), human adenovirus (dsDNA), and PhiX-174 phage (dsDNA), with MS2 being the most susceptible to indirect sunlight inactivation, followed by adenovirus and then PhiX-174 (Kohn and Nelson 2007; Kohn et al. 2007; Mattle et al. 2015; Romero et al. 2011). Based on data reported in the literature (Kohn and Nelson 2007; Romero et al. 2011), the S_{90} values under sunlight exposure for MS2 in WSP water and in $NaHCO_3$ buffer (with 10 mg C L^{-1}) are estimated to be 4.1 MJ m^{-2} and 0.9 MJ m^{-2}, respectively. When the UV portion of the sunlight is blocked, the S_{90} values increase to 7.9 and 6.1 MJ m^{-2}, respectively. These results imply that the rate of solar inactivation for MS2 coliphage by the indirect exogenous mechanism is slightly less efficient than its rate of inactivation by the direct mechanism under sunlight. The rate of indirect sunlight inactivation for MS2 is more efficient with higher temperatures (Romero et al. 2011) and higher total organic carbon (TOC) concentrations (Kohn et al. 2007). It also increases when the phage is located in closer physical proximity to reactive species (and presumably in closer physical proximity to photosensitizer particles in WSPs). Other factors that can affect the rate

of indirect sunlight inactivation are pond water characteristics (e.g. hardness) and the types of algal species present (Kohn et al. 2007).

Not all enteric viruses are as vulnerable to indirect sunlight inactivation as MS2 coliphage. Bosshard et al. (2013) demonstrated that the exposure of human adenovirus to sunlight (without the UVB portion) in the presence of a photosensitizer caused damage to fiber and hexon proteins, which are necessary for host cell binding and virus infectivity. However, adenovirus is still more resistant to indirect sunlight inactivation than MS2 coliphage (Mattle et al. 2015). Rotavirus is likely even more resistant. Romero et al. (20110) found that exogenous sunlight inactivation does not significantly contribute to the inactivation of porcine rotavirus, even in the presence of 20 mg C/L of Suwannee River NOM (Romero et al. 2011).

In summary, the mechanisms that contribute to sunlight-mediated virus inactivation in WSPs are dependent on the strength of solar radiation, the optical and physicochemical characteristics of WSP water, and the properties of the virus (e.g. genome type and size, and the vulnerability of capsid proteins and the virus genome to reactive species).

3.3 Disinfection of Waste Stabilization Pond System Effluent

The effluent of WSP systems can also be disinfected using a variety of different processes, such as chlorination, ultraviolet (UV) lamps, and tertiary treatment processes (e.g. rock filters, constructed wetlands). Ozone and peracetic acid are also used to disinfect wastewater, but no information was found for this review regarding the effectiveness of these disinfectants for viruses in WSP systems.

Chlorination is one approach that is used for the disinfection of treated wastewater from some WSP systems. Indeed, several states in the U.S. issue discharge permits that require WSP system operators to chlorinate effluent prior to discharge or reuse (US EPA 2011). Residual chlorine should not be discharged to rivers, so WSP systems with chlorination processes also include dechlorination. In New Zealand, the chlorination of WSP system effluent had been common practice in the past, but is no longer used due to concerns with the formation of chlorinated organic compounds, and because of its higher cost compared to the use of UV lamps (New Zealand Ministry for the Environment 2005). The European Union's Wastewater Treatment Directive 91/271/EEC does not specifically require chlorination of treated wastewater (European Union 1991), and chlorination of WSP system effluent does not appear to be common practice in most European countries.

There are a number of challenges associated with chlorinating WSP system effluent. High concentrations of algae, suspended solids, and sulfide in ponds increase chlorine demand, requiring chlorine doses as high as 20 to 30 mg/L in some systems (White and Clifford 1974). The relationship between algae concentrations and the effectiveness of chlorine-induced coliform inactivation has been shown to be inversely proportional (Polprasert and Rajput 1984), but no literature was identified about the effect of algae concentrations on the chlorine-induced inactivation of viruses. In one study, the chlorination of WSP system effluents decreased coliforms by five orders of magnitude, but the concentration of enteroviruses decreased by only 37% (Kott 1973).

The chlorination of WSP effluent may have some operational advantages, such as the reduction of suspended solids (US EPA 1983). There are also financial, safety, environmental and public health tradeoffs. Low doses of free chlorine can oxidize suspended particles, increasing the concentration of soluble COD (Johnson et al. 1978). Higher doses kill algal biomass, causing the release of nutrients in addition to the increase in BOD_5 and COD (Echelberger et al. 1971). High levels of dissolved organic matter and ammonia in WSP system effluent may also lead to the production of toxic chloramines (US EPA 1983).

The use of commercial UV disinfection lamps is the most common form of disinfecting the effluent of WSP systems in New Zealand (New Zealand Ministry for the Environment 2005). However, the efficiency of virus inactivation by UV lamps is contingent on the transmittance of radiation (typically at 254 nm) through the water column, which may present challenges. Bio-fouling of UV lamps can be caused by algae and other suspended solids (Malley, Jr. and Burris 2001), and may contribute to the shielding of viruses from harmful UV radiation. In New Zealand, rock filters and rock groynes are commonly used with WSP systems (New Zealand Ministry for the Environment 2005) to remove suspended solids. They may also help control bio-fouling on UV lamps. Apart from the works reviewed about direct UV inactivation (Section 2.3.1), no other literature was found that specifically addresses virus inactivation in WSP systems using UV lamps.

The use of additional unit processes for tertiary treatment and disinfection, such as constructed wetlands (Kivaisi 2001) and rock or plant filters (Mara and Johnson 2007), has been proposed as an effective way to upgrade WSP system effluent and achieve additional pathogen reduction. Submerged constructed wetland systems, plant filters, or reed bed systems can reduce coliphage and culturable enterovirus levels in wastewater by 2 or more $\log_{10}$ units(Williams et al. 1995). Rock filters can also be added to the end of WSP systems, to polish pond effluents and provide additional virus removal. Alcalde et al. (2003) reported a 1.7 $\log_{10}$ reduction in F-specific bacteriophage in

rock filters treating effluent from a WSP system in Israel during the summer, but less than 0.5-$\log_{10}$ removal was reported in the winter; lower reductions of somatic coliphage were reported.

The mechanisms for virus removal in free-water surface constructed wetlands will be similar to those that occur in maturation ponds. The main difference between the two is that in constructed wetlands, emergent macrophytes may block sunlight from reaching the water, potentially decreasing the efficiency of sunlight-mediated inactivation mechanisms. However, the root zone of these plants may also provide a site for attached biomass growth, which can potentially assist with the removal of viruses (Jackson and Jackson 2008). Mara (2006) argued that constructed wetlands do not necessarily provide an added benefit as an addition to WSP systems because they generally require the same amount of land area as additional maturation ponds. Nevertheless, constructed wetlands with emergent plants may have other environmental and social advantages, such as nutrient uptake and the restoration of wildlife habitats (Muga and Mihelcic 2008).

New technological developments in constructed wetlands systems may enable improved virus removal efficiencies. Modular wetland treatment units designed to naturally select for the growth of periphyton species are currently being developed with the objective to optimize the removal of pathogens (Nelson et al. 2013) and emerging trace contaminants (Jasper et al. 2013). These units, which naturally select for periphyton species that grow close to the bottom of the water profile, allow for greater sunlight penetration, which increases the efficiency of virus inactivation.

3.4 Virus-Particle Interactions

Viruses in WSPs may be enmeshed within bio-flocs or adsorbed to suspended particles such as NOM, algae, bacteria, colloidal materials, and biological or chemical flocs (Templeton et al. 2008). The association with particles as small as 7 μm can shield viruses from UV exposure and reduce their vulnerability to direct sunlight inactivation (Templeton et al. 2005); however, the association of MS2 coliphage with NOM may actually enhance its vulnerability to indirect sunlight inactivation (Kohn et al. 2007). Adsorption of viruses to a surface is dependent on the characteristics of the virus, the surface, and the surrounding environment (Gerba 1984). Bacteriophages (especially MS2 coliphage) have been used previously as surrogates for enteric viruses in particle-association studies (e.g. Dika et al. 2011; Templeton et al. 2005). One important difference between the adsorption of

bacteriophages and enteric viruses in WSP systems is that phages adsorbed to bio-aggregates containing their bacterial host can multiply, while enteric viruses cannot multiply outside of the human host.

If viruses become associated with larger particles in wastewater treatment ponds, they may be removed via sedimentation. Authors from earlier publications have reported that virus-particle association and sedimentation is one of the main mechanisms responsible for virus removal in WSPs (Feachem et al. 1983; Mara 2003; Shuval 1990). However, there is limited evidence in the literature to support this theory.

3.4.1 Theoretical Considerations for Virus-Particle Interactions

Mechanisms driving virus-particle interactions in water are governed in part by the principles of extended Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, which considers van der Waals forces, electrostatic double layer forces, hydrophobic interactions (Wong et al. 2012), and osmotic interactions between particles (although osmotic interactions are negligible for microorganisms (Hermansson 1999)). Steric interactions and hydration forces may also hinder the association of viruses with particles (Gutierrez and Nguyen 2012).

Electrostatic interactions between viruses and particles are influenced by the overall surface charge of the virus capsid and of the particle surface (Templeton et al. 2008). The different carboxyl groups and amines in a virus capsid may ionize at different pH levels (Flint et al. 2009), giving the virus an electrical charge which can vary with respect to the location on the capsid surface (Templeton et al. 2008). Results from recent work suggest that virus genetic material may also play a role in the overall net charge of viruses (Dika et al. 2011). The isoelectric point of a virus is defined as the pH at which ionization of virus capsid functional groups is such that the net charge of the virus is neutral. In general, viruses have a net positive charge at pH levels below the isoelectric point, and a net negative charge at pH levels above the isoelectric point (Templeton et al. 2008). Suspended and dissolved particles in WSPs (e.g. NOM, humic materials, algal solids, bioflocs) should have a net negative charge at the pH levels typically encountered in WSPs. The pH level in anaerobic ponds is typically neutral; the pH in facultative ponds may be slightly higher; maturation ponds typically have the highest pH levels (often as high as 9). The isoelectric point of most waterborne viruses is generally between 3.5 and 7 (Michen and Graule 2010). It would follow that viruses in WSPs would tend not to be particle-associated. However, this is not necessarily true in all circumstances because other mechanisms may favor virus attachment (Templeton et al. 2008). Increased ionic strength and hardness of water favors increased virus attachment to particles (Kohn et al. 2007; Schijven and Hassanizadeh

2000), and these conditions may vary in WSPs due to differences in climate, geology, and human behavior (water use practices).

Hydrophobic interactions between viruses and particles are also believed to play an important role in virus-particle associations (Schijven and Hassanizadeh 2000). Proteins in virus capsids can contain spans of hydrophobic amino acids, which may explain why some viruses (e.g. rotavirus) aggregate at pH levels above their isoelectric point (Gutierrez et al. 2010). The increased hydrophobicity of a virus particle would make it more likely to interact with other hydrophobic surfaces. Similarly to electrostatic interactions, hydrophobic interactions are dependent on the characteristics of the virus, the particle surface, and the water (Schijven and Hassanizadeh 2000). Steric effects, such as steric hindrance, have been implicated in several studies on the interaction of microorganisms with particles or surfaces (Foppen et al. 2008). The presence of divalent cations can alter the surface of NOM, in some cases reducing the steric effects and allowing for better adsorption of viruses to organic matter (Pham et al. 2009).

3.4.2 Particles in Waste Stabilization Ponds

Virus-particle interactions are dependent on the characteristics of the particles. Suspended particles and dissolved NOM in untreated sewage differs structurally and chemically from that which is found in natural water reservoirs (Ma et al. 2001). Specifically, wastewater NOM contains less humic material and more hydrophilic material than NOM from natural aquatic systems, and provides higher quantum yields of reactive oxygen species (Mostafa and Rosario-Ortiz 2013) which can inactivate viruses. WSP suspended solids mostly consist of algae (Mara 2003), which is different from the suspended solids in untreated sewage and in wastewater treated by other processes. Maturation ponds will typically contain higher concentrations of algae than other pond types, especially anaerobic ponds. Anaerobic ponds may also change the composition and characteristics of wastewater in ways that are different from aerobic ponds. Barker et al. (1999) found that the majority of the soluble residual COD in the effluent of anaerobic reactors consisted of material with lower molecular weight distributions (<1 kDa) than those found in the effluent of aerated systems (Barker and Stuckey 1999). In a manure soil amendment study, Davis et al. (2006) reported that MS2 coliphage in anaerobically-treated dairy manure absorbed to soil more readily than MS2 coliphage in untreated manure; however, the treatment did not affect poliovirus in the same way. Metals are also reduced in this environment and in aerobic conditions, they can become mobile. Metals such as Fe may also coat particles in wastewater, forming and dissolving under different redox conditions (Leppard et al. 2003).

3.4.3 Microbial Communities in Waste Stabilization Pond Systems

The microbial communities present in WSPs also influence virus-particle associations. Viruses in WSPs display reversible adsorption to suspended algae and bacteria (Sobsey and Cooper 1973), which may depend on the level of dissolved oxygen in the water (Ohgaki et al. 1986). Viruses may also be internalized by larger organisms (e.g. nanoflagellates), and could potentially settle to the bottom of ponds with these larger organisms (see Section 3.2.2). The influence of the overall microbial communities present in WSP systems may be especially relevant for polishing ponds treating the effluent of different types of anaerobic or aerobic reactors. Anaerobic reactors are frequently used in combination with polishing ponds, especially in tropical regions and developing countries. The microbial communities in some reactors, such as upflow anaerobic sludge blanket reactors are subjected to conditions that stimulate the production of granular sludge. Viruses may become enmeshed within these substances (see Chapter 4 of this dissertation). Anaerobic granular sludge is also quite sensitive to abrupt changes in the characteristics of wastewater, and as a result these biogranules sometimes disintegrate (Liu et al. 2004). If viruses are enmeshed within biogranules that subsequently disintegrate into smaller pieces and exit a reactor, viruses may enter polishing ponds associated with smaller particles.

3.5 Relationship between Pond Hydraulics and Virus Removal

WSPs are non-ideal reactors, and just as the hydraulic efficiency of a pond can influence the removal of parasites (Verbyla et al. 2013) and bacteria (von Sperling 2005), it can also influence the efficiency of virus removal by creating short circuits and dead zones, resulting in HRT that is lower than the theoretical value (Herrera and Castillo 2000). Overall virus removal in ponds has been assumed to be pseudo-first order, and the rate of exogenous sunlight-mediated virus inactivation in WSP waters has second-order kinetics (Kohn and Nelson 2007; Mattle et al. 2015). Therefore, individual pond geometry and hydraulics plays an important role in virus removal. Many different modeling approaches have been used to estimate hydraulic efficiency and the degree of mixing in WSPs, but very few of them have been correlated with pathogen removal (Sah et al. 2012), and even fewer studies combine assessments of WSP hydrodynamics with virus analyses (e.g. Frederick and Lloyd 1996; Herrera and Castillo 2000; Macdonald and Ernst 1986; Pedahzur et al. 1993). Still, these four studies indicate that the reduced hydraulic efficiency can decrease the efficiency of virus or phage removal (see Table 2). In fact, using the theoretical HRT, the observed virus or phage removals are less than the removal rates reported by Shuval (1990); however, when the

actual mean HRT values are used, the observed virus or phage removal is either within the range of rates reported by Shuval (1990) or very close to this range.

Predicting the extent of hydraulic short-circuiting in ponds (without a tracer study) may assist the prediction of virus removal, but this is not a simple task. Computational fluid dynamics (CFD) models may be accurate, but require a large computational demand. Dispersed flow and tanks-in-series (TIS) models are computationally less demanding, but have other limitations, especially for WSP systems; for example, the dispersed flow model cannot account for back-mixing, and neither the dispersed flow nor the TIS model is capable of incorporating dead zones or recirculating fluxes, which can considerably impact the hydraulics of WSPs (Alvarado et al. 2012). Persson (2000) used CFD modeling to demonstrate that ponds with equal volumes, but different configurations, can have different HRT distributions, despite having similar theoretical mean HRT values. The results from dye tracer studies of full-scale WSP systems also reflect this potential high variation in HRT distribution. Torres et al. (1999) concluded that three facultative ponds in Spain exhibited hydraulic behavior that was similar to a completely mixed reactor. Macdonald and Ernst (1986) however, reported a mean HRT that was 25% lower than its theoretical value in an Australian system consisting of four ponds in series, and detected dye in the effluent of the system after less than 5% of the theoretical HRT.

3.6 Virus Removal in Field Studies

3.6.1 Development of a Database for Virus Removal in Wastewater Pond Systems

A systematic review of field studies (see Appendix A) yielded a total of 48 publications describing virus or bacteriophage removal from 70 different WSP systems (Alcalde et al. 2003; Bausum et al. 1983; Benyahya et al. 1998; Betancour 2013; Botero et al. 1997; Campos et al. 2002; Ceballos et al. 1995; Davies-Colley et al. 2005b; Donnison and Ross 1995; El-Deeb Ghazy et al. 2008; Emparanza-Knörr and Torrella 1995; England et al. 1967; Fattal et al. 1998; Hadley 2013; Herrera and Castillo 2000; Hodgson and Paspaliaris 1996; Iriarte et al. 2013; Jenner 2009; Klock and John 1971; Kott et al. 1973, 1978; Lewis et al. 1986; Lijklema et al. 1986; Lucena et al. 2004; Macdonald and Ernst 1986; Malherbe and Strickland-Cholmley 1967a; b; Morris 1984; Nupen 1970; Nupen et al. 1974; Ohgaki et al. 1986; Omura et al. 1985; Oragui et al. 1987, 1995; Pearson et al. 1995; Pedahzur et al. 1993; Rao et al. 1981; Reinoso et al. 2008b, 2011; Salter et al. 1999; Sheladia et al. 1982; Shuval 1970; da Silva et al. 2008; Silverman et al. 2013; Soler et al. 1995; Turner and Lewis 1995; Zhenbin et al. 1993).

Table 2. Studies reporting virus removal and hydraulic efficiency in the same pond system.

Location	Number of Ponds in Series	Type of Virus/Phage	Theoretical Overall HRT[a] (Actual Mean HRT) (days)	Observed Overall Virus/Phage Removal (log_{10} reduction)	Days of theoretical HRT (actual HRT) for each log_{10} removal of viruses	Notes	Reference
West Camden, Australia	2	Culturable enteric virus using cell line inoculations	34 (14.1)	1.2[b]	28.3 (11.8)	Both ponds were baffled, but authors observed dye leaking through baffle joints	MacDonald & Ernst (1986)
Windsor, Australia	4	Culturable enteric virus using cell line inoculations	16 (12.0)	1.2[b]	13.3 (10.0)	These ponds were not baffled, and treated the effluent of a trickling filter system	MacDonald & Ernst (1986)
Sha'alvim, Israel	1	F+ coliphage (using host *S. typhimurium* WG-49)	5 (2.5 - 4.5)	0.8	6.3 (3.1 - 5.6)	For this experiment, the pond had four horizontal plastic-cloth baffles	Pedahzur et al. (1993)
Cayman Islands	1[c]	Bacteriophage (using host *Serratia marcescens*SM24)	11.5 (1.7)	Not Reported (phage used as tracer)	n/a	Authors reported that prevailing wind direction increased short-circuiting	Frederick & Lloyd (1996)
Melipilla, Chile	2	Coliphage (authors do not report which *E. coli* strain was used as host)	29.2 (11.1)	1.3[d]	22.4 (8.5)	Experiments were carried out in the winter	Herrera & Castillo (2000)
Melipilla, Chile	2	Coliphage (authors do not report which *E. coli* strain was used as host)	38.3 (13.0)	2.9[d]	13.2 (4.5)	Experiments were carried out in the summer	Herrera & Castillo (2000)

Notes:

[a] HRT = hydraulic retention time

[b] The authors reported virus concentrations after 16 and 10 days of retention for the West Camden and Windsor systems, respectively, which do not correspond exactly to the reported mean overall hydraulic retention times for these systems

[c] This system actually had two facultative ponds in parallel, followed by two maturation ponds in series, but the authors only studied one of the first two facultative ponds

[d] The removal of phage in this pond system was not directly measured by the authors; inferred removal values are reported here based on the coliphage decay coefficient reported by the authors based on batch experiments with the same pond water and the dispersed flow model using dispersion coefficients based on the authors' results from the dye tracer study.

Results for WSP systems in Yungas, Bolivia (see Section 4.2) and a municipal WSP system in Michigan (see Section 4.3) were also used in the analysis, bringing the total number of systems up to 71. One of the publications presented data from a high-rate pond system (Davies-Colley et al. 2005b), including ponds with mechanical mixing and other unique design aspects; this data was not used for subsequent analyses. References were found for eight additional publications that may have contained useful data, but the journals were not accessible. If data were presented from different times throughout the year, the mean $\log_{10}$ removal and HRT values were used. If data were presented for several different types of viruses in the same WSP system, a unique value for $\log_{10}$ removal was used for each virus type. Figure 8 contains histograms showing the distribution of characteristics of the WSP systems included in this analysis (latitude, number of ponds in series, viruses analyzed, and theoretical HRT). Information about the types of ponds in each system (anaerobic, facultative, maturation, polishing, surface-aerated) was also recorded (Appendix A, Figure A1), although some authors did not report this information. The final set of data included 106 values for theoretical HRT paired with an equal number of values for mean $\log_{10}$ virus or bacteriophage removal.

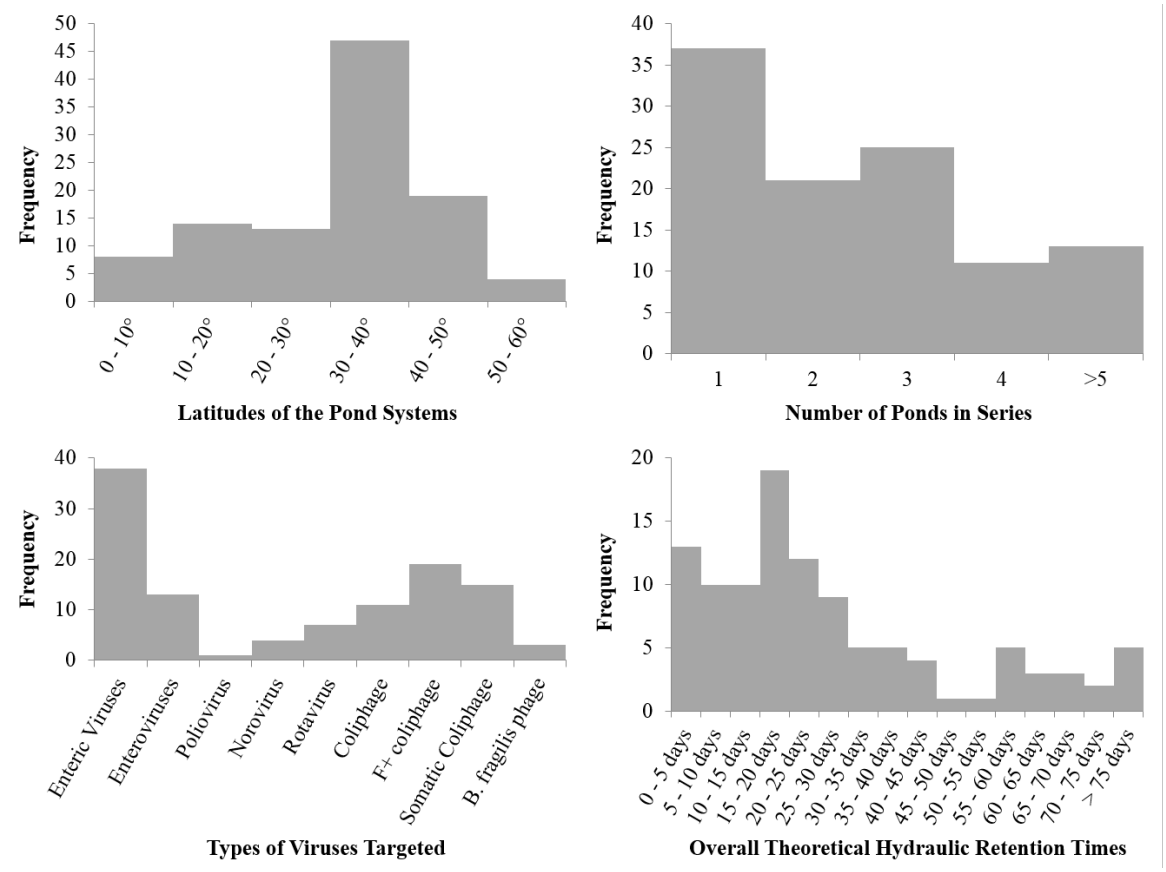


Figure 8. Histograms showing the characteristics of the pond systems and viruses targeted in the studies.

A variety of methods were used by the authors of the different studies to quantify viruses and bacteriophages in wastewater samples (Appendix A, Figure A2). Of the 106 points in the database, six of them came from studies where authors only reported the presence or absence of viruses in replicate samples. For these data, virus concentrations were calculated using a maximum likelihood estimator (Jarvis et al. 2010, see Appendix A).

A ratio (termed here as the HRT-removal ratio) was calculated for each system by dividing the theoretical HRT of the overall system by the overall $\log_{10}$ reduction of viruses (the difference between concentrations reported at the influent and effluent of each system). This ratio represents the number of days of HRT required for each $\log_{10}$ reduction in the concentration of viruses in the WSP systems analyzed. A probability plot correlation coefficient test for normality (NIST/SEMATECH 2012) indicated that the HRT-removal ratios are log-normally distributed, so the natural logarithms of the HRT-removal ratios were used in subsequent statistical analyses. Six data points were found to be outliers based on their positions in a Q-Q plot. They were removed from the data set. The remaining 100 points were used in the analyses.

3.6.2 Statistical Analysis and Discussion of Virus Removal

The theoretical hydraulic retention times and the corresponding mean $\log_{10}$ virus removals reported in the literature are plotted in Figure 9. As mentioned previously, the overall consensus about virus removal in WSPs has changed considerably throughout the years. Feachem et al. (1983) reported that enteric viruses would be reduced by 1- to 2-$\log_{10}$ units for every five days of retention in ponds with temperatures exceeding 25°C; but only 22% of the total observations from the literature (28% for phages, 19% for enteric viruses) fell within this range (70% are lower than this range). Shuval et al. (1986) and Shuval (1990) concluded that virus removal in a 20-day pond system with temperatures above 20°C should be between 2- and 4-$\log_{10}$ units, a claim recently cited by Drechsel et al. (2010); however only 32% of observations (30% for phages, 34% for enteric viruses) fell within this range and 37% were lower than this range. Maynard et al. (1999) reported that overall removal in ponds was generally less than one $\log_{10}$-unit; however virus removal was greater than one $\log_{10}$ in 62% of the field studies (65% for phages, 59% for enteric viruses).

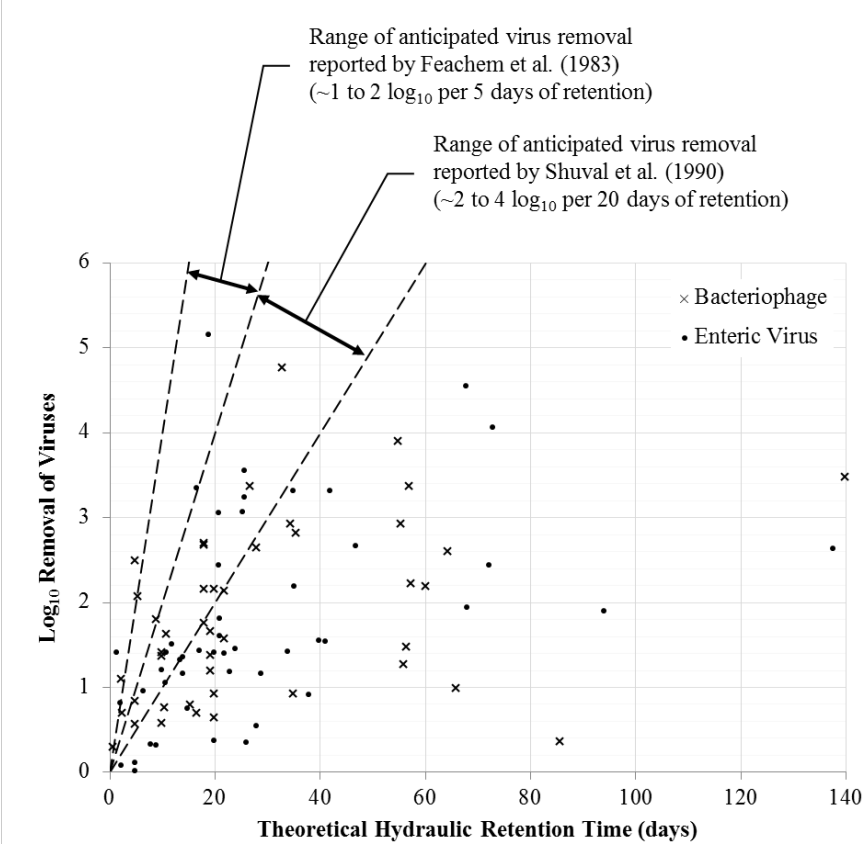


Figure 9. Theoretical hydraulic retention times versus mean $\log_{10}$ virus removals from the literature.

There do not appear to be any trends that adequately describe the removal of viruses in WSP systems based on the theoretical HRT alone. There is only a weak to moderate correlation between the theoretical HRT and the virus removals reported (Pearson's r = 0.34, p = 0.001). Additionally, the correlation between HRT and bacteriophage removal (Pearson's r = 0.37, p = 0.017) is nearly the same as the correlation between HRT and enteric virus removal (Pearson's r = 0.32, p = 0.013). The best-fit log-linear function only explains 12% of the variance in the data, and the best-fit log-log function explains only 18% of the variance. A log-linear regression using two factors (HRT and number of ponds in series) only explains 23% of the variance in the data. None of these models would be adequate for the specification of a design equation or guidelines for virus removal based on theoretical HRT alone.

In order to evaluate virus removal in systems with different numbers of ponds in series, data were organized into groups based on the number of ponds in series. Welch's one-way ANOVA and the Games-Howell post-hoc test (Wilcox 1987) were performed on the log-transformed HRT-removal ratios for pond systems with a different number of ponds in series (α = 0.01), and results indicate that systems with more ponds in series do not

have significantly higher HRT-removal ratios. There was also no significant correlation between the number of ponds in series and the HRT-removal ratios, and the correlation coefficients for phages were the same as they were for enteric viruses (Pearson's r = -0.1, p = 0.32 and 0.25 for phages and enteric viruses, respectively). Ninety-five percent of the HRT-removal ratios (for all systems and all virus types) are less than 54 days (see Figure 10); and the geometric mean of these ratios is 14.5 days. However, using Cox's method for log-normally distributed variables (Zhou and Gao 1997), the mean HRT-removal ratio for all virus types in all WSP systems (regardless of the number of ponds in series) is estimated to be 20.9 days (95% C.I.: 17.2, 25.5). The correlation between virus removal and solar insolation was also investigated (using average daily solar insolation values for the latitude of each pond system, gathered from the United States National Aeronautics and Space Administration (NASA 2014)). For pond systems with one, two, three, and four ponds in series, there was no significant correlation between the HRT-removal ratios and solar insolation. For systems with more than four ponds in series, Pearson's r was equal to -0.62 (see Appendix A, Table A1, Figure A3).

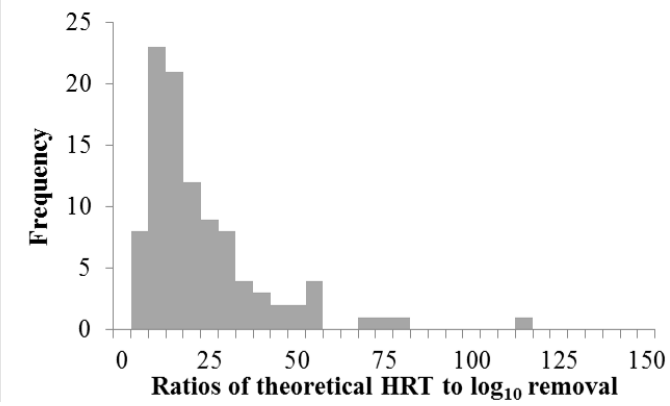


Figure 10. Histogram of the ratios of the overall theoretical hydraulic retention time and the log_{10} removal of viruses in the waste stabilization pond systems.

3.6.3 Limitations of the Analysis of Data from Field Studies

The results associated with this analysis of virus removal in field studies have the following limitations: 1) much of the data used in the analysis are from studies that used bacteriophage as a surrogate for human enteric viruses; some phages (somatic coliphage in particular) have poor specificity for predicting human versus animal fecal contamination (Harwood et al. 2013); 2) the data represent measurements made using a variety of methods to quantify virus concentrations (Appendix A, Figure A2), which may have different sensitivities and specificities (Flint et al. 2009); 3) the data represent observations made on systems with a mixture of different pond types (e.g. anaerobic, facultative, maturation/polishing), and virus removal efficiency may be greater in maturation ponds than

it is in other types of ponds; and, 4) the data only represent the mean $\log_{10}$ removal of viruses and the mean theoretical HRT of the overall WSP system (the variability or uncertainty associated with such estimates is often not reported in the literature). Values reported for theoretical HRT in each of the studies may not even be based on actual flow rates measured during the time of virus sample collection. It is possible that the flow rates or HRT values reported by the authors of the studies reviewed in Section 6.2 were only estimates (rather than measured values).

3.7 Summary, Knowledge Gaps, and Conclusions

This review presents the first comprehensive analysis of overall virus removal in 70 WSP systems located throughout the world, demonstrating that there is only weak to moderate (but significant) correlation between the theoretical HRT of pond systems and the $\log_{10}$ removal of viruses. The virus removal rates from these field studies are highly variable and are generally lower than the rates reported previously by Feachem et al. (1983), Shuval et al. (1986), Shuval (1990), Maynard (1999), and Drechsel et al. (2010). In the past decade, much progress has been made toward increasing the understanding of the mechanisms responsible for virus removal in WSP systems. Recent research is helping to elucidate the relative importance of direct versus indirect sunlight-mediated mechanisms. While efficiency differs between virus species, sunlight disinfection efficiency may be correlated to virus genome type and length (e.g. Lytle and Sagripanti 2005), and the characteristics of virus capsid proteins (e.g. Bosshard et al. 2013). Another major finding, which contradicts some earlier conclusions about virus removal in ponds, is that virus associations with particles in WSPs do not necessarily result in virus removal by sedimentation (da Silva et al. 2008; also, see Chapter 4 of this dissertation). Bacteriophages have been used as surrogates to help understand enteric virus removal in WSP systems. MS2 coliphage in particular is recommended as a surrogate to study sunlight-mediated virus inactivation in WSPs (Mattle et al. 2015). The results from the analyses used in this review do not indicate any statistically significant difference between the removal of bacteriophages and the removal of enteric viruses in WSP systems, however more research may be needed to confirm this.

There are still many gaps in the literature. For example, there is no model that adequately and accurately describes virus removal in WSP systems. There is a need to develop a design equation for virus removal in WSP systems that can be used for water reuse planning, and can be incorporated into the next edition of the World Health Organization Guidelines for Wastewater Use in Agriculture (WHO 2006). A better understanding about the relationship between virus removal and the hydraulic efficiency of ponds may be needed in order to develop such a

design equation. Another major area where future research is needed is on the interaction of viruses with particles and with other micro- and macro-organisms in WSPs. It is still unclear whether virus internalization by higher-trophic organisms has a protective or a detrimental effect on virus viability in WSP systems. Also, the effect of virus-particle associations on sunlight-mediated inactivation mechanisms is still unclear. Recent advances in the development of genomic and proteomic methods provide unique opportunities for future research to help elucidate the fundamental mechanisms responsible for virus removal in WSP systems (e.g. Bosshard et al. 2013). Also, the recent development of the periphyton oxidation pond (Nelson et al. 2013) also provides a promising outlook for a new WSP technology that may improve the efficiency of virus removal.

4 VIRUS-PARTICLE ASSOCIATIONS IN WASTE STABILIZATION PONDS AND UASB REACTORS[4]

4.1 Overview

There is insufficient evidence in the literature to determine whether or not virus settling (after association with larger particles) is an important removal mechanism in WSPs (see Chapter 3). Virus-particle associations in WSP systems have also not been well-studied. The use of different wastewater treatment technologies in combination with WSPs may change the dynamics of virus-particle associations and overall virus removal efficiency. The use of UASB reactors to treat domestic wastewater is rapidly gaining acceptance in low-income countries and in countries with emerging economies (Lettinga 2010). In Latin America and South Asia, new wastewater treatment systems are often designed with UASB reactors followed by WSPs, and UASB reactors are also commonly added to the front of existing WSP systems to increase the overall treatment capacity in cities and towns with growing populations. Given that UASB reactors are specifically designed to allow for the production of granular sludge, it is important to understand whether or not the use of UASB reactors in combination with WSPs changes virus-particle associations or affects the overall efficiency of virus removal.

The objective of this chapter is to measure the removal of viruses and their association with particles in WSPs, UASB reactors, and systems with UASB reactors and WSPs. Section 4.2 includes a comparative case study of virus removal in the same two WSP systems presented in Section 2.1, which serve two similar small towns in the Yungas region of Bolivia. One system consists of three ponds in series, and the other consists of a UASB reactor followed by two ponds in series. Quantities of several enteric viruses and proposed viral surrogate PMMoV are measured in water samples from both systems, and the associations of these viruses with wastewater particles are measured using a cascade filtration study. The additional virus removal required for safe reuse for irrigation is estimated for both systems using QMRA. The study presented in Section 4.3 expands upon the preliminary findings

[4] Sections 4.2 and 4.3 are written as stand-alone documents and formatted as manuscripts. The reason for this is because Section 4.2 was published in *Water Research* in 2014. The research presented in Section 4.3 builds off of the findings from Section 4.2, and an abstract for the material presented in Section 4.3 was accepted for presentation at the 11th IWA Specialist Group Conference on Wastewater Pond Technologies, in Leeds, UK, on March 21 – 23, 2016. Section 4.3 (or a slightly shorter version of it) will be submitted to the conference program committee by December 11, 2015, for publication in the conference proceedings.

from the virus-particle association study presented in Section 4.2 by measuring the quantities of an additional enteric virus (human adenovirus (AdV)) in the Yungas system samples, and in samples collected from three other wastewater treatment systems in the United States and Brazil with WSPs, UASB reactors, or a combination of both technologies.

4.2 Enteric Virus Removal and the Risk of Water Reuse for Two Waste Stabilization Pond Systems[5]

4.2.1 Introduction

Freshwater resources are limited and a growing number of watersheds may be affected by freshwater scarcity because of changes in climate, urbanization, population, and land use (Fry et al. 2008). Accordingly, it has become increasingly important to assess wastewater for its reuse potential, especially in expanding small cities and towns that are geographically and economically connected to agricultural areas (Chapter 2 of this dissertation). Despite the potential health concerns associated with human pathogens in domestic wastewater, this water source is rich in biologically-available nutrients and is currently used to irrigate 20 million hectares globally (Raschid-Sally and Jayakody 2008). However, 1.5 billion people worldwide still discharge untreated wastewater (Baum et al. 2013) and 3.5 million hectares of crops are irrigated with untreated wastewater (Drechsel et al. 2006). In places where wastewater reuse for agriculture is a priority, it is necessary to identify practical treatment technologies for removal of pathogens.

Waste stabilization ponds (WSPs), which consist of shallow engineered basins, stabilize and treat wastewater via natural processes driven by sunlight and biological flora proliferation and predation. WSPs are one of the most prevalent treatment technologies in the world, particularly for small towns and developing communities (Maynard et al. 1999; Oakley 2005a). There are many types of ponds (e.g. high-rate algal ponds, aerated ponds); however, a common configuration consists of a facultative pond followed by one or more maturation ponds. Facultative ponds are typically designed to reduce biochemical oxygen demand (BOD) and total suspended solids (TSS), whereas maturation ponds are designed to remove pathogens (Maynard et al. 1999). While some WSP

[5] Reprinted from *Water Research*, Volume 65, E.M. Symonds, M.E. Verbyla, J.O. Lukasik, R.C. Kafle, M. Breitbart, J.R. Mihelcic, "A case study of enteric virus removal and insights into the associated risk of water reuse for two wastewater treatment pond systems in Bolivia." Pages 257 – 270. Copyright 2014, with permission from Elsevier. Permission is included in Appendix F.

The two lead authors for this publication, E.M. Symonds (EMS) and M.E. Verbyla (MEV), were credited as having made equal contributions to the work. EMS and MEV both determined the research questions, which were reviewed by JRM and MB; EMS took the lead on designing the sampling and laboratory methods with support from MB; EMS performed the laboratory work with assistance from MEV; the culturable EV analysis was done by JOL; MEV took the lead on the quantitative microbial risk assessment with support from RCK. EMS and MEV both wrote the manuscript and the supporting information (found in Appendix B), with review and input from all other authors.

systems utilize chemical disinfectants for pathogen reduction, many rely on natural mechanisms that are complex and poorly understood (particularly for virus removal). Previous investigations have demonstrated that WSP systems consisting of a facultative pond followed by two maturation ponds can provide > 4-$\log_{10}$ removal of bacterial indicators (von Sperling 2005); however, the extent of enteric virus removal in WSPs is not as well understood.

Enteric virus removal in WSPs can occur through sedimentation (when viruses are attached to settleable particles), predation, or disinfection (e.g. nucleic acid damage by UV light; capsid damage via high temperatures, high pH levels, and exposure to reactive oxygen species). However, the relative importance of each of these mechanisms is not clear, and dominant removal mechanisms may vary for different viral types (Maynard et al. 1999). Virus settling in WSPs is contingent upon virus-particle adsorption, which is driven by a combination of electrostatic, hydrophobic, and steric interactions and is dependent on environmental conditions and the characteristics of both particle surfaces and viruses (Templeton et al. 2008). Some studies have shown significant virus adsorption to non-settleable particles (<180-μm), implying that sedimentation may not be the driving virus removal mechanism (da Silva et al. 2008; Templeton et al. 2008). Furthermore, virus association with non-settleable particles may affect their vulnerability to disinfection, either by protecting them from direct sunlight inactivation or by making them more vulnerable to indirect sunlight-mediated inactivation mechanisms (Kohn et al. 2007; Templeton et al. 2008). Previous studies that have addressed enteric virus removal in pilot-scale WSP systems in Brazil (Oragui et al. 1987, 1995) and in full-scale WSP systems in Egypt (El-Deeb Ghazy et al. 2008), France (da Silva et al. 2008), India (Rao et al. 1981), and the United States (Bausum et al. 1983), have demonstrated highly-variable removal, ranging from 1- to 5-$\log_{10}$.

Over the past 30 years, high-rate anaerobic reactors, such as upflow anaerobic sludge blanket (UASB) reactors, have become more widely accepted for the treatment of domestic wastewater, particularly in warm climates (Lettinga 2010). Unlike facultative ponds, UASB reactors allow for biogas recovery. However, UASB reactor effluent requires post-treatment for pathogens, which is frequently accomplished with maturation (polishing) ponds (Chernicharo 2007). UASB reactors also differ from other wastewater treatment reactors because they are optimized for the formation of large biological flocs and granular sludge (biogranules), which may enmesh viruses. However, the influence of particle association on virus removal in UASB-pond systems is not well-understood.

The two systems analyzed in this study are located in the remote, tropical Yungas region of Bolivia. The first system (three-pond system) consists of a facultative pond followed by two maturation ponds; the other (UASB-

pond system) consists of a UASB reactor followed by two polishing ponds (Table 3). These systems have been evaluated previously for their overall treatment performance (Muga et al. 2009b), appropriateness (Fuchs and Mihelcic 2011), resource recovery potential (Chapter 2 of this dissertation), parasite removal, and hydraulic efficiency (Verbyla et al. 2013). A recent study also quantified the reduction in environmental impacts (e.g. eutrophication potential, global warming potential, embodied energy) if the treated wastewater from these systems was reused for irrigation (Cornejo et al. 2013). Water reuse is a particular priority for these communities, given that water stress in this region has been linked to climate change (Fry et al. 2012). In Bolivia, acute diarrhea contributes to 5% of all deaths and a disease burden of 0.015 disability-adjusted life years (DALYs) per person annually (Prüss-Üstün et al. 2008).

Table 3. The waste stabilization pond systems analyzed in this study.

System (population served in 2012)	**Sampling Sites**	**Pond Dimensions** (L × B × D, m)	**Theoretical hydraulic retention (days)**	
			June 17, 2012	**June 19, 2012**
Three-Pond (780 people)	A Untreated sewage[a] B Facultative pond (effluent) C Maturation pond 1 (effluent) D Maturation pond 2 (effluent)	 50x27.5x1.8 39x13x1.5 39x13x1.5	 16.8 4.6 4.6 **Total = 26.0**	 16.5 4.5 4.5 **Total = 25.5**
UASB-Pond (1,310 people)	F Untreated sewage[b] G UASB reactor (effluent) H Polishing pond 1 (effluent) I Polishing pond 2 (effluent)	 60x21x1.5 60x23x1.5	 1.2 26.8 29.6 **Total = 57.6**	 0.6 12.4 13.7 **Total = 26.7**

[a] Sample collected at the influent of the facultative pond (this system has no grit chamber)
[b] Sample collected at the influent of the UASB reactor (effluent of the grit chamber)

Because human enteric viruses pose a threat to public health, are resistant to wastewater treatment, and can persist in the environment, the primary objective of this investigation was to measure the removal of culturable enteroviruses (EV), as well as the removal of human norovirus genotype I (NoVGI), human rotavirus (RV) group A, and pepper mild mottle virus (a recently proposed surrogate for enteric viruses; PMMoV (Rosario et al. 2009)) using quantitative polymerase chain reaction with reverse transcription (RT-qPCR). Secondarily, virus-particle associations were studied to help elucidate possible virus removal mechanisms. Finally, the additional virus removal required for safe reuse of effluent from these two systems for restricted irrigation was evaluated using a quantitative microbial risk assessment (QMRA) model. The effluent from these two systems is not currently reused, but wastewater reuse is common practice in Bolivia. More than 7,000 hectares are currently irrigated with wastewater in

Bolivia, and the government has proposed a strategy to increase wastewater-irrigated agriculture to 10,000 hectares by 2025 (Viceministerio de Recursos Hídricos y Riego 2013). Furthermore, local farmers have expressed interest in restricted reuse for irrigating fodder crops or high-growing fruit trees in light of increasing water scarcity.

This study is among a handful of investigations in which enteric virus removal is evaluated in full-scale WSP systems. Despite the limited sampling period, this study also represents the first time, to our knowledge, that virus removal is compared in a conventional full-scale stabilization pond system and a WSP system with a UASB reactor and polishing ponds. Furthermore, measurements of virus concentrations are used in the QMRA model to predict the additional virus removal required to protect adult farmers and children at play, providing important perspective on the suitability of treated wastewater reuse in agriculture.

4.2.2 Materials and Methods

4.2.2.1 Wastewater Composite Samples and Sludge Collection

Composite, 24-hour wastewater samples were collected simultaneously from both systems on two different days in June 2012 (Table 3). Samples from sites B, C, D, G, H, and I (described in Table 3) were collected during the first sampling event and analyzed for virus-particle associations. Samples from all sites were collected during the second sampling event in order to analyze the removal of viruses from the systems. The second set of samples from the UASB-pond system was limited to a 13-hour collection, such that sampling did not occur overnight due to unexpected rainfall substantially increasing flow rates; however, the 13-hour collection encompassed both known morning and evening peaks in flow. Sludge core samples were collected from the UASB reactor and from the facultative pond (near the influent pipe) after the second sampling event, using a ½-inch PVC pipe, and were placed in sterile, opaque, 500-ml HDPE bottles. All samples were maintained in the dark on ice during collection and transport to the laboratory.

4.2.2.2 Quantification of Culturable Enteroviruses

Composite wastewater and sludge core samples were maintained in the dark on ice until they were received and processed (within 72 hours of collection) by BCS Laboratories (US National Environmental Laboratory Accreditation Program (NELAP), FL DOH Lab ID E82924; US EPA Lab ID FL01147). Analysis was performed as per US EPA/600/R-95/178 and US EPA 1615 for total culturable enteric viruses and specifically selecting for EV by

the use of the Buffalo Green Monkey (BGM) cell line (Rodriguez et al. 2008; US EPA 2001a, 2012b). Sample extracts/concentrates were analyzed by monitoring for cytopathic development on recently passaged, BGM monolayers (passage 130-155). Concentrates from sludge samples were isolated per US EPA/625/R-92/013 Appendix H (ASTM method 4994-89, approved 2002) (US EPA 2003). Given the concentrated and turbid nature of the wastewater, wastewater extracts were not concentrated via US EPA method 1615 because that method was developed for relatively well-treated, dilute, low-turbidity, and low-pH wastewater (US EPA 2012b). Alternately, wastewater samples were diluted with 1% beef extract (Neogen, Lansing, MI), homogenized for 15 min, centrifuged at 10,000×g for 15 min, and the resulting supernatants were filtered through a protein pretreated 0.22-µm filter (Corning Inc., Corning, NY) and supplemented with penicillin/streptomycin and amphotericin B (Corning Inc., Corning, NY) prior to inoculation onto cell monolayer (US EPA 2003).

Foci of infection of culturable EV were observed and counted on the BGM monolayers within 7 days of inoculation. The cells were incubated for 14 days. Flasks demonstrating negative cytopathic effects (CPE) were freeze thawed at -80°C and inoculated onto fresh BGM cells for a second passage of 14 days. Positive and negative CPE results were used to calculate the Most Probable Number (MPN) of culturable EV. In all wastewater and sludge samples, the MPN had a NELAC data qualifier indicating the actual value was known to be greater than the observed value, except for the three-pond system effluent (site D in Table 3), where the observed value was between the laboratory method detection limits and the laboratory practical quantitation limit.

4.2.2.3 Virus Isolation and Concentration for Molecular Analyses

Because adsorption-elution methods require smaller sample volumes and have been shown to effectively isolate and concentrate intact viruses from wastewater for genomic quantification analyses (Haramoto et al. 2008), a modified absorption-elution method was used to concentrate the viruses. To measure virus concentrations in each of the eight composite wastewater samples collected during the second sampling event at points A – D and F – I, 60-ml samples were acidified to pH 3 using 1M acetic acid and filtered onto a 0.45-µm, mixed cellulose ester, 47-mm diameter filter (Type HAWP; Millipore, Billerica, MA) in triplicate using a modified can crusher (Appendix B, Figure B1), sterile syringe, and syringe filter holder. Filter holders, initially sterile and dedicated to each WSP system, were rinsed 3x with distilled water between samples, and all samples were processed from most treated (WSP effluent) to least treated (WSP influent) to minimize cross contamination. As an additional precaution,

process blanks, using distilled water, were collected from each filter holder after all samples had been processed to examine a worst-case contamination scenario. The filters containing the viruses were aseptically folded in half, placed in bead beating tubes (lysis matrix E; MP Biomedical, Solon, OH) containing 600 µl of buffer RLT Plus (Qiagen, Valencia, CA), and stored in the dark at 4°C for three days prior to storage at -80°C.

To investigate virus-particle associations, a series of filters were employed to capture viruses attached to particles of different sizes as described previously (da Silva et al. 2008). In order to minimize contamination, filter holders were dedicated to a particular WSP system and a single filter type (e.g. 180-µm filters were never placed in a holder designated for 0.45-µm filters). Furthermore, process controls were collected as described above. Triplicate 80-ml samples were isolated from the influent and effluent points of the maturation and polishing ponds from composite samples. Each sample was split into two 40-ml subsamples, one of which was filtered in the field through the following cascade of 47-mm diameter filters: 180-µm nylon net (Millipore, Billerica, MA), 0.45-µm mixed cellulose ester (Type HAWP; Millipore, Billerica, MA), and a positively charged (+) 0.45-µm LifeAssure® membrane (Regal-Brown, Daphine, AL). The other 40-mL subsample was acidified and collected onto 0.45-µm mixed cellulose ester filters, as described previously. All filters were aseptically placed into bead beating tubes (lysis matrix E; MP Biomedical, Solon, OH) containing 600 µl of buffer RLT Plus (Qiagen, Valencia, CA) and stored in the dark at 4°C for 10 days prior to storage at -80°C.

4.2.2.4 RNA Purification and Reverse Transcription

For RT-qPCR analyses, all filters were thawed on ice prior to RNA purification. Once thawed, 100 µl of buffer RLT plus (Qiagen, Valencia, CA) was added to each sample. Filters were mechanically disrupted via bead beating (Pewee Boxer model 3115RS-12T-B20; Biospec Products, Bartlesville, OK) for a total of 3 min (in 1-min intervals) at maximum speed. RNA was purified from the resulting lysate using the AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA), following manufacturer's instructions, and eluted with 50 µl molecular grade water. RNA was immediately reverse transcribed with random hexamers using the Superscript III First Strand Synthesis for RT-PCR (Invitrogen, Carlsbad, CA) per manufacturer's instructions. Extraction blanks, containing only the kit reagents, were processed alongside samples to ensure the absence of cross-contamination. RNA and cDNA were stored at -80°C and -20°C, respectively. No contamination was observed during RNA purification and reverse transcription for any of the molecular targets.

4.2.2.5 RNA Purification and Reverse Transcription Control

To ensure adequate purification of sample RNA and reverse transcription of cDNA, each sample as well as a calibrator sample containing molecular grade water, was spiked with 5×10^9 copies of a synthetic equine arteritis virus (EAV) RNA oligonucleotide prior to bead beating (see Appendix B). Before the synthetic EAV oligonucleotide was added, the absence of EAV in the wastewater samples was confirmed through qPCR analysis. The efficiency of RNA purification and reverse transcription was determined by comparing the average qPCR-determined concentration of EAV in the calibrators to the average concentration of EAV in each spiked sample.

Of the 96 wastewater samples collected, the vast majority of the RNA purification and reverse transcription efficiencies were >10%, with the exception of a few samples from both WSP systems collected on (+) 0.45-µm filters (4 of 18 samples) and a 180-µm filter (1 of 18 samples), which had efficiencies between 1% and 10%. In addition, one acidified sample from the UASB-pond system collected on a 0.45-µm filter and three samples from the UASB-pond system collected on a (+) 0.45-µm filter had efficiencies <1% and were excluded from further analyses.

4.2.2.6 Molecular Quantification of EAV, NovGI, RV, and PMMoV

Quantitative PCR, using published primers and probes, was used as previously described with minor modifications to determine the concentrations of RV, NoVGI, PMMoV, and the EAV purification control in each sample (Appendix B, Table B1) (Rosario et al. 2009; Svraka et al. 2009). Briefly, all samples, process controls, calibrators, and purification blanks for each target virus and extraction control were assayed alongside manufactured plasmids, containing the appropriate target sequence, in order to generate standard curves. Using 7500 Software v2.0.6 (Life Technologies), default settings were used to determine the quantification cycle (Cq) values. For each 96-well plate, a standard curve was generated by linear regression of the relationship between the Cq value and copy number. Only data collected from plates whose standard curve had R^2 values >0.97 and qPCR efficiencies between 90% and 110%, were accepted (Bustin et al. 2009).

The limit of quantification (LOQ), defined as the lowest standard dilution within the linear dynamic range of the standard curve, was 10 copies/reaction for EAV, NoVGI, and RV, and 100 copies/reaction for PMMoV. For NoVG1, PMMoV, and RV, the sample concentration was considered ‘less than the limit of detection’ (<LOD) if no increase in fluorescence was observed after 40 cycles. For composite samples whose mean Cq value was less than 40 and greater than the corresponding Cq of the LOQ, the composite sample was categorized as ‘positive but below

the LOQ' (+BLOQ). Based upon the observation that the undiluted sample Cq value was never greater than the Cq value of its 1:10 dilution, no significant PCR inhibition was observed. In order to account for the stochastic inter-plate variability inherent to qPCR analyses, the pooled approach was used to estimate the number of copies in each unknown sample from the corresponding mean Cq value for plasmid standards and unknown samples for each assay (Sivaganesan et al. 2010) (see Appendix B). Using the estimated number of copies of EAV, NoVGI, PMMoV, and RV detected per qPCR reaction, the concentrations in the original composite samples from the two wastewater treatment systems were back-calculated to reflect all sample dilutions (e.g. nucleic acid purification through qPCR detection) and the original sample volume.

The theoretical process LOQ represents the minimum concentration of viruses in the original composite sample that will yield a quantifiable qPCR measurement and optimistically assumes 100% recovery of the target virus throughout the concentration, RNA purification, and reverse transcription processes. For the 60-ml composite samples analyzed for virus concentrations, the theoretical process LOQ was 11 copies/ml for NoVGI/RV and 109 copies/ml PMMoV. Similarly for the 40-ml composite samples analyzed for virus-particle associations, the theoretical process LOQ was 16 copies/ml for NoVGI/RV and 164 copies/ml for PMMoV. It is likely that actual process LOQ is much higher than the theoretical process LOQ given the inevitable losses associated with each step prior to RT-qPCR analysis.

4.2.2.7 Quantitative Microbial Risk Assessment for Restricted Irrigation

Following guidelines recommended by the World Health Organization (WHO) (Mara et al. 2010a; WHO 2006) and the approach used by Barker et al. (2013b), a QMRA model was developed to estimate the additional $\log_{10}$ reduction of NoVGI, RV, and EV required for restricted irrigation with treated wastewater from these two systems (i.e. the irrigation of crops that are not eaten raw by humans). Assumptions for the parameters used in the QMRA model are provided in Table 4.

Table 4. QMRA model parameter assumptions.

Parameter	Units	Value or Distribution	References
Concentration of viruses in treated wastewater			
Rotavirus concentration (c_{eff_RV})	copies/mL	Three-pond system: lognorm(1622,3.55) UASB-pond system: unif(6,11)	This study
Norovirus concentration (c_{eff_NoV})	copies/mL	Three-pond system: lognorm(363,1.86) UASB-pond system: lognorm(219,2.04)	This study
Enterovirus concentration (c_{eff_EV})	iu/mL	Three-pond system: 0.0037 UASB-pond system: 9.1	This study
Exposure			
Volume of water ingested per day (V)	mL	1.0	(Ottoson and Stenström 2003)
Days of exposure per year (n)	days	Adult farmers: 75 Children at play: 150	(Mara et al. 2007; Seidu et al. 2008)
Susceptibility fraction: $S_f = 1 - (p_r + e \cdot p_v)$			
Population with acquired/genetic resistance (p_r)	proportion	NoV: unif(0, 0.2) RV: 0 (children <5) EV: 0	
Proportion of population that is vaccinated (p_v)	proportion	NoV: 0 RV: 0.78 EV: 0	(WHO 2014)
Vaccine efficacy (e)	proportion	RV: unif(0.54, 0.79)	(Patel et al. 2013)
Dose-Response models			
Norovirus (8fIIa & 8fIIb inocula)		Hypergeometric model: $\alpha = 0.04$, $\beta = 0.055$, $\eta_{NV} = 0.00255$, $r_{NV} = 0.086$, $a = 0.9997$	(Teunis et al. 2008)
Rotavirus		"Exact" Beta-Poisson model: $\alpha = 0.167$, $\beta = 0.191$	(Teunis and Havelaar 2000)
Enterovirus		Exponential: k = 0.00374	(Cliver 1981; Huang 2013)
Illness:Infection (I)	proportion	NoV: $p_{Ill\|inf} = 1 - (1 + \eta_{NV} c_{NV} V)^{-r_{NV}}$ RV and EV: 0.9	(Teunis et al. 2008) (Havelaar and Melse 2003)
Disease burden (B)	DALYs lost per case of illness	NoV: unif(3.71×10^{-4};6.23×10^{-3}) RV: unif(1.50×10^{-2},2.60×10^{-2}) EV: unif(1.50×10^{-2},2.4×10^{-3})	(Mok et al. 2014) (Havelaar and Melse 2003; Prüss-Üstün et al. 2008) (Howard et al. 2007; Prüss-Üstün et al. 2008)

For restricted irrigation, farmers and children playing in wastewater-irrigated fields have the greatest exposure to pathogens (Mara et al. 2007). While NoV, RV, and EV are common disease agents in Bolivia, adult farmers are only susceptible to illness from NoV and EV; children may be susceptible to all virus groups (Appendix B). Exposure to farmers was modeled, assuming the involuntary ingestion of 1 ml of irrigation water per day, for 75 irrigation days per year (Ottoson and Stenström 2003; Seidu et al. 2008). Considering children may travel to fields while their parents work or play there during non-work days, exposure was also modeled for children, assuming the ingestion of 100 mg of soil per day for 150 days per year (Mara et al. 2007, Seidu et al. 2008). The virus dose associated with the ingestion of 100 mg of soil was considered approximately equivalent to the dose received from ingesting 1 ml of water, based on typical bulk densities and moisture saturation points for agricultural soils in Bolivia and the potential adsorption of viruses to soils (Appendix B).

In some cases, a certain portion of the population may not be susceptible to infection. For this QMRA model, the susceptible fraction of the population was defined by the equation

$$Sf = 1 - (pr + e \cdot pv) \quad (1)$$

where p_r is the proportion of the (non-vaccinated) population with acquired or genetic resistance, p_v is the proportion of the population that has been vaccinated, and e is the efficacy of the vaccine.

The WHO has recently recommended that, in developing countries with high existing disease burdens, wastewater irrigation should not cause an annual disease burden exceeding 10^{-4} DALYs per person (Mara et al. 2010). The disease burden per case of illness (B) was assumed to be uniformly distributed based on a range of values reported in the literature (Table 4). The annual disease burden (DB) resulting from illness caused by viral infection, expressed in DALYs per person per year, was calculated as

$$DB = P_{ill} \cdot B \cdot S_f \quad (2)$$

where P_{ill} is the annual probability of illness, B is the estimated burden of disease per case of illness (DALYs per case), and S_f is the susceptible fraction of the population. The annual probability of illness (P_{ill}) was calculated as

$$P_{ill} = 1 - (1 - p_{ill})^n \quad (3)$$

where p_{ill} represents the probability of illness per day of exposure, and n represents the number of days of exposure per person per year. The daily probability of illness was modeled as

$$p_{ill} = p_{inf} \cdot p_{ill|inf} \quad (4)$$

where p_{inf} is the probability of infection per day of exposure (Equations B1 through B3) and $p_{ill|inf}$ is the conditional probability of illness, given a positive infection (Equations B4 and B5). Previously-published dose-response models (Equations B1 and B2) and best-fit values for model parameters were used for NoV and RV (Teunis and Havelaar 2000, Teunis et al. 2008, see Appendix B). The RV dose-response model was also used as a surrogate dose-response model for EV (Schijven and de Roda Husman 2006). For comparison, an exponential dose-response model (Equation B3) was also used for EV, with fit parameter k determined using data from a study where pigs were challenged with porcine EV type 7 (Cliver 1981, Huang 2013). The dose (λ) was defined as

$$\lambda = c \cdot V \quad (5)$$

where c is concentration and V is volume of water ingested.

The additional $\log_{10}$ reduction value (LRV) required to ensure that the calculated disease burden is less than or equal to 10^{-4} DALYs per person per year was calculated (Barker et al. 2013b) as

$$LRV = log_{10}(c_{eff}) - log_{10}(c) \quad (6)$$

where c represents the maximum tolerable concentration of RV, NoV or EV (estimated using Equations 1 through 5, and B1 through B5), and c_{eff} represents the concentration of RV, NoVGI, or culturable EV in the effluent of the two WSP systems studied. For NoVGI and RV, where two composite samples were collected in triplicate, the $\log_{10}$-transformed data was assumed to be normally distributed (Tanaka et al. 1998), with the mean and standard deviations equal to the mean and standard deviations of the $\log_{10}$-transformed virus data. In the case of RV, which was detected in the UASB-pond system as positive, but below the assay limit of quantification, concentrations were assumed to be uniformly distributed between the assay limit of detection and the limit of quantification. The data used to develop the dose-response relationships for RV was quantified in terms of focus-forming units (FFU), using culture-based methods (Ward et al. 1986). In this study, molecular methods were used to measure RV concentrations. In order to correct for this harmonization issue, a genome:FFU ratio of 1000 to 1900 was assumed

(McBride et al. 2013; Mok and Hamilton 2014). The data used to develop the dose-response relationships for NoV was quantified using the same molecular methods used in this study; therefore, no such adjustment was made. Likewise, no harmonization adjustments were made for EV.

To account for uncertainty and variability in the model parameters, a set of 10,000 random values was sampled for each parameter with a distributional assumption, and the 1%, 10%, 25%, median, mean, 75%, 90%, and 99% values of the maximum concentration satisfying the WHO recommended limit of 10^{-4} DALYs were calculated. All modeling was performed in 'R' Version 3.1.0 (The R Foundation for Statistical Computing). A sample of the code used is provided in Appendix B.

4.2.3 Results

4.2.3.1 Culturable Enteroviruses

The culturable EV results for the wastewater samples are presented in Table 5. A 3.1-$\log_{10}$ removal and 0.8-$\log_{10}$ removal of culturable EV was observed from the three-pond and UASB-pond systems, respectively. The fraction of dry solids in sludge was 14.9% for the UASB reactor and 10.3% in the facultative pond. Sludge samples collected from the facultative pond and UASB reactor had infectious EV concentrations of 27 and 2000 infectious units (iu)/4 g dry weight, respectively.

Table 5. Concentration of total culturable enteroviruses (infectious units (iu) per mL) in wastewater from the three-pond and UASB-pond systems.

System	Site	Site Description	Total Culturable Enteroviruses (iu/mL)
Three-Pond System	A	Untreated Sewage	4.2E+01
	B	Effluent of Facultative Pond	1.1E+00
	D	Effluent of Second Maturation Pond	3.7E-02
UASB-Pond System	F	Untreated Sewage	6.2E+01
	G	Effluent of UASB Reactor	2.0E+01
	I	Effluent of Second Polishing Pond	9.1E+00

4.2.3.2 Molecular Quantification of EAV, NovGI, RV, and PMMoV

No measurable reduction of NoVGI, RV, and PMMoV genomes was observed in triplicate, composite samples from either WSP system using RT-qPCR (Figure 11), with the exception of the $\geq$1-$\log_{10}$ reduction of RV in the UASB-pond system (between sites G and H in Table 3). Concentrations of PMMoV were consistently several orders of magnitude higher than concentrations of NoVGI and RV in both systems. Throughout the three-pond

system, NoVGI, RV, and PMMoV concentrations remained relatively constant, at approximately 1×10^3 NoVGI or RV copies/ml and 1×10^6 PMMoV copies/ml. In the UASB-pond system, concentrations of RV decreased to levels +BLOQ, while concentrations of NoVGI and PMMoV increased slightly.

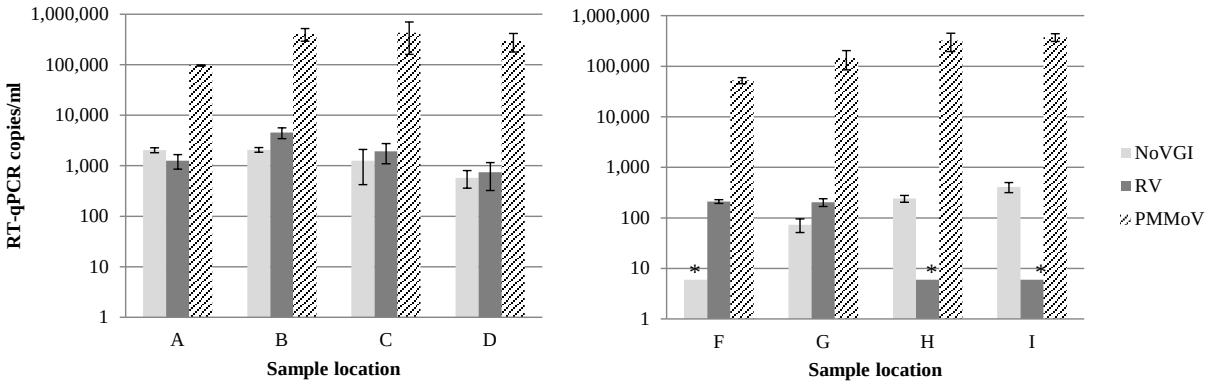


Figure 11. RT-qPCR derived concentrations of norovirus genotype I (NoVGI), rotavirus group A (RV), and pepper mild mottle virus (PMMoV) in the three-pond (left) and UASB-pond (right) systems. All samples with an asterisk (*) were positive but below the limit of quantification and consequently, they are displayed as the theoretical limit of detection (6 copies/mL).

4.2.3.3 Virus-Particle Associations

Overall, the concentrations of NoVGI, RV, and PMMoV detected on the different-sized filters in the cascade were much lower than the corresponding concentrations detected in acidified samples captured on 0.45-µm filters, which is taken to represent the "total" number of viruses in each sample (Tables 6 – 8). In addition, concentrations of all viruses retained on the 180-µm filters were generally lower than the corresponding concentrations collected serially on the 0.45-µm and (+) 0.45-µm filters throughout both WSP systems.

The ratio of viruses retained on each of the filters in the cascade (180-µm, 0.45-µm, and (+) 0.45-µm) to those retained on the 0.45-µm filters in the acidified samples indicates the proportion of viruses associated with different-sized particles. In both WSP systems, at all sampling locations, the ratios of NoVGI, RV, and PMMoV retained on the 180-µm filters to those detected in the acidified samples were generally <5%. In the three-pond system, concentrations of all virus types retained on 0.45-µm and (+) 0.45-µm filters were less than half of the corresponding concentrations in acidified samples. The ratios of NoVGI and PMMoV detected on 0.45-µm and (+) 0.45-µm filters to NoVGI and PMMoV detected in the acidified samples were greater in the UASB reactor effluent (site G) than they were at all other sample sites in both systems, and these ratios decreased progressively at sample sites H and I (the effluent of each subsequent polishing pond).

Table 6. Quantities (copies/mL) of norovirus genotype I on a series of filters without acidification and on a 0.45 µm filter after acidification from three sample sites within the three-pond and UASB-pond systems. The asterisk (*) indicates <1% extraction efficiencies.

Three Pond System					UASB-Pond System				
Site:Replicate	**180 µm**	**0.45 µm**	**0.45 µm (+)**	**Acidified 0.45µm**	**Site:Replicate**	**180 µm**	**0.45 µm**	**0.45 µm (+)**	**Acidified 0.45µm**
B:1	+BLOQ	1.50E+02	2.60E+02	9.70E+02	G:1	+BLOQ	1.90E+01	+BLOQ	* <LOD
B:2	+BLOQ	8.50E+01	6.60E+01	3.40E+02	G:2	+BLOQ	+BLOQ	+BLOQ	1.80E+01
B:3	1.10E+01	3.10E+02	+BLOQ	6.90E+02	G:3	+BLOQ	1.30E+01	<LOD	3.50E+01
C:1	+BLOQ	1.20E+02	5.20E+01	5.90E+02	H:1	+BLOQ	2.20E+01	<LOD	4.20E+01
C:2	+BLOQ	5.50E+01	1.50E+01	2.40E+02	H:2	<LOD	+BLOQ	+BLOQ	7.90E+01
C.3	1.80E+02	1.30E+02	+BLOQ	3.50E+02	H:3	+BLOQ	3.20E+01	* <LOD	1.40E+02
D:1	+BLOQ	5.90E+01	<LOD	3.50E+02	I:1	+BLOQ	+BLOQ	* <LOD	8.70E+01
D:2	<LOD	1.60E+01	+BLOQ	1.30E+02	I:2	+BLOQ	+BLOQ	* <LOD	1.80E+02
D:3	+BLOQ	3.50E+01	<LOD	3.00E+02	I:3	8.20E+01	+BLOQ	+BLOQ	1.10E+02

Table 7. Quantities (copies/mL) of rotavirus group A on a series of filters without acidification and on a 0.45 µm filter after acidification from three sample sites within the three-pond and UASB-pond systems. The asterisk (*) indicates <1% extraction efficiencies.

Three Pond System					UASB-Pond System				
Site:Replicate	**180 µm**	**0.45 µm**	**0.45 µm (+)**	**Acidified 0.45µm**	**Site:Replicate**	**180 µm**	**0.45 µm**	**0.45 µm (+)**	**Acidified 0.45µm**
B:1	1.60E+02	1.60E+03	4.30E+03	3.10E+04	G:1	<LOD	5.70E+01	<LOD	* <LOD
B:2	4.00E+02	4.90E+02	6.40E+02	1.70E+04	G:2	<LOD	1.70E+01	<LOD	4.30E+01
B:3	3.40E+01	2.70E+02	2.40E+01	6.40E+03	G:3	<LOD	1.50E+01	<LOD	+BLOQ
C:1	2.60E+02	1.10E+03	8.30E+02	2.20E+04	H:1	<LOD	<LOD	<LOD	<LOD
C:2	2.20E+02	3.00E+02	4.90E+02	1.50E+04	H:2	<LOD	<LOD	5.40E+01	3.30E+01
C.3	<LOD	1.20E+02	+BLOQ	2.20E+03	H:3	<LOD	2.80E+01	* <LOD	1.50E+01
D:1	1.60E+02	1.60E+02	<LOD	1.00E+04	I:1	<LOD	1.90E+01	* <LOD	3.00E+01
D:2	4.50E+01	5.40E+01	<LOD	5.00E+03	I:2	<LOD	+BLOQ	* <LOD	<LOD
D:3	1.70E+02	1.20E+02	<LOD	1.40E+03	I:3	+BLOQ	<LOD	<LOD	+BLOQ

Table 8. Quantities (copies/mL) of pepper mild mottle virus on a series of filters without acidification and on a 0.45 µm filter after acidification from three sample sites within the three-pond and UASB-pond systems. The asterisk (*) indicates <1% extraction efficiencies.

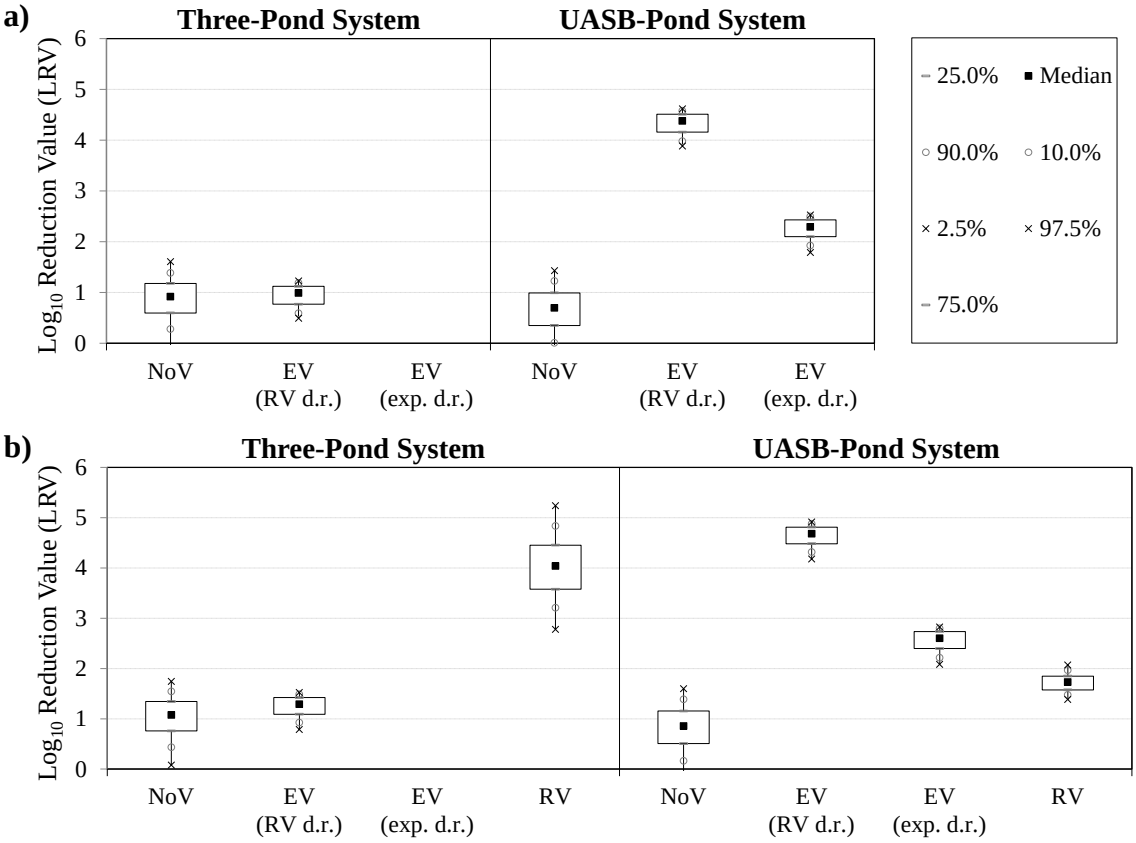
Three Pond System					UASB-Pond System				
Site:Replicate	**180 µm**	**0.45 µm**	**0.45 µm (+)**	**Acidified 0.45µm**	**Site:Replicate**	**180 µm**	**0.45 µm**	**0.45 µm (+)**	**Acidified 0.45µm**
B:1	1.97E+03	8.13E+03	1.57E+05	6.48E+05	G:1	6.81E+03	1.65E+05	4.78E+04	* 1.02E+04
B:2	2.80E+03	2.83E+04	9.35E+04	7.36E+05	G:2	6.76E+03	3.10E+05	1.98E+05	3.67E+05
B:3	2.42E+03	3.01E+04	2.51E+03	5.31E+05	G:3	1.40E+04	2.05E+05	7.26E+03	2.70E+05
C:1	1.60E+03	1.60E+04	5.02E+04	6.59E+05	H:1	3.06E+03	2.48E+04	2.00E+02	2.05E+05
C:2	2.88E+03	3.24E+04	3.55E+04	7.52E+05	H:2	1.14E+03	6.51E+04	1.41E+03	1.75E+05
C.3	<LOD	2.61E+04	1.58E+03	2.56E+05	H:3	1.02E+03	3.20E+04	* 4.86E+02	3.75E+05
D:1	1.43E+03	2.55E+04	<LOD	5.75E+05	I:1	1.22E+03	3.31E+04	* <LOD	4.18E+05
D:2	2.30E+03	1.58E+04	2.58E+02	5.28E+05	I:2	1.42E+03	2.25E+04	* <LOD	2.70E+05
D:3	6.15E+02	1.85E+04	1.41E+02	4.24E+05	I:3	5.82E+04	4.79E+03	1.29E+03	4.97E+05

Despite efforts to minimize contamination during sample collection, minimal contamination was observed for a few filter types. In the process blank from the second sampling event (investigating overall system removal), NoVGI was observed as positive but below the limit of quantification (+BLOQ), and 2,746 PMMoV copies/ml (equivalent to less than 3% of the lowest observed mean concentration detected in other samples) were measured. In the process blank for the first sampling event (analyzing virus-particle associations), acidified and non-acidified composite samples collected on 0.45-µm filters showed +BLOQ for NoVGI, as well as 4,125 PMMoV/ml and 551 PMMoV/ml, respectively (still lower than the observed standard deviation). The low levels of contamination observed likely arose due to the re-use of filter holders; however, the observed contamination represents a worst case scenario because process blanks were collected after sample collection and samples were processed in order of most treated (effluent) to least treated (untreated sewage).

4.2.3.4 Quantitative Microbial Risk Assessment for Restricted Irrigation

The results of the QMRA model are shown in Figure 12. The model demonstrated that, for adult farmers irrigating 75 days per year with effluent from the three-pond system, in order to satisfy the recommended limit of 10^{-4} DALYs per person per year, the additional required LRV is 0.9 (2.5%: -0.1, 97.5%: 1.6) for NoVGI, and 1.0 (2.5%: 0.5, 97.5%: 1.2) for culturable EV (based on the RV dose-response curve). For the UASB-pond system, the LRV is 0.7 (2.5%: -0.4, 97.5%: 1.4) for NoVGI, but 4.4 (2.5%: 3.9, 97.5%: 4.6) for culturable EV (using the RV dose-response curve). If the exponential dose-response curve is used for EV instead of the RV model (Appendix B, Figure B3), the predicted LRV is 2.3 (2.5%: 1.8, 97.5%: 2.5) for the UASB-pond system, and <0 for the three-pond system (indicating that no further treatment would be required).

For the scenario of children at play, the QMRA model estimated LRVs for RV of 4.0 (2.5%: 2.8, 97.5%: 5.2) for the UASB-pond system and 1.7 (2.5%: 1.4, 97.5%: 2.1) for the three-pond system. The magnitude of uncertainty associated with the estimates is quite large; the differences between the 2.5% and 97.5% LRVs is as high as 2.5 (for RV in the three-pond system). The LRVs for NoV for the two WSP systems were not significantly different, but the LRVs for culturable EV for the UASB-pond system were significantly greater than the corresponding LRVs for culturable EV for the three-pond system ($p < 0.01$). Likewise, the LRV for RV for the three-pond system was significantly greater than the corresponding LRV for RV for the UASB-pond system.



NoV = norovirus; RV = rotavirus; EV = enteroviruses; exp. = exponential; d.r. = dose-response model

Figure 12. Additional log_{10} reduction required for treated wastewater to be reused for restricted irrigation a) in fields where only adult workers are present and b) in fields where children <5 years are present.

4.2.4 Discussion

4.2.4.1 Virus Removal in WSP Systems

The extent of enteric virus removal measured in the two WSP systems differed depending on whether culture- or molecular-based methods were used; little removal was observed using molecular-based methods. Given that free viral RNA can disappear within minutes (Limsawat and Ohgaki 1997), it is unlikely that the molecular-based results were biased by the detection of free viral RNA. However, it is possible that the qPCR-derived results underrepresent NoVGI and RV removal because it does not differentiate between infectious and non-infectious virus particles. Since many viruses are non-culturable (e.g. norovirus) or not easily cultured (e.g. rotavirus), the use of complementary culture- and molecular-based approaches allowed for a thorough, short-term investigation of enteric virus removal.

In contrast to a previous study that reported significant removal of NoVGI genomes in a three-pond system with a theoretical hydraulic retention time (HRT) of several weeks (Da Silva et al. 2008), no reduction in NoVGI was observed in the two WSP systems here, despite the fact that the theoretical HRTs for these two systems exceeded 25 days. Possible explanations for this apparent discrepancy between the treatment efficiencies of the three-pond system in France (da Silva et al. 2008) and the three-pond system from this study might include differences in wastewater characteristics, hydraulic efficiency, or environmental conditions. Overall removal of total suspended solids, five-day biochemical oxygen demand, and chemical oxygen demand in the WSP systems from this system (see Chapter 2) was similar to that reported by da Silva et al. (2008); however, the pH levels of the WSP systems in this study were generally between 6.5 and 7.5 (data not shown), which are slightly lower than the system in France (da Silva et al. 2008).

To our knowledge, this investigation constitutes the first study of culturable EV removal in a UASB reactor system where ponds are used for post-treatment. The removal of culturable EV in the three-pond system (3.1 $\log_{10}$) was greater than or equal to enteric virus removal reported in conventional WSP systems from other regions of the world (Table 9). Removal of culturable EV in the UASB-pond system from this study was considerably lower, despite the similar overall theoretical HRTs of both WSP systems. There was greater removal of culturable EV in the facultative pond (between sites A and B) than in the UASB reactor (between sites F and G), which is to be expected since the UASB reactor has a much shorter theoretical HRT than the facultative pond (Table 3). However, there was less removal of culturable EV in the polishing ponds of the UASB-pond system (0.3-$\log_{10}$ reduction between sites G and I) than there was in the maturation ponds in the three-pond system (1.5-$\log_{10}$ reduction between sites B and D). This was unexpected, especially considering that the theoretical HRT of the polishing ponds treating the UASB reactor effluent was 17 days longer than the theoretical HRT of the maturation ponds treating the facultative pond effluent on the day of sampling (Table 3).

While it is possible that the virus concentrations determined in the UASB-pond system were biased by a shorter collection period, it is unlikely that the lack of sample collection overnight (when flow rates are the lowest) greatly influenced the results. The volumes collected for the hourly sub-samples were proportional to the hourly flow rates measured (i.e. larger samples collected during higher flow rate periods). Rainfall at the UASB-pond system on the day of sample collection may have resulted in a slight underestimation in virus concentrations, due to dilution.

Table 9. Reported reduction of total culturable enteroviruses in pond systems from different locations, configurations, and overall hydraulic retention times (HRT; defined as the volume divided by the average daily flow rate reported) in comparison to those observed in this study.

Reference	Location	Configuration	Overall HRT (days)	Log_{10} Reduction
Rao et al. (1981)	Maharashtra, India	Three different pilot-scale ponds	10.8 – 13.5	0.2 – 2.4
	Maharashtra, India	Single full-scale pond	17.2	0.9 – 1.9
	Chhattisgarh, India	Single full-scale pond	2.7	1.3 – 1.6
Oragui et al. (1987)	Paraiba, Brazil	Five pilot-scale ponds in series (anaerobic, facultative, three maturation)	20.9	3.0
Bausum et al. (1983)	Mississippi, USA	Two full-scale facultative ponds in series	138	2.0 – 3.4
	Mississippi, USA	Two full-scale ponds in series (facultative, maturation)	72	1.9 – 3.0
	Texas, USA	Two full-scale facultative ponds in parallel, followed by maturation pond	35.2	1.8 – 2.8
	Texas, USA	Two full-scale ponds in series (anaerobic, facultative)	24.0	1.0 – 2.1
	South Dakota, USA	Two full-scale facultative ponds in series	62	1.3 – 2.3
	South Dakota, USA	Two full-scale facultative ponds in parallel, followed by maturation pond	72	1.3 – 2.7
This study	Sud Yungas, Bolivia	Three full-scale ponds in series (facultative, two maturation)	25.5	3.1
		UASB reactor, followed by two polishing ponds in series	26.7	0.8

This study is also the first to demonstrate high concentrations of PMMoV in domestic wastewater in South America (10^5-10^6 targets/ml). These concentrations are similar to those reported in the United States (Rosario et al. 2009). The unique dietary origin of PMMoV and its high concentrations make it an attractive surrogate that could circumvent the difficulties in molecular detection of seasonally variable and less abundant enteric viruses. As in other wastewater treatment systems, PMMoV concentrations did not greatly decrease in the WSP systems in this study (Kitajima et al. 2014; Rosario et al. 2009). The lack of PMMoV removal is consistent with the trend in NoVGI concentrations, but differs from that of culturable EV, for which decreases were observed. Because little is known about PMMoV's persistence in wastewater or its correlation with infectious enteric viruses, future research is needed in order to fully understand the utility of PMMoV as a surrogate for enteric viruses.

Given the methodological and logistical challenges of conducting research in a remote region, it was not possible to analyze samples over time or under different seasonal or operational conditions. Thus, the extent of virus removal observed in this study represents only a temporal snapshot and may not reflect system performance under differing conditions. The removal of viruses in ponds is driven by highly complex and heterogeneous physical, chemical, and biological processes, which depend upon environmental conditions (e.g. temperature, wind, precipitation, sun irradiation) as well as system operation and maintenance. Future research should also examine enteric virus removal and particle associations in WSP systems under a variety of environmental and maintenance scenarios. Nevertheless, from a water reuse perspective, the virus concentration data were collected at an ideal time because June is the driest month and the peak of the irrigation season in this region. Also, RV and NoV outbreaks in this region of South America are associated with winter months, with RV peaks common from April to June (Romero et al. 2007) and NoV peaks common from June to October (O'Ryan et al. 2010).

Other methodological constraints may have further influenced the results of this study, possibly leading to underestimations of actual virus concentrations using either method. These constraints include sample storage prior to analysis and inefficient virus concentration, nucleic acid purification, and reverse transcription. Furthermore, variability across sampling sites and/or for different viruses may also have influenced the results. Concentrations of NoVGI and PMMoV measured in this system actually increased slightly from the untreated sewage to the treated wastewater. This may be a result of variable NoVGI and PMMoV shedding by the community over time. Even though samples were composites, the retention times of these systems are several weeks and samples collected on the same day at the influent and effluent do not represent the same "plug" of wastewater. This means that the results may be affected by the day-to-day heterogeneity of viruses in wastewater.

4.2.4.2 Sedimentation of Viruses in Maturation Ponds

Overall, a very small proportion of the viruses were associated with particles ≥180 μm throughout the two WSP systems. Wastewater flocs with diameters >180 μm could potentially settle to the bottom of the maturation and polishing ponds in this study, given their depths and retention times (Appendix B). Thus, this investigation supports previous suggestions that sedimentation is not the primary removal mechanism in maturation ponds (da Silva et al. 2008; Templeton et al. 2008). However, these results also contradict earlier conclusions made by Feachem et al. (1983), who stated that viruses adsorb to larger particles that settle to the bottom of ponds, and by Shuval (1990),

who reported that up to 90% of viruses could be removed by sedimentation in pond systems. Interestingly, PMMoV-particle associations in this study were similar to those of NoVGI and RV, suggesting that PMMoV may be a suitable surrogate for enteric viruses in particle-association studies. PMMoV-particle associations are easier to measure given overall higher concentrations in wastewater (up to 4-$\log_{10}$ greater) compared to NoVGI and RV. It is possible that virus isolation and nucleic acid purification may have varied slightly between the different filter types. However, it is unlikely that the measured virus-particle associations were heavily biased by filter type (with the exception of (+) 0.45-µm filters) in regards to sample RNA yield/efficiency of reverse transcription.

In almost all samples and sample locations, more viruses were detected on the 0.45-µm and (+) 0.45-µm filters than on the 180-µm filters. This suggests that viruses in these WSP systems may be more frequently attached to smaller particles, unicellular algae, or bacterial flocs that generally will not settle. Furthermore, these associations may actually shield viruses from direct UV inactivation, potentially slowing the disinfection process in maturation or polishing ponds. This may be particularly relevant to the UASB-pond system where virus-particle associations were greatest in the UASB reactor effluent. The difference in virus-particle associations in the effluent of the facultative pond (site B) and the UASB reactor (site G) may be a result of inherent differences in the untreated sewage composition in both systems, and shorter HRT of the UASB reactor compared to the facultative pond. Alternatively, these observed differences might be an inherent result of the UASB reactor design, which is optimized to select for microbial communities that form large flocs and biogranules. Virus removal in the UASB reactor may be due to the entrapment of viruses within these flocs and biogranules, while virus removal in the facultative, maturation, and polishing ponds may be primarily due to disinfection mechanisms driven by the sunlight, not by association with particles and sedimentation. This is supported by the higher concentration of culturable EV detected in the UASB reactor sludge compared to the facultative pond sludge. The sludge from the UASB reactor had a higher concentration of total solids and a slightly lower fraction of volatile solids than the sludge from the facultative pond (Appendix B, Table B4). The volumetric density of culturable EV in the facultative pond (based on the total solids and virus concentration per dry weight) is ~0.03 iu/ml, lower than the culturable EV concentration from the water sample at point A. In the UASB reactor sludge, the volumetric density of culturable EV is ~143 iu/ml, greater than the corresponding concentrations measured in water samples from sites F and G. This indicates that viruses in the UASB reactor may have a higher affinity to associate with sludge particles in the UASB reactor than they do in the facultative pond.

Biogranules in UASB reactors are sensitive to abrupt changes in the composition of wastewater, and may suddenly disintegrate without any apparent reason (Liu et al. 2004). If viruses are enmeshed within these granules and the granules disintegrate, the viruses may exit the reactor still bound to smaller particles, as suggested by the increased virus-particle associations observed at site G. Further investigations of virus-particle associations in WSPs and UASB reactors are presented in Section 4.3 of this Chapter.

4.2.4.3 Effluent Risk to Human Health and Potential for Wastewater Reuse

The effluent of the three-pond system only requires an additional virus reduction of 1- to 2-$\log_{10}$ units, which can be easily achieved through the implementation of simple engineered farm-based treatment measures, such as on-farm reservoirs or bank filtration systems (Keraita et al. 2014; Silverman et al. 2015). The UASB-pond system requires an additional 2.5- to 4.5-$\log_{10}$ reduction of EV, implying that the three-pond system effluent may be more suitable for water reuse than the UASB-pond system effluent.

The results also demonstrate that, despite Bolivia's vaccination program, risks for RV illness may be significant for children at play. In this scenario, both systems would require an additional 3- to 5-$\log_{10}$ reduction of viruses. In this study, the same assumptions were made for the disease burden per case of illness (parameter *B* in Table 4) for adult farmers and children at play. The actual disease burden per case of illness may be much higher for children than it is for adults, which would mean that the required LRV for the "children-at-play" scenario may actually be underestimated. This is an indication that children should not be allowed to play in fields with restricted wastewater irrigation.

While the LRV predictions provide a general idea of the feasibility of water reuse, there are limitations associated with the use of QMRA. First, the choice of dose-response model can significantly influence the results. The use of a different dose-response model for EV resulted in a difference of 1.8 in the predicted LRVs (Appendix B, Figure B3). Second, many of the parameter estimates come from the literature, and may not be representative of agricultural practices in Bolivia. For example, the volume of wastewater or soil ingested per day of exposure, and the number of days of exposure per year, are contingent upon highly-variable factors that are difficult to verify (e.g. climate, crop choice, farmer behavior, etc.). Seidu et al. (2008) assumed that farmers in Ghana may ingest up to 5 ml of irrigation water on a given day due to the increased wastewater contact resulting from the use of watering cans.

In Bolivia, it is common practice for farmers in some regions to chew coca leaves while working, which results in increased hand-to-mouth contact and potentially higher viral doses. A sensitivity analysis demonstrated that doubling the volume ingested (*V*) per work day results in an increase of 0.3 for the predicted LRV for NoVGI (Appendix B, Figure B2). Another limitation of the QMRA results is that the assumed distributions of virus concentrations in the WSP systems come from only two composite sampling events. A NoV or RV outbreak in a small town like the ones from this study may significantly increase the LRV required for water reuse, as has been demonstrated by Barker et al. (2013b). Nevertheless, by assuming distributions for the concentrations instead of point estimates, some of this variability and uncertainty was incorporated into the model and propagated to the results.

4.2.4.4 Possible Implications of UASB Reactors used in Pond Systems

This investigation highlights the importance of considering potential microbiological quality tradeoffs associated with energy recovery versus water reuse, as those tradeoffs may influence the selection of wastewater treatment technologies in different contexts. Given the increased popularity of UASB reactors (especially in Latin America) and the importance of energy recovery from wastewater (McCarty et al. 2011), additional research is needed to fully understand if and why virus removal is less efficient in ponds treating UASB reactor effluent. In this short-term investigation, the three-pond system removed culturable EV more effectively than the UASB-pond system. A previous study found that the same three-pond system also removed helminth eggs and indicator bacteria more efficiently than the UASB-pond system (Section 2.1 of this dissertation). However, other UASB-pond systems may provide better pathogen removal than the system from this study. Although the removal of fecal indicator organisms is not necessarily indicative of virus removal, overall *E. coli* reductions of 4- to 5-$\log_{10}$ units have been reported for polishing ponds treating the effluent of three different UASB reactor systems (von Sperling and Mascarenhas 2005; von Sperling et al. 2005). If reduced viral removal is inherent to UASB-pond systems, it would be necessary to determine methods for improving the efficiency of virus removal in these systems.

4.2.5 Conclusions

- While there was limited removal of virus genome targets in both WSP systems, there was <1-$\log_{10}$ removal of culturable EV in the UASB-pond system and >3-$\log_{10}$ removal of culturable EV in the three-pond system.

- There was limited evidence of virus association with particles >180 μm; thus, it is unlikely that sedimentation is a major mechanism of virus removal in both WSP systems.
- Viruses in the effluent of the UASB reactor were more highly associated with particles <180 μm than viruses in any other sample location; these particles may protect viruses from UV inactivation mechanisms in the subsequent polishing ponds, which may explain the limited removal of culturable EV in this system.
- In order for the effluent from these WSP systems to be safely reused for restricted irrigation (with respect to the risk of viral illness to farmers), it would be necessary to reduce virus concentrations by an additional 1- to 2-$\log_{10}$ in the three-pond system and an additional 2.5- to 4.5-$\log_{10}$ in UASB-pond system.
- If children play in the irrigation fields, both systems would have to reduce virus levels by an additional 3- to 5-$\log_{10}$ units.

4.3 Virus Associations with Particles in Upflow Anaerobic Reactors and Waste Stabilization Ponds

4.3.1 Introduction

The removal of viruses in WSPs may occur via several mechanisms, including sunlight-mediated inactivation mechanisms (direct and indirect), predation, and sedimentation (see Chapter 3 of this dissertation). However, since viruses are too small to settle to the bottom of ponds by themselves, they will only settle when associated with larger particles. Despite earlier reports that sedimentation is an important virus removal mechanism in ponds (Feachem et al. 1983; Shuval 1990), there is limited evidence that actually documents the importance of this mechanism (see literature review in Chapter 3). Furthermore, there have been very few publications about the association of viruses with particles in WSPs (Ohgaki et al. 1986; Sobsey and Cooper 1973).

WSP particles appear to have limited adsorptive capacity for viruses, and the adsorption is likely reversible. In one study, 30% of poliovirus inoculated into water from a pilot-scale WSP system in California, USA became associated with particles within 3 min (Sobsey and Cooper 1973). The adsorption conformed to a Freundlich isotherm, and the proportion of particle-associated viruses remained steady for 122 min, indicating that an equilibrium (or pseudo-equilibrium) had been reached. Furthermore, poliovirus maintained infectivity after 45 minutes of contact with WSP particles, implying that association with these particles was reversible and did not

result in a greater rate of inactivation (Sobsey and Cooper 1973). In another study, naturally-occurring coliphages (forming plaques on host strain *Escherichia coli* B) recovered from a small canal receiving effluent from a WSP system in Bangkok, Thailand were found to adsorb to microbial particulates harvested from a WSP when samples were aerated, and then desorb after aeration ceased and samples became anaerobic (Ohgaki et al. 1986). The coliphage concentration in the supernatant (after centrifugation at 12,000 rpm for 10 min) returned to the same original concentration, even after two cycles of aeration and no aeration. In facultative ponds, the water surface is generally aerobic, but oxygen concentrations decrease with increasing pond depth, meaning that if viruses desorb from settleable WSP particles under anaerobic conditions may never make it to the pond sediment before being detached from those particles.

In the study presented in Section 4.2 of this dissertation, and in another recent study from a WSP system in France (da Silva et al. 2008), only a very small percentage of human enteric viruses in WSP systems were retained on 180 µm filters, suggesting that they may not be susceptible to sedimentation. Greater concentrations of viruses passing through 180-µm filters and retained on 0.45-µm and positively-charged 0.45-µm filters were detected in samples from the effluent of a UASB reactor, relative to samples from the WSPs (see Section 4.2 of this dissertation).

Finally, though it is not a WSP system, Sima et al. (2011) detected three different strains of norovirus (GI, GII, GIV) in wastewater samples entering a membrane bioreactor (from an aeration basin), and in sludge samples collected from the membrane, but not in water samples from the effluent of the membrane bioreactor. Based on these results, they concluded that the viruses became associated with particles in the aeration basin and as a result, were removed by the membrane.

Microbial activity in WSP particles may result in the production of extracellular proteolytic enzymes that have antiviral properties, while association with microalgae in WSPs may actually have a protective effect on viruses (Nasser et al. 2002; Sobsey and Cooper 1973). Viruses in WSPs may also be internalized by organisms of higher trophic levels, but it is still unclear how or if this affects virus removal or inactivation in WSPs (see Chapter 3 of this dissertation). Sobsey & Cooper (1973) found that the poliovirus inactivation rate was significantly greater when indigenous bacteria and algae from WSPs were grown together in culture than it was when viruses were in flasks with only WSP algae, indicating potential antiviral activity associated with bacterial growth. They also found that the poliovirus inactivation rate in culture with a particular WSP algal species (*Scenedesmus quadricauda*) was

significantly lower than it was for the control, indicating a protective effect for viruses associated with this species of algae (Sobsey and Cooper 1973).

In summary, there are uncertainties surrounding the importance of sedimentation as a removal mechanism for viruses in WSPs; the mechanisms contributing to virus-particle associations; and the overall impact of virus-particle interactions on virus removal. Because of the high cost associated with virus analysis, wastewater facilities are generally not required to comply with effluent discharge limits for virus concentrations (fecal bacterial indicators are used instead); however, virus-particle interactions may affect enteric virus removal in wastewater treatment systems, as well as the efficacy of downstream disinfection processes. This has important implications for water reuse, which is a resource recovery priority that may be more important than energy recovery for small cities and towns (see Chapter 2 of this dissertation). Some newer anaerobic technologies that are optimized for energy recovery (*e.g.*, the UASB reactor) are intentionally designed to select for microbial communities forming biogranules with good settling properties that are retained in the reactor sludge. Based on results reported in Section 4.2, these sludge particles may entrap viruses, causing them to be present at high concentrations in UASB reactor sludge; viruses may also potentially leave reactors associated with smaller, non-settleable particles. Thus, the objective of this study was to investigate the association of viruses with particles in domestic wastewater treatment systems using WSPs, upflow anaerobic treatment units, or a combination of these technologies. The viruses analyzed include AdV, PMMoV, and F+ coliphage.

4.3.2 Materials and Methods

One mesocosm experiment, and five field studies were carried out in order to investigate the association of viruses with particles. The field sites included one pilot-scale and four full-scale wastewater treatment plants. Details about the system are provided in Table 10. Treatment units listed in Table 10 are all single units operating in series, unless otherwise noted. The first two sites are full-scale WSP systems located in the Yungas region of Bolivia. The third site is a full-scale WSP system located in Belding, Michigan, USA. The fourth site is a pilot-scale WSP system located at the Universidade Federal de Minas Gerais (UFMG) and Companhia de Saneamento de Minas Gerais (COPASA) Center for Research and Training in Sanitation (CePTS) in Belo Horizonte, Brazil. The final site is a full-scale wastewater treatment plant located in the state of Minas Gerais, Brazil.

Table 10. Information about the wastewater treatment systems.

System (Population Served)	Treatment Units and Dimensions[a] (L × B × D, meters)	Average Flow Rate (m^3 d^{-1})	Volume[a] (m^3)	Theoretical Retention Time (days)
Yungas WSP System (780 people)	Facultative Pond (50 × 27.5 × 1.8)	121	2,000	17
	Maturation Pond 1 (39 × 13 × 1.5)		550	4.5
	Maturation Pond 2 (39 × 13 × 1.5)		550	4.5
	Overall Theoretical Hydraulic Retention Time			**26 days**
Yungas UASB-Pond System (1,310 people)	Preliminary Treatment	Screening and Grit Chamber		
	UASB Reactor	124	71	0.6
	Polishing Pond 1 (60 × 21 × 1.5)		1,540	12
	Polishing Pond 2 (60 × 23 × 1.5)		1,700	14
	Overall Theoretical Hydraulic Retention Time			**27 days**
Belding WSP System (~5,000 people)	Preliminary Treatment	Screening and Grit Chamber		
	Anaerobic Pond[b] (90 × 40 × 3)	5,640	11,000	2
	Facultative Pond 1 (68,800 m^2 × 2)[c]		164,000	29
	Facultative Pond 2 (360 × 170 × 2)		122,000	22
	Storage Pond 1 (170 × 160 × 2)		54,000	10
	Storage Pond 2 (200 × 160 × 2)		63,000	11
	Overall Theoretical Hydraulic Retention Time			**74 days**
CePTS UASB-Pond System (250 person-equivalents)	Preliminary Treatment	Screening and Grit Chamber		
	UASB Reactor	30	14	0.5
	Polishing Pond 1 (25 × 5.3 × 0.8)		105	3.5
	Polishing Pond 2[d] (25 × 5.3 × 0.8)		105	3.5
	Horizontal Rock Filter[e] (25 × 5.3 × 0.8)		15	0.5
	Overall Theoretical Hydraulic Retention Time			**8 days**
Minas Gerais UASB Reactors (~86,000 people)	Preliminary Treatment	Screening and Grit Chamber		
	UASB Reactors (four in parallel)[f]	10,500	1,200 (× 4)	0.5
	Overall Theoretical Hydraulic Retention Time			**0.5 days**

[a] Dimensions are approximate; sludge accumulation is not considered; L = length, B = width, D = depth

[b] This is an upflow anaerobic pond (the influent wastewater enters at the bottom of the pond)

[c] This pond has six surface aerators located near the pond inlet (two were in use at the time of sampling); the pond is also not rectangular (surface area and depth reported)

[d] This pond has two longitudinal baffles (all other ponds do not have baffles)

[e] More information about the system have been described in detail previously (11), however when that study was published, the rock filter only occupied 1/3 of the pond bed. For the present study, the rock filter occupied the entire pond bed. The first section of the rock filter contains fine stone, the second contains medium stone, and the third contains coarse stone.

[f] Only three were operating at the time of sampling (one had been taken off line for maintenance)

4.3.2.1 Field Studies

Cascade filtration experiments were performed with wastewater samples from all field sites, using different combinations of at least two or more of the following 47-mm diameter filters: 180-µm nylon net filter (Millipore, Billerica, MA, USA); 10-µm nylon net filter (Millipore); 0.45-µm mixed cellulose ester filter (Type HAWP, Millipore); 0.45-µm positively-charged (+) Life-Assure® membrane (Regal-Brown, Daphine, AL, USA); and 0.20-µm positively-charged (+) Life-Assure® membrane (Regal-Brown). Because of the availability of materials at the different field sites, slightly different methods were used at each site (see Table 11).

Table 11. Cascade filtration experimental designs used for the mesocosm study and at the different field sites.

System	Cascade Filtration Set-Up	Final Filtrate Analyzed?	Total Sample Concentrations Analyzed?	Virus(es) Analyzed
Yungas Systems	180-µm > 0.45-µm > 0.45-µm(+)[d]	No	Yes[a]	AdV
Mesocosm Study	180-µm	Yes (sediment)[a]	Yes (supernatant)[a]	PMMoV, AdV
Belding System	180-µm > 0.45-µm > 0.20-µm(+)	Yes[b]	No	AdV, F+ coliphage
CePTS System	10-µm	Yes[a]	Yes[a]	AdV
Minas Gerais UASB Reactor System	10-µm > 0.45-µm	Yes[a]	No	AdV
	Centrifugation study also performed to separate supernatant from pellet after 10 min at 1157×*g*		No	AdV

[a] Samples were acidified and viruses were retained on 0.45-µm filters
[b] Viruses were concentrated from samples using PEG precipitation
[c] PMMoV, norovirus genotype I (NoVGI), and rotavirus (RV) were also previously analyzed in the same cascade filtration samples (see Section 4.2 of this dissertation)
[d] 0.45-µm (+) filters were not analyzed for AdV, but were analyzed for PMMoV, NoVGI, and RV (see Section 4.2 of this dissertation)

The sampling procedure, cascade filtration set-up, sample preservation methods, and nucleic acid extraction/purification protocol for samples collected from the WSP and UASB-Pond systems in Yungas, Bolivia, were described in Section 4.2. Briefly, the cascade set-up consisted of a 180-µm nylon net filter, a 0.45-µm mixed cellulose ester filter, and a 0.45-µm positively-charged (+) Life-Assure® membrane (Regal-Brown). Sample DNA was extracted and purified using the AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA, USA) with bead-beating, as described for the CePTS UASB-Pond system samples, and stored at -20°C until further analysis. Due to limitations with access to the site, it was not possible to return to the Yungas WSP systems to collect additional samples.

Grab samples were collected at the Belding WSP system at the influent and effluent of each of the five ponds on 6/22/2014, during four different time points throughout the day (between 10:00 am and 3:00 pm). Grab samples from each sample point were composited to form a single sample. These samples were then passed in triplicate through the following cascade filtration set-up: 180-µm nylon net filters (Millipore, Billerica, MA, USA), 0.45-µm mixed cellulose ester (Type HAWP, Millipore), and 0.20-µm positively-charged (+) Life-Assure® membrane (Regal-Brown). Viruses in the final filtrate were concentrated by PEG precipitation as described previously (da Silva et al. 2008). Briefly, PEG (10% w/v) and NaCl (0.01752 g/mL) were added to each sample, and samples were shaken for 60 s, and then stored at 4°C overnight. Lysis reactions were performed directly on each of the filters to extract sample DNA as follows: sample filters were aseptically placed into 50-mL centrifuge tubes with the sample side pointing inward. Then, 600 µL PBS and 60 µL Proteinase K were added to each centrifuge tube. After vortexing for 15 s, 600 µL of Buffer AL was added to each tube and tubes were vortexed again for 0.5 – 2 min, until the sample appeared to be visually released from the filter. Tubes were spun at 13,000×g for 20 s, then, ~140 ng of salmon sperm DNA (used as extraction control) were added to each of the samples and to a calibrator sample. All tubes were incubated at 56°C on a shaker table for 10 – 15 min, allowing the lysis buffer to wash over the filters. Then, 600 µL of ethanol (96–100%) were added to all samples, and samples were pulse vortexed for 15 s, and then spun down for 20 s. Liquid from each sample was transferred to clean 2-mL tubes (filters were discarded). Tubes were spun at 13,000×g for 5 min to remove sediment and particles and supernatant from all tubes was added, 700 µL at a time, to spin columns provided in the QIAamp DNA Mini Kit. The manufacturer's instructions were followed from here on. All DNA was stored at -20°C until further analysis.

Grab samples from the CePTS UASB-Pond system were collected on 3/19/2015, 4/9/2015, 4/23/2015, 5/14/2015, 5/28/2015, and 6/18/2015, between 9:00 and 11:00 am, from the following four locations: untreated sewage (after preliminary treatment); the effluent of the UASB reactor; the effluent of the second (baffled) pond; and the effluent of the rock filter. Additional grab samples from the effluent of the second pond and the effluent of the rock filter were collected during morning hours on 7/14/2015. Samples collected on 3/19/2015, 4/9/2015, and 7/14/2015 were concentrated by acidification and adsorption-elution with bead-beating (samples acidified to a pH of 3.0 to 3.5 with 1.0 M glacial acetic acid, and passed through 0.45-µm mixed cellulose ester filters (Millisul)). Viruses from samples collected on the other dates (n=4) were separated into two fractions: those that were retained on 10-µm nylon net filters (Millipore), and those that were present in the filtrate from these filters (and concentrated

by acidification adsorption-elution on 0.45 μm filters with bead-beating, as described above). All filters were folded aseptically, placed in Beadbug tubes with 700 μL of lysis buffer RLT Plus (Qiagen), and stored at -80°C for up to six months. Once thawed, samples were subjected to mechanical disruption via bead-beating (Pewee Boxer model 3115RS-12T-B20, Biospec Products, Bartlesville, OK, USA) for a total of 3 min, in 60 s intervals. After centrifugation at 13,000×g for 5 min, sample DNA was purified using the AllPrep DNA/RNA Mini Kit (Qiagen), following the manufacturer's recommended protocol.

Grab samples were collected from the Minas Gerais UASB Reactor system on 5/26/2015, 6/3/2015, 6/9/2015, and 6/25/2015, between the hours of 9:00 am and 12:00pm, at the following locations: untreated sewage (after screening and grit removal); the effluent of the UASB reactors; the sludge blanket of one of the UASB reactors; and the bottom layer of sludge from one of the UASB reactors. Grab samples were collected instead of composite samples due to limited access to the site. Samples were transported to the applied microbiology laboratory at the Federal University of Itajubá (UNIFEI) and processed the same day, using the following protocol. Untreated sewage (40 – 45 mL) and UASB reactor effluent (58 – 125 mL) samples collected on 5/26/2015 and 6/3/2015 were passed consecutively through a cascade of the following 47-mm diameter filters: 10-μm nylon net (Millipore, Billerica, MA, USA) and 0.45-μm mixed cellulose ester (Type HAWP, Millisul, Porto Alegre, RS, Brazil). The final filtrate from this cascade was then concentrated via acidification and adsorption-elution with bead beating. All filters were added to 2-mL homogenization tubes with 0.1 mm glass beads (Beadbug™ tubes, Labscience, Belo Horizonte, MG, Brazil) with 700 μL of Solution PV1 lysis buffer (Biotika, São Paulo, SP, Brazil) with 1% β-mercaptoethanol (βME) (Thermo Fisher Scientific, São Paulo, SP, Brazil), and stored at -20°C for up to 6 weeks.

Untreated sewage (50 – 70 mL) and UASB reactor effluent (50 – 100 mL) samples collected on 6/9/2015 and 6/25/2015 were centrifuged at 1,157×g for 10 min with a swinging-bucket rotor. The supernatants were acidified and passed through 0.45-μm filters, which were transferred to Beadbug tubes with 700 μL of PV1 lysis buffer with βME. The pellets were resuspended in 700 μL of PV1 lysis buffer with βME and transferred to Beadbug tubes. UASB sludge blanket samples were diluted to a factor of 0.1 and then 50 mL of this dilution was centrifuged at 1157×g for 10 min and the supernatants and pellets were preserved in Beadbug tubes with 700 μL of PV1 lysis buffer with βME as described above. Approximately 0.5 mL of bottom sludge samples (~50 mg dry solids) were added directly to Beadbug tubes with 700 μL of PV1 lysis buffer with βME. Additional 50-mL aliquots from the

untreated sewage and UASB reactor effluent samples collected on 6/25/2015, as well as the 0.1 dilution of the UASB sludge blanket sample, were centrifuged at 1157×g for 10 min. After decanting the supernatants, the pellets were resuspended in 40 mL of 0.05 M glycine, 3% beef extract (GBE) buffer (pH 9), vortexed for 5 min, and centrifuged again at 1157×g for 10 min. The supernatants were acidified, passed through 0.45-µm filters, and the filters were transferred to Beadbug tubes with 700 µL of PV1 lysis buffer with βME. The pellets were resuspended in 700 µL of PV1 lysis buffer with βME before being transferred to Beadbug tubes. All Beadbug tubes were stored at -20°C for up to 6 weeks.

After thawing, samples were subjected to mechanical disruption for 3 min, in 60 s intervals, with a Mini Beadbeater (Biospec Products Model 3110BX, Bartlesville, OK, USA). After centrifugation at 13,000×g for 5 min, DNA and RNA in sample supernatant were simultaneously purified using the MoBio PowerViral™ Environmental RNA/DNA Isolation Kit (Biotika), and RNA was immediately reverse-transcribed using the Superscript III First Strand Synthesis Kit (Invitrogen via Thermo Fisher Scientific) with random hexamers, in accordance with the manufacturers' recommended protocols. All DNA and cDNA were stored at -20°C until further analysis.

4.3.2.2 Mesocosm Study

An 80-L plastic trash bin was rinsed several times with water from the effluent of the first polishing pond in the Yungas UASB-Pond system, and then filled to the top and placed in an ambient temperature outdoor water bath next to the pond in an area with sunlight exposure. A 40-mL sample of this water was collected in duplicate as the bin was being filled (Day 1). On Day 6, a 40-mL grab sample was collected in duplicate from the supernatant by skimming the water surface with a sterile centrifuge tube. A 40-mL sample of the bin sediment was collected in duplicate by first siphoning off the supernatant, then homogenizing and collecting the water left at the bottom of the bin. Surface samples were immediately concentrated via acidification with 1.0 M acetic acid to a pH of 3.0 to 3.5, and adsorption-elution on 0.45-µm mixed cellulose ester filters (Type HAWP, Millipore) with bead-beating. Sediment samples were first passed through 180-µm nylon net filters (Millipore), then the filtrates were concentrated using acidification and adsorption-elution on 0.45-µm filters with bead-beating. All filters were placed in Lysis Matrix E bead tubes (MP Biomedical, Solon, OH, USA) containing 600 µL of lysis buffer RLT plus with 1% β-mercaptoethanol, as described in Section 4.2. Bead tubes were stored at 4°C in the dark for a week, and then stored at -80°C until nucleic acid extraction. Once thawed, samples were subjected to mechanical disruption via

bead-beating (Pewee Boxer model 3115RS-12T-B20, Biospec Products) for a total of 3 min, in 60 s intervals. After centrifugation at 13,000×*g* for 5 min to remove filter debris and sample sediments, DNA and RNA were purified with the AllPrep DNA/RNA Mini Kit (Qiagen), in accordance with the manufacturer's recommended protocol. RNA was immediately reverse-transcribed using the Superscript III First Strand Synthesis Kit (Invitrogen, Carlsbad, CA, USA) with random hexamers, in accordance with the manufacturers' recommended protocol. All DNA and cDNA were stored at -20°C until further analysis.

4.3.2.3 F+ Coliphage Plaque Assay

The double agar layer (DAL) plaque assay method, performed by Becca Ives and Tim Stieve at Michigan State University, was used to measure the concentrations of F+ coliphage in water samples. This assay is based on the protocol describe in U.S. Environmental Protection Agency (EPA) Method 1602 (US EPA 2001b). In summary, host cells of *E. coli* Famp (ATCC#700891) were grown to log-phase in a tryptic soy broth (TSB) culture with ampicillin and streptomycin. Then, 0.5 mL of these host cells and 2 mL of the samples or sample dilutions were added to 1.5% tryptic soy agar (TSA) overlay tubes, tempered in a 50°C water bath. The tubes were mixed by rolling between palms and then the contents dispensed immediately onto pre-hardened 1.5% TSA plates, which were swirled to evenly distribute the overlay. After 10 to 15 minutes, when the agar overlay was set, the plates were inverted and then incubated overnight at 36 ± 1.0°C. After 16 – 24 h of incubation, plates were removed and circular zones of clearing in the bacterial host lawn were counted as plaque-forming units (pfu). Concentrations were calculated using average plaque counts from five replicate plates using the dilution that produced between 20 and 200 pfu per plate. Precision and recovery, method blanks, positive and negative process controls, TSA plate controls, and TSA plate plus overlay tube controls were analyzed for quality assurance and quality control (US EPA 2001b).

4.3.2.4 Molecular Methods

Quantitative PCR with reverse transcription (RT-qPCR) was used to measure concentrations of PMMoV in cDNA samples, using a previously-published assay (Rosario et al. 2009) with primers (5' to 3') GAG TGG TTT GAC CTT AAC GTT TGA (forward), TTG TCG GTT GCA ATG CAA GT (reverse), and probe [FAM]CCT ACC GAA GCA AAT G[TAMRA]. The 25-µL reaction mixtures contained 400 nM of each primer, 125 nM of probe,

and Taqman Environmental Master Mix 2.0 (Life Technologies, Grand Island, NY, USA) at a final concentration of 1×. A quantitative PCR (qPCR) assay was used to measure concentrations of AdV, using previously-published primers (5' to 3') GGA CGC CTC GGA GTA CCT GAG (forward), ACI GTG GGG TTT CTG AAC TTG TT (reverse) and probe, [FAM]CTG GTG CAG TTC GCC CGT GCC A[MGBNFQ] (Jothikumar et al. 2005). The 20-µL reaction mixtures contained 250 nM of each primer, 150 nM of probe, and Taqman Environmental Master Mix 2.0 at a final concentration of 1×.

Using the ABI7500 Real Time PCR System (Life Technologies), 2-µL samples were analyzed in duplicate, and in order to identify (RT-)qPCR inhibition, an additional replicate for each sample was spiked with 6×10^5 copies of purified pIDTSMART plasmids with ampicillin resistance (Integrated DNA Technologies, Coralville, IA, USA), containing the corresponding target sequences for AdV 40 and PMMoV (plus 6 bp extra on each side), derived from GenBank sequences (AdV: accession number L19443.1; PMMoV: accession number AB550911.1). The (RT-)qPCR cycling conditions were as follows, with data collected on the elongation step: 95°C for 10 min, then 45 cycles of 95°C for 30 s, 53°C for 1 min, and 72°C for 1 min (for PMMoV RT-qPCR); 95°C for 10 min, then 40 cycles of 95°C for 15 s, 55°C for 20 s, and 60°C for 1 min (for AdV qPCR). Data from the (RT-)qPCR were analyzed using the 7500 software v2.0.6 (Life Technologies), with the default settings, to determine the quantification cycle (Cq) values for each qPCR well. The threshold values were set to 0.05 (for AdV) and 0.04 (for PMMoV).

The qPCR process limit of detection (pLOD), defined as the lowest concentration at which amplification occurs before 40 cycles for 95% of positive sample replicates (i.e., the concentration for which <5% failed reactions occur), was determined using an exponential survival function with standard curve data (see Appendix C), following methods used to derive parameters for dose-response models (Haas et al. 2014). If the measured fluorescence for a sample did not pass the threshold value before 40 cycles, the sample was labeled as "NA" or "no amplification observed", and the concentration was considered to be lower than the limit of detection (<LOD).

For a subset of samples with AdV concentrations below the qPCR process limit of detection (pLOD), a nested polymerase chain reaction (PCR) assay was used to determine the presence or absence of AdV, with the following previously-published primers (Allard et al. 1992) (5' to 3'): GCC GCA GTG GTC TTA CAT GCA CAT C (forward, round 1), CAG CAC GCC GCG GAT GTC AAA GT (reverse, round 1), GCC ACC GAG ACG TAC TTC AGC CTG (forward, round 2), TTG TAC GAG TAC GCG GTA TCC TCG CGG TC (reverse, round 2). The

25-µL reaction mixtures contained 1000 nM of each primer, and GoTaq® Green Master Mix (Promega, Madison, WI, USA) at a final concentration of 1×. Cycling conditions (for both cycles of the nested AdV assay) were 4 min at 94°C, followed by 40 cycles of 92°C for 30 s, 60°C for 30 s, and 72°C for 1 min and a final incubation step at 72°C for 5 min. 5 µL of the product from the first round of PCR was used as a template for the second reaction. 15 µL of the final PCR products were added to a 2% agarose gel with ethidium bromide and analyzed using electrophoresis for a total of 90 min at 80 – 90 V. A positive control (untreated sewage), a negative (blank) control, and 100 bp DNA ladders (ThermoFisher Scientific, Grand Island, NY, USA) were analyzed simultaneously with samples. This nested PCR assay can detect as few as 100 copies per reaction (Symonds et al. 2009).

4.3.3 Results and Discussion

4.3.3.1 Yungas WSP and UASB-Pond Systems

The data from the Yungas WSP and UASB-Pond systems for AdV are summarized in Table 12, and the data for NoVGI, RV, and PMMoV are summarized in Tables 6, 7, and 8, respectively (Section 3.2). Samples were collected serially on 180-µm, 0.45-µm, and positively-charged (+) 0.45-µm filters to separate viruses associated with different-sized UASB and WSP particles. The data reported here for AdV are from the same DNA used for reverse transcription and detection of RV, NoVGI, and PMMoV, with the only exception that the 0.45-µm (+) filter samples were not analyzed for AdV due to the low recovery of the DNA extraction and purification control (see Appendix E). The concentrations of PMMoV, RV, and NoVGI retained on 180-µm filters were generally less than 5% of the corresponding concentrations detected in acidified samples captured on 0.45-µm filters (assumed to be the total concentrations in the samples), and were also lower than the corresponding concentrations retained on the 0.45-µm and 0.45-µm (+) filters from the cascade (see Section 4.2.3.3). The concentrations of AdV retained on 180-µm filters were all below the pLOD for all samples, except for all three sample replicates collected from the effluent of the UASB reactor (and one of three replicates from the second polishing pond in the Yungas UASB-Pond system).

Concentrations of NoVGI and PMMoV retained on 0.45-µm and 0.45-µm (+) filters were greater in the UASB reactor effluent than they were in all of the pond samples from both systems (see Section 4.2.3.3), indicating the possibility that viruses leaving the UASB reactor were more likely to be associated with small (<180 µm) particles than viruses in the WSPs. However, the AdV concentrations retained on 0.45-µm filters from the cascade (receiving filtrate from the 180-µm filters) for the UASB reactor sample were only slightly greater than they were at

other sample sites, and were also similar to the corresponding concentrations on 0.45-µm filters from acidified samples. This was not the case for PMMoV, which could indicate that AdV may be more associated with particles than PMMoV. Also, contrary to PMMoV, RV, and NoVGI, AdV leaving the UASB reactor was more associated with particles larger than 180 µm.

Table 12. Log_{10}-transformed quantities of human adenovirus (copies mL^{-1}) retained serially on a cascade of 180-µm and 0.45-µm filters without acidification, and on a 0.45-µm filter after acidification, from three samples points each in the Yungas WSP and UASB-Pond systems. Mean values of log_{10}-transformed sample triplicates are shown in bold and in parentheses. Concentrations marked with an asterisk (*) are below the process limit of detection. NA = no qPCR amplification. †

WSP (Three-Pond) System						
Sample Site	**180 µm**		**0.45 µm**		**0.45 µm (acidified)**	
Facultative Pond	1.8*		3.2		3.8	
	1.9*	**(2.2)**	4.1	**(3.9)**	3.1	**(3.7)**
	3.0*		4.4		4.2	
Maturation Pond 1	2.4*		4.2		3.5	
	2.2*	**(2.3)**	4.1	**(4.1)**	2.6*	**(3.4)**
	2.5*		4.0		4.2	
Maturation Pond 2	2.1*		3.3		3.0*	
	1.5*	**(1.9)**	3.9	**(3.8)**	3.1	**(3.4)**
	2.0*		4.2		4.0	
UASB-Pond System						
Sample Site	**180 µm**		**0.45 µm**		**0.45 µm (acidified)**	
UASB Reactor	3.1		4.0		3.4	
	3.4	**(3.4)**	4.8	**(4.6)**	4.6	**(4.2)**
	3.9		5.2		4.6	
Polishing Pond 1	1.6*		3.7†		3.1	
	1.8*	**(1.9)**	4.4	**(4.2)**	3.9	**(3.8)**
	2.2*		4.4		4.2	
Polishing Pond 2	NA†		2.8*†		2.6*	
	1.8*	**-**	3.7	**(3.3)**	3.6	**(3.2)**
	3.7		3.4		3.5	

† Recovery of extraction control was between 1% and 10%.

4.3.3.2 Mesocosm Study

In order to further study particle associations and the settling of viruses in WSP water, a mesocosm study was carried out at the Yungas UASB-Pond system. The results of the mesocosm study are shown in Figure 13. At the beginning of the experiment, the average AdV concentration in the WSP water was 1.5×10^4 copies mL^{-1}. After

five days, the concentration of AdV retained on 180-µm filters from the sediment sample was 8.2×10^2 copies mL^{-1}, and the average concentration passing through this filter was 2.5×10^3. The concentration in the supernatant was only 2.7×10^2 copies mL^{-1}. The measured Day 6 concentrations were all close to the assay pLOD; concentrations in the two sediment samples were slightly above this limit, while the concentration in the supernatant sample was slightly below it. Given that the virus counts in the two sediment samples are cumulative (filtration was performed in series), the sum of the two measured AdV concentrations on Day 6 in the sediment samples is only 0.6-$\log_{10}$ lower than the initial concentration on Day 1, and was greater than the concentration in the supernatant. The PMMoV results showed a different trend. On Day 1, the PMMoV concentration in the mesocosm was 3.8×10^4 copies mL^{-1}, and after five days, the concentration in the supernatant was nearly the same (4.7×10^4 copies mL^{-1}). The concentrations in the sediment samples, however, were two or more orders of magnitude lower.

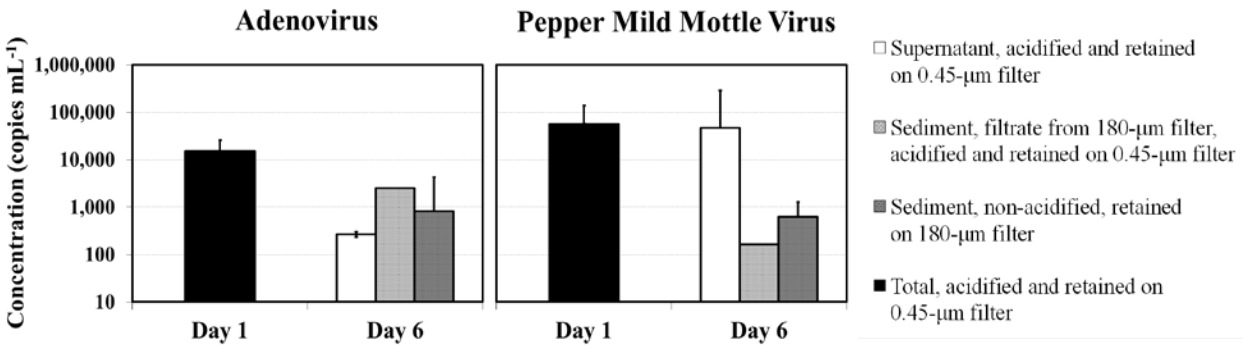


Figure 13. Mesocosm experiment results for adenovirus and pepper mild mottle virus. Error bars represent standard deviations from duplicate samples (duplicate samples were not analyzed for non-acidified sediment samples retained on 180-µm filters; these data represent single samples, and thus they have no error bar).

Thus, AdV genome targets were either removed or degraded more than PMMoV genome targets. Also, particle-associated AdV may also have been removed via sedimentation, or alternatively, AdV at the water surface may have degraded more rapidly than AdV at the bottom of the mesocosm. However, PMMoV likely did not settle in this mesocosm, and its genome did not degrade at the water surface after five days. PMMoV genome targets appear to have degraded more rapidly at the bottom of the mesocosm than they did at the surface. Based on these results, PMMoV may be a conservative surrogate for AdV transport at the surface of WSPs, but not necessarily in pond sediments.

4.3.3.3 Belding WSP System

The AdV concentrations measured in the cascade-filtration samples collected at the Belding WSP system are presented in Table 13. DNA extraction efficiencies, as measured by Salmon Sperm DNA recovery (Appendix E) were all greater than 1%, and with the exception of a few samples from the untreated sewage, anaerobic pond, and final two storage ponds, were all above 10% (see Table 13).

Table 13. Log_{10}-transformed quantities of human adenovirus (log_{10}(copies) mL^{-1}) retained serially on a cascade of 180-µm, 0.45-µm, and positively-charged (+) 0.20-µm filters and in the PEG-concentrated final filtrate, for samples from the Belding WSP system. Mean values of log_{10}-transformed sample triplicates are shown in bold and in parentheses. If mean values were estimated using the survival model, they are shown in parentheses and in italics, but not in bold. NA = no qPCR amplification.

Sample Site	180 µm		0.45 µm		0.20 µm (+)		PEG-concentrated final filtrate	
Influent (Untreated Sewage)	1.5*†		2.2*†		1.8*		2.6*	
	1.6*†	*(1.7)* +	2.8	**(2.6)** +	2.0*	*(2.0)* +	2.3*	**(2.5)** +
	NA		2.9		2.1*		2.5*	
Upflow Anaerobic Pond Effluent	1.5*		4.5		1.7*		2.2*	
	NA†	*(1.9)* +	4.2	**(4.3)** +	4.6	**(3.6)** +	3.9	**(3.3)** +
	2.4*		4.3		4.6		3.7	
Facultative Pond 1 Effluent	NA		1.8*		1.7*		NA	
	NA	*(1.3)* +	1.6*	*(2.3)* +	1.7*	*(2.0)* +	NA	*(1.2)*
	1.7*		2.4*		1.7*		0.4*	
Facultative Pond 2 Effluent	NA		1.2*		NA		2.2*	
	NA	+	NA	*(1.3)*	NA		1.4*	*(1.6)* +
	NA		NA		NA		NA	
Storage Pond 1 Effluent	1.7*		2.4*†		NA		1.4*	
	NA†	*(1.1)*	NA†	*(2.0)* +	NA		NA	*(1.5)*
	NA		NA†		NA		NA	
Storage Pond 2 Effluent (Final Discharge)	NA		NA†		NA		NA	
	NA		NA†		NA	*(1.3)* +	NA	
	NA		NA		0.8*		NA	

* Below the process limit of detection

+ Positive signal detected via nested PCR

† Recovery of extraction control was between 1% and 10%. Recovery was > 10% for all other samples.

The AdV concentrations retained on 180-µm filters were all below the pLOD, but still showed qPCR amplification for one or two out of three replicates from four out of the six sample locations. Red water mites were visibly observed in the samples from the effluent of Storage Pond 1, and appeared to be the only material retained on

the 180-µm filter from that sample. Untreated sewage samples yielded a mean $\log_{10}$-transformed concentration of 2.6 on the 0.45-µm filter, and 2.5 in the PEG precipitate of the final filtrate (though both mean values were below the pLOD). Concentrations retained on the 180-µm and 0.20-µm (+) filters from untreated sewage samples were also below the pLOD, but were estimated to be approximately 1.7 $\log_{10}$ units based on the survival function (Appendix C). The highest AdV concentrations were detected in the anaerobic pond effluent, retained on 0.45-µm and 0.20-µm (+) filters. The anaerobic pond effluent was also the only location where AdV was consistently detected at levels above the pLOD. This may be an indication that the anaerobic pond is causing a large proportion of AdV to become associated with small (<180 µm) and possibly negatively-charged or colloidal particles.

The results for F+ coliphage for samples collected from the Belding WSP system are presented in Figure 14. All negative controls, including 100-mL samples of phosphate-buffered water passed through the filtration equipment after washing and disinfecting filter holders and Erlenmeyer flasks (a test for cross-contamination between samples), resulted in the absence of plaque-forming units. Initially, the positive (spot plate) and precision and recovery (spiked) controls failed (no plaque-forming units or blotches detected after incubation). However, after a second run, the positive control passed (blotches were present), and the precision and recovery control yielded a concentration of 3.7 pfu mL^{-1}, which is approximately half of the concentration in the spiked reagent water used for the test (i.e., ~50% recovery).

In the wastewater samples, F+ coliphage was only detected at quantifiable concentrations (>10 pfu per plate) in samples from the untreated sewage, the anaerobic pond, and the first facultative pond. In samples from the effluents of the second facultative pond and the first storage pond, F+ coliphage was detected at concentrations < 1 pfu mL^{-1}. At the final effluent and discharge point (effluent of Storage Pond 2), F+ coliphage was not detected at all—the total concentration in this sample was < 0.1 pfu mL^{-1}, meaning that the overall removal was greater than 3.2-$\log_{10}$ units. The fractions of F+ coliphage concentrations measured in filtrates from the different filters were very similar for the samples from the untreated sewage, anaerobic pond, and first facultative pond samples, indicating that F+ coliphage-particle associations may not have changed much in the anaerobic and facultative ponds. Furthermore, differences in the $\log_{10}$ reductions in concentrations in the filtrates from each of the different filters were not significant, meaning that F+ coliphage inactivation rates in the first two ponds were probably similar for coliphage retained on each of the filter sizes, and for the coliphage passing through the final 0.20-µm (+) filter.

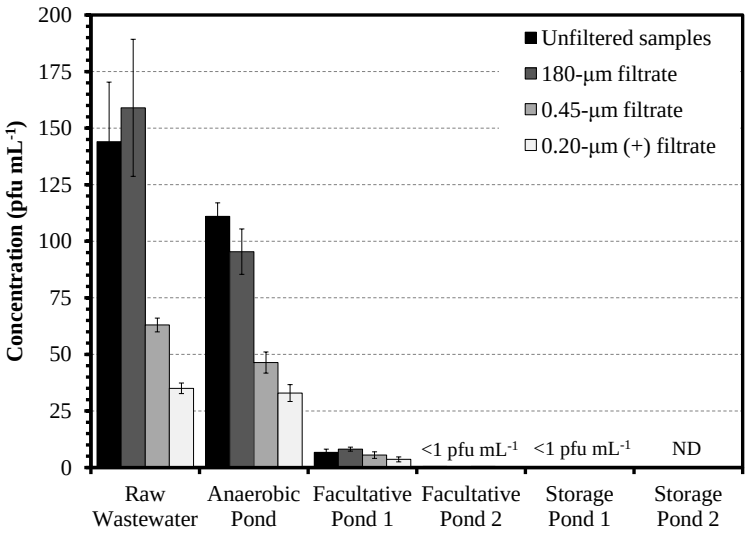


Figure 14. F+ coliphage concentrations in unfiltered samples and in the consecutive filtrates of 180-µm, 0.45-µm, and positively-charged 0.20-µm filters, from samples of untreated sewage and from the effluent of each pond in the Belding WSP system. Error bars represent standard deviations of 5 replicates. ND = not detected.

When comparing results for AdV and F+ coliphage, it is important to note that there are fundamental differences in the data based on the methods that were used, notably that the methods used to detect and quantify AdV (nested PCR and qPCR) do not distinguish viable viruses from non-viable ones, and the method used to quantify F+ coliphages (double agar layer plaque assay) only measures viable coliphages that are capable of forming plaques on the lawn of *E. coli* Famp strain (ATCC #700891).

4.3.3.4 CePTS UASB-Pond System

The results of the CePTS UASB-Pond system samples are presented in Figure 15. In the untreated sewage samples, the concentrations of AdV retained on the 10-µm filters and passing through the 10-µm filters were similar. However, while the concentrations passing through the 10-µm filters from the UASB reactor, second pond effluent, and rock filter effluent samples were all above the pLOD, the concentrations retained on the 10-µm filters reduced throughout the treatment train. The difference between the median $\log_{10}$-transformed AdV concentration retained on the 10-µm filters in the untreated sewage and the median $\log_{10}$-transformed AdV concentration retained on the 10-µm filters in the rock filter effluent is 3.4 ($\log_{10}$ units). The difference between the same two points for the 10-µm filter filtrate (viruses passing through the 10-µm filters) is only 1.0 $\log_{10}$ units. This indicates that many of the AdV that are associated with particles greater than 10 µm are either settling to the bottom of the ponds, or are degrading more rapidly than the non-particle-associated AdV and the AdV associated with particles smaller than 10 µm.

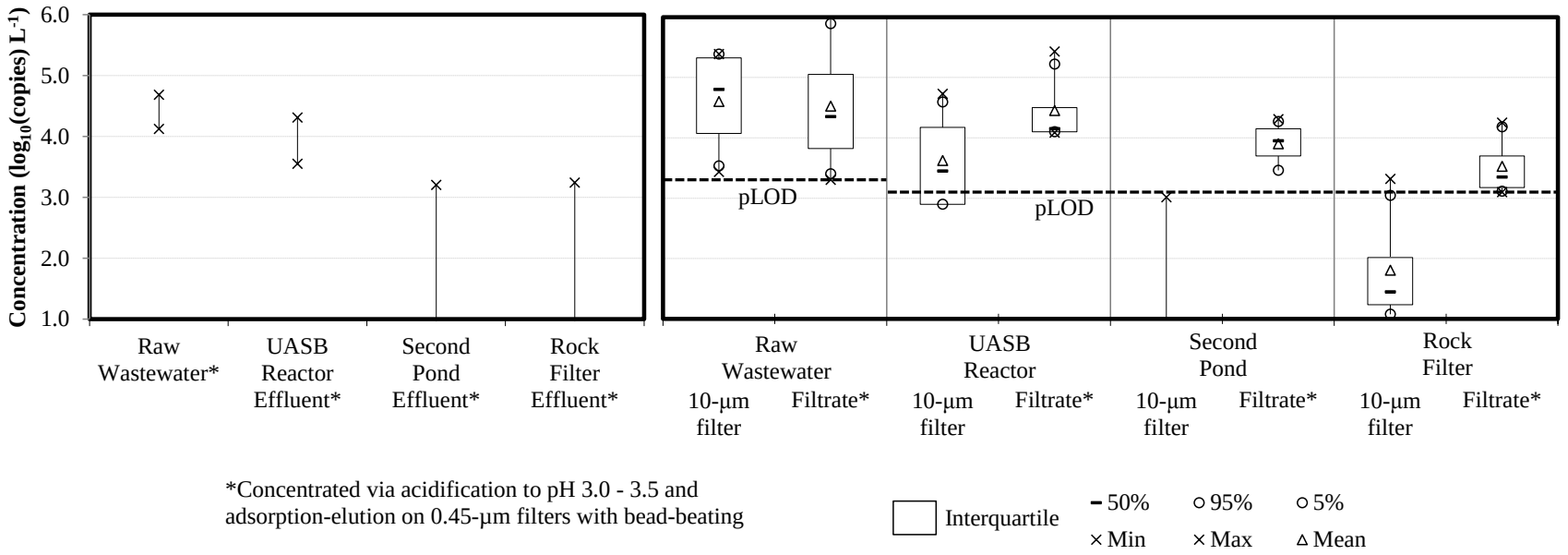


Figure 15. Total human adenovirus concentrations (copies mL^{-1}) (left); and human adenovirus concentrations retained on 10-µm filters and in the filtrate from the 10-µm filters (right), in samples from the untreated sewage, UASB reactor effluent, second pond effluent, and rock filter effluent.

4.3.3.5 Minas Gerais UASB Reactor System

The results of the Minas Gerais UASB reactor system samples are presented in Figure 16. For the cascade filtration study, the concentrations of AdV detected on the 10-µm and 0.45-µm filters for the untreated sewage and UASB reactor effluent samples were similar. However, the concentration detected in the final filtrate for the UASB reactor sample was approximately an order of magnitude greater than the corresponding concentration in the final filtrate of the untreated sewage sample. This implies that AdV in wastewater leaving this UASB reactor may be less associated with particles than AdV in the untreated sewage.

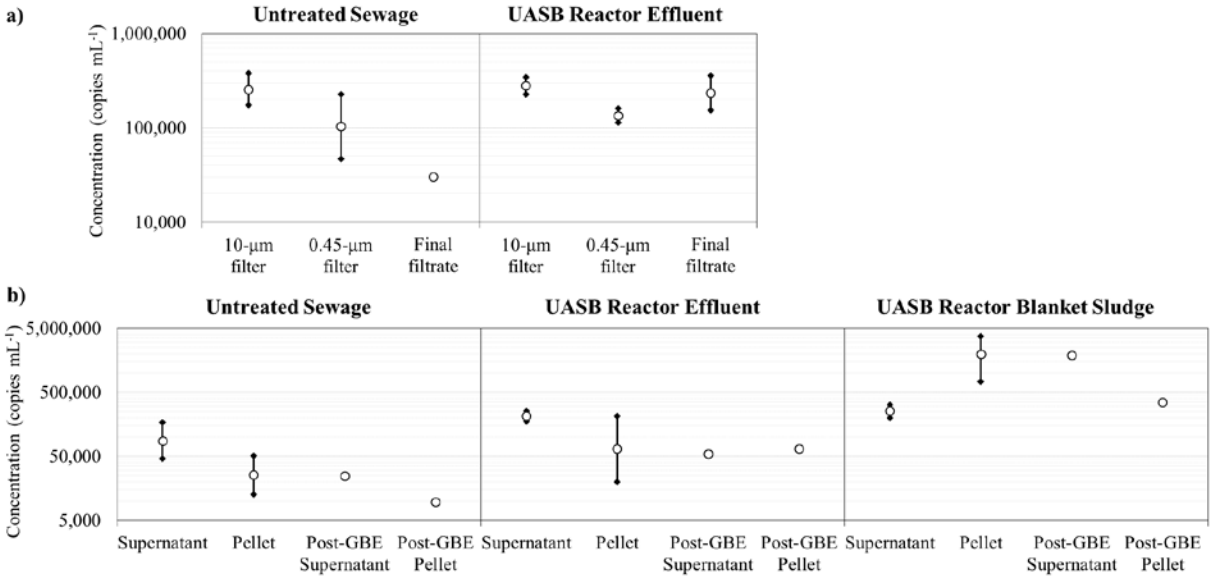


Figure 16. Concentrations of human adenovirus a) retained on a cascade of filters: 10-µm filters, 0.45-µm filters, and in the final filtrate (final filtrate acidified and viruses retained on 0.45-µm filters); and b) in sample supernatants and pellets after centrifugation at 1,157×g for 10 min, for samples from the Minas Gerais UASB reactor system. The circles represent the geometric mean concentration and the error bars represent the minimum and maximum concentrations measured. "Post-GBE" refers to the supernatant and pellet portions separated from the original pellet, after resuspension 40 mL of 0.05 M glycine, 3% beef extract (GBE) buffer

After centrifugation at 1,157×g for 10 min, the proportions of AdV detected in sample supernatants compared to the pellets were similar for the untreated sewage and UASB reactor samples. The overall concentrations in the untreated sewage were slightly lower than the overall concentrations in the UASB reactor effluent, however this may have been because grab samples were collected instead of composite samples. The overall rate of AdV entering the wastewater treatment plant in the untreated sewage may fluctuate throughout the day, depending on how many people with AdV infections are discharging wastewater to the sewerage system at a

given time. The proportion of AdV in the supernatant of the sludge blanket sample after centrifugation was much lower, compared to the concentration detected in the pellet. Not surprisingly, the concentrations of AdV detected in the supernatants of the UASB reactor sludge blanket sample and the sample from the effluent of the UASB reactor were similar. Viruses not attached to settleable particles, in the liquid fraction of the water in the UASB reactors would be expected to exit through the outlet at the top of the reactor. Interestingly, the geometric mean concentration of AdV in the final filtrate of the UASB reactor samples was similar to the AdV concentrations in the supernatants of the samples from the UASB reactor sludge blanket and the UASB reactor liquid effluent, after centrifugation. According to Stoke's law, assuming a particle density of 1,200 g L^{-1}, the centrifugation conditions used in this experiment should separate out all particles larger than 1.2 μm, which is not much larger than the 0.45-μm pore size of the filter. One possible explanation for the greater concentration of AdV in the final filtrate from the UASB reactor effluent samples is that the viruses may be desorbing from wastewater particles when exposed to the anaerobic conditions, as reported previously for coliphage and WSP particles (Ohgaki et al. 1986). However, it is plausible that some AdV are still entrapped within small flocs or granular sludge, and therefore exiting the UASB reactor associated with particles that are too small to settle, as was observed in the Belding system (Section 4.3.1.3, Table 13).

After resuspension of sample pellets in the GBE buffer and a second round of centrifugation at 1,157×*g*, 72 to 77% of AdV in the untreated sewage sample pellets and 78 to 85% of AdV in UASB blanket sludge sample pellets were desorbed and recovered in the supernatant after the second round of centrifugation. On the contrary, for the UASB reactor effluent sample pellet, the percentage recovered in the supernatant after the second round of centrifugation was only 45 to 47%. This might be an indication that AdV-particle associations in untreated sewage and UASB granular sludge were largely reversible, while approximately half of particle-attached AdV exiting the UASB reactor exhibited non-reversible adsorption to these smaller particles.

The median volumetric concentration of AdV in samples collected from the bottom sludge layer in the UASB reactor is 2.6×10^{7} copies mL^{-1}, which is one order of magnitude greater than the median sum of AdV concentrations in the pellets and supernatants from the UASB reactor sludge blanket samples. This is an indication that AdV is indeed enmeshed within UASB granular sludge and a fraction of the viruses are removed from the reactor in the sludge. In the Minas Gerais UASB reactor system, sludge was discharged to an open sludge drying bed with a pervious floor that allowed drain water to percolate out of the sludge, allowing the sludge to become

desiccated. While samples of the sludge drying bed drain water were not collected for this study, the higher concentration of AdV in UASB reactor sludge demonstrates the possible need for treatment or disinfection of sludge and drying bed drain water from these systems.

4.3.3.6 Implications for Wastewater Treatment, Resource Recovery and Future Research

In summary, the results from the mesocosm study and the field studies of virus-particle associations demonstrate that UASB reactors and WSPs affect virus-particle associations in potentially different ways, and this effect may be different for different virus types (see Table 14). While PMMoV detected in the effluent of the Yungas UASB reactor and WSP mesocosm was more associated with smaller particles and showed no indication of settling, AdV in the effluent of three different UASB reactors was associated with small and large particles, and showed evidence of possible settling in downstream WSPs. PMMoV genome targets appeared to degrade more rapidly in pond sediments than they did at the pond surface. Also, AdV genome targets were more volumetrically concentrated in UASB reactor sludge than they were in UASB reactor influent and effluent water samples, which indicates that AdV may become entrapped in granular UASB reactor sludge; culturable EV was also found to be more volumetrically concentrated in UASB sludge than it was in WSP sediments and in wastewater samples from the UASB reactor effluent or any of the WSPs from the two Yungas systems (see Section 4.2). Viruses are likely removed from UASB reactors when sludge is evacuated. As such, precautions should be taken when handling UASB reactor sludge.

The results of the centrifugation study for samples collected from the Minas Gerais UASB reactor system indicate that AdV particle-associations in the liquid effluent of UASB reactors may be less reversible than the AdV associations with granular sludge suspended in the UASB sludge blanket. Some of the particle-associated AdV in untreated sewage may also dissociate under anaerobic conditions in UASB reactors, which follows observations made by Ohgaki et al. (1986) for indigenous coliphage from a WSP system in Thailand. WSPs and UASB reactors may create conditions that alter the surface properties of wastewater solids or the ionic conditions in the water, which could in turn, affect virus-particle adsorption (Sobsey and Cooper 1973). The concentrations and characteristics of suspended solids in these systems can also affect virus-particle adsorption. The average total suspended solids (TSS) concentration in the untreated sewage from the Belding WSP system during the month of sample collection was 145 mg L^{-1} (SD = 56), while the average TSS concentration in the anaerobic pond effluent

was 43 mg L^{-1} (SD = 6.4), and this concentration decreased throughout the system, with a concentration of only 3.9 mg L^{-1} (SD = 1.3) in the final effluent of storage pond 2. In the Yungas WSP system, TSS concentrations were similar, with 240 mg L^{-1} in the influent, 53 mg L^{-1} in the facultative pond effluent, 50 mg L^{-1} in the effluent of the first maturation pond, and 36 mg L^{-1} in the effluent of the second maturation pond. However, TSS concentrations in the two UASB-Pond systems studied were greater, with average TSS concentrations of 83 mg L^{-1} and 74 mg L^{-1} detected in the effluents of the UASB reactors of the Yungas and CePTS systems, respectively. AdV in the UASB-Pond system were also more highly-associated with particles than AdV in the Belding WSP system, but it is unclear if the higher TSS concentrations had anything to do with this.

The results from the particle-association studies presented here have important implications for wastewater treatment and resource recovery from WSP systems. Viruses that are not removed from WSP systems, and retain viability, can present a risk for farmers using the treated wastewater for irrigation, or for vendors and consumers of wastewater-irrigated crops. The sedimentation of viruses in WSP systems does not appear to be an important mechanism for removal. However, when UASB reactors are incorporated at the beginning of WSP systems, some viruses, such as AdV, may be removed more readily, possibly via sedimentation. Other viruses, such as PMMoV, may not. AdV that is in closer proximity to small, non-settleable particles may be vulnerable to capsid damage caused by indirect sunlight mechanisms. A previous study found that AdV capsid proteins are damaged by reactive intermediates formed when sunlight interacts with sensitizer molecules in wastewater (Bosshard et al. 2013). On the other hand, small non-settleable particles may protect viruses from direct sunlight inactivation. The qPCR methods used to detect AdV in this study only measure the quantity of a targeted region of virus genomes present in the samples. The quantities measured likely provide an over-estimation of the quantities of viable AdV.

To better understand the nature of virus-particle associations in UASB reactors, the objectives of future work should be to determine if virus-particle adsorption is reversible, results in loss of viability, or protects the virus from inactivation or disinfection mechanisms. It is also important to understand if aggregated or particle-associated viruses leaving UASB reactors pose different risks than dispersed (non-aggregated, non-particle-associated) viruses, since infectivity may change based on the virus' state of aggregation. For example, aggregated norovirus in an inoculum used in a human volunteer study was found to have a higher infectious dose than disaggregated (dispersed) norovirus in a different inoculum (Teunis et al. 2008).

Table 14. Summary of results and potential implications for wastewater treatment and resource recovery.

System	Methods	Summary of Results	Possible Implications
Yungas WSP System Yungas UASB-Pond System	Cascade filtration: 180-µm > 0.45-µm > 0.45-µm (+)[a]	PMMoV, RV, and NoVGI in UASB reactor effluent more highly-associated with particles <180 µm (Section 4.2); high AdV concentrations retained on 180-µm filter in UASB reactor effluent; minimal change in concentrations of PMMoV, NoVGI, and AdV throughout system; RV detected in UASB reactor but not in polishing ponds	• Virus removal via sedimentation appears to be unlikely for PMMoV in particular • AdV in UASB reactor effluent are more associated with particles >180 µm; PMMoV, RV, and NoVGI are more associated with particles <180 µm
Mesocosm Experiment	Separation of supernatant and sediment by gravity for 5 days; supernatant analyzed separately and sediment analyzed with cascade filtration: 180-µm > filtrate[b]	PMMoV concentration in supernatant on Day 6 was similar to overall concentration on Day 1, but PMMoV concentrations in sediment were more than an order of magnitude lower; AdV concentration in the supernatant on Day 6 was several orders of magnitude lower than the overall concentration on Day 1, and lower than concentration in the sediment on Day 6.	• AdV may attach to particles and settle under quiescent conditions • PMMoV appears to not settle • PMMoV genome targets may degrade faster in WSP sediments
Belding System	Cascade filtration: 180-µm > 0.45-µm > 0.20-µm (+) > filtrate[b]	Concentrations of F+ coliphage passing through to the final filtrate were similar for untreated sewage and anaerobic pond samples; F+ coliphage concentrations in unfiltered samples similar to concentrations passing through 180-µm filters; high AdV concentrations on 0.45-µm, 0.20-µm (+) filters and in final filtrate for anaerobic pond samples	• F+ coliphages were removed within first three ponds • AdV in the anaerobic pond effluent may be largely associated with particles <180 µm and unassociated (dispersed)
CePTS System	Cascade filtration: 10-µm > filtrate[c]	Concentrations of AdV retained on 10-µm filters decreased progressively throughout the system (>3-$\log_{10}$ reduction); minimal reduction of AdV passing through 10-µm filters	• AdV attached to particles >10-µm may be removed via sedimentation in subsequent WSPs
Minas Gerais UASB Reactors	Cascade filtration: 10-µm > 0.45-µm > filtrate[c]	AdV concentrations on 10-µm and 0.45-µm filters were similar for both untreated sewage and UASB reactor effluent samples; AdV concentration passing through both filters was order of magnitude greater in UASB reactor effluent	• UASB reactor may cause some particle-associated AdV in untreated sewage to dissociate • AdV-particle associations in UASB reactor effluent may be less reversible than particle associations in granular sludge from UASB sludge blanket • Some of the AdV retained in granular sludge appear to settle to the bottom of the UASB reactor and are removed during sludge evacuation
	Separation by centrifugation at 1,157×*g* for 10 min; separate analysis of supernatant and pellet; resuspension of pellet in glycine beef extract (GBE) buffer, repeated centrifugation, separate analysis of supernatant and pellet	Similar AdV concentrations in supernatants of UASB reactor effluent and "blanket" sludge; greater proportion of AdV in pellet from "blanket" sludge released after contact with GBE buffer, compared to effluent of UASB reactor; AdV concentrations per dry weight in sludge "blanket" and bottom of the UASB reactor were similar	

[a] 0.45-µm (+) filters analyzed for PMMoV, NoVGI, and RV (Section 4.2), but not for AdV
[b] Viruses concentrated using PEG precipitation
[c] Samples were concentrated via acidification and adsorption-elution on 0.45-µm filters with bead-beating

5 WATER REUSE AND MICROBIAL RISK[6]

5.1 Overview

Globally, 1.5 billion people (the majority in developing countries) are connected to sewerage collection systems without any form of wastewater treatment (Baum et al. 2013). Many of the wastewater treatment systems in developing countries that do exist are overloaded, have major operation and maintenance problems, or are completely abandoned altogether (Libhaber and Orozco-Jaramillo 2012). With increasing population growth and water scarcity, the direct reuse of wastewater treated with overloaded or malfunctioning wastewater treatment plants and the indirect reuse of untreated wastewater discharged to water bodies, is becoming increasingly common for farmers in urbanizing watersheds. With the new SDG targets related to increasing wastewater treatment coverage and expanding safe water reuse by 2030, many of these communities with non-existent or ineffective wastewater treatment plants are likely to invest in wastewater treatment infrastructure. In order to establish effective wastewater treatment and safe water reuse practices, it is necessary to understand the fate and transport of pathogens and fecal indicators in wastewater reuse systems. Also, because the installation of new wastewater treatment plants can take years to complete, intermediary solutions (such as the use of RBF on farms) are necessary to reduce the existing health burden associated with many current water reuse practices that may be unsafe.

The purpose of this chapter is to evaluate direct and indirect wastewater reuse for irrigation a) by comparing the fate and transport of pathogens and fecal indicators in WSPs and in on-farm RBF systems treating sewage-impacted river water, and b) by estimating health burdens from consuming wastewater-irrigated lettuce using a QMRA model that incorporates parameter uncertainty. Section 5.2 is a study of the performance of two overloaded WSPs in two small cities located in the Cochabamba Valley (Bolivia), and the fate and transport of pathogens and fecal indicators in these systems and in the fields irrigated directly with treated wastewater from these systems. Section 5.3 is a study of the use of RBF to treat surface water that is highly-contaminated with untreated sewage and is used to irrigate lettuce crops. Quantitative microbial risk assessment is used to assess the estimated

[6] Sections 5.2 and 5.3 are written as stand-alone manuscripts for the journals *Water Science and Technology* and *Environmental Science and Technology*, respectively. Section 5.3 was submitted to *Environmental Science and Technology* on November 2, 2015.

health burdens in Cochabamba resulting from the consumption of lettuce irrigated with river water, and the consumption of lettuce irrigated with river water that is treated on the farm by RBF.

5.2 Pathogens and Fecal Indicators in Waste Stabilization Pond Systems with Direct Reuse for Irrigation: Fate and Transport in Water, Soil and Crops

5.2.1 Introduction

WSPs are frequently used for wastewater treatment in rural areas, small communities, and regions with limited financial resources, partly because they are resilient systems that perform well and remove pathogens with limited maintenance requirements (Mara 2003). However, most studies in the literature are limited to WSP systems that are well-maintained and in good condition. Data is seldom collected from systems that are overloaded and that have started to malfunction due to the lack of maintenance and sludge accumulation. The social, economic, and environmental benefits of wastewater reuse have been well-documented (Cornejo et al. 2013; Mo and Zhang 2012). However, the majority of direct wastewater reuse for irrigation in the world likely occurs in places with treatment systems that are bypassed, abandoned, malfunctioning, or overloaded. For example, nearly 2.4 billion m^3 of treated wastewater are directly used for irrigation each year in North Africa and the Middle East (FAO 2015), where many wastewater facilities are heavily overloaded (Ghneim 2010; McIIwaine and Redwood 2010). The Latin America and the Caribbean region has more land area equipped for direct wastewater irrigation than the Middle East and North Africa combined (FAO 2015), but less than 10% of the wastewater in this region receives treatment, partially due to the fact that the majority of wastewater treatment plants are either abandoned or experience operational problems and are malfunctioning (CReW 2014; Patiño Gómez and Lara-Borrero 2012; UN-HABITAT 2008). This lack of operation and maintenance for wastewater treatment systems poses a serious problem to safe wastewater reuse in many developing regions of the world.

Little is known about pathogen removal efficiency in these overloaded wastewater treatment systems. Data about pathogens in water, soil, and crops from wastewater irrigation operations in developing countries with overloaded systems are also often not available. Few laboratories in developing countries are equipped to measure the concentrations of pathogens in environmental samples, and sometimes only fecal indicators (e.g., thermotolerant coliforms) are the only organisms monitored. As a result, many developing countries still operate with water reuse policies based on the old 1989 World Health Organization Guidelines for Wastewater Reuse (WHO 1989). These

old guidelines required thermotolerant coliform concentrations to be lower than 1,000 cfu per 100 mL for unrestricted reuse (e.g., irrigation of crops consumed raw, sports fields, public parks), and suggested that WSP systems with 8 – 10 days of retention provide sufficient treatment for restricted reuse (e.g., irrigation of fodder crops, pasture, cereal crops).

The 2006 WHO Guidelines (WHO 2006) do not establish limits for fecal indicators, but rather recommend the use of QMRA to establish appropriate concentrations of pathogens in treated wastewater used for irrigation. There are several challenges associated with the implementation of these guidelines, which are much more complex than the 1989 guidelines. This requires knowing (or assuming) actual pathogen concentrations in treated wastewater, which is often done based on the concentrations in untreated sewage and an assumption about the pathogen reduction achieved for a given wastewater treatment process (e.g., use of WSPs). However, the pathogen reduction efficiency may be quite different in new or well-performing WSP systems than it is in overloaded and under-maintained systems. Because of this, it is necessary to understand the performance of overloaded WSP systems, especially those systems where sludge has accumulated for years without removal, which is too often the case for WSP systems in small towns and low income regions. Another challenge associated with the implementation of water reuse policies based on the 2006 WHO Guidelines is the lack of knowledge about the concentration of pathogens in soil or on crops (which would be used to estimate the risks to field workers and consumers of crops). In most exposure assessments, the quantities of pathogens on irrigated crops are often estimated based on pathogen concentrations in irrigation water and an assumption about the retention of water by the crop after irrigation (e.g., Mok and Hamilton 2014; Shuval et al. 1997); this approach was also used for an example presented in the 2006 WHO Guidelines for unrestricted water reuse for irrigation (WHO 2006). Concentrations of pathogens and fecal indicator organisms in irrigation water are seldom compared with the respective concentrations in irrigated soil and on irrigated crops.

Therefore, the objective of this study was to assess the fate and concentrations of a broad range of human pathogens and fecal indicator organisms in water samples from two overloaded WSP systems, and in soil and crop samples from fields irrigated with the treated effluent of those systems. The organisms chosen for this study were *E. coli*, coliphage, *Giardia*, *Cryptosporidium*, and helminth eggs. Two WSP systems located in the upper Cochabamba Valley of Bolivia were used as case examples; in this region, wastewater is used to irrigate at least 5,700 hectares of crops (Ministerio de Medio Ambiente y Agua 2013). National wastewater reuse guidelines do not exist, but local

norms established in Cochabamba are based on the 1989 WHO guidelines. At the time of sampling, the two WSP systems were overloaded and had not been maintained. This is not uncommon in Bolivia, where 37% of wastewater treatment plants are not functioning at all, 22% provide only primary treatment, and the majority of wastewater treatment plants that are in operation perform poorly due to operation and maintenance problems (Ministerio de Medio Ambiente y Agua 2013). The two WSP systems described in this study are officially managed by the respective municipal wastewater companies, but in practice they have been largely abandoned, and are informally maintained by cooperatives of local farmers who use the effluent for irrigation.

5.2.2 Methods

5.2.2.1 Waste Stabilization Pond and Irrigation Systems

The wastewater treatment systems in Arani and Punata, located in the Upper Cochabamba Valley, consisted of anaerobic, facultative, and maturation ponds (Figure 17). The system in Arani, which received an average flow rate of 748 m^3/day, contained two 425 m^3 anaerobic ponds (in parallel), followed by two 1,500 m^3 facultative ponds (in parallel), two 950 m^3 primary maturation ponds (in parallel), and two 950 m^3 secondary maturation ponds (in parallel). This system was constructed in 2000, and at the time of sampling, it had been operating for more than 13 years without sludge ever having been removed. The total theoretical HRT, based on the original pond volumes (not considering sludge accumulation) and the flow rate measured at the time of sampling, was 10 days. However, sludge had accumulated to the point where it blocked the entrance to many of the ponds, causing a situation where wastewater only flowed through one of each of the ponds in parallel. The sludge that had accumulated in the ponds that were receiving wastewater was estimated to occupy roughly 80% of the pond volumes (data not available). Considering that wastewater was only flowing through one of each of the duplicate ponds in series, and considering the estimated volume lost due to sludge accumulation, the total theoretical HRT for this system at the time of sampling was probably closer to only one day (Iriarte et al. 2013; Mercado et al. 2013).

The WSP system in Punata received an average flow of 2,730 m^3/day, and consisted of three 1,300 m^3 anaerobic ponds (in parallel), followed by two 7,000 m^3 facultative ponds (in parallel), and three 3,000 m^3 maturation ponds (in parallel). The total theoretical HRT, based on the original pond volumes (not considering sludge accumulation) and the flow rate measured at the time of sampling, was also 10 days. However, flow patterns had also been disturbed in this system, causing wastewater to only flow through one of each of the ponds in parallel.

This unequal flow distribution may have occurred due to one or more of the following factors: 1) modifications made to the final outlet channel to divert treated water to irrigation fields, which altered the level of water upstream in the ponds; 2) poor construction of the inspection boxes, causing unequal distribution of flow to ponds connected in parallel; or 3) the lack of maintenance in the ponds, causing uneven sludge accumulation. Sludge in these ponds had not accumulated to the same extent as it had in the Arani system, but still had never been evacuated since the construction of the system in 1995. Considering the observed flow patterns and the estimated volume lost due to sludge accumulation, the theoretical HRT for this system at the time of sampling was estimated to be 2.5 days (Iriarte et al. 2013; Mercado et al. 2013). The final effluent from each system flowed to adjacent irrigation fields in open channels (Figure 18) and was applied to crops using a border irrigation technique with small rectangular plots, known locally as *melgas*, *platabandas*, or *cajetas* (Figure 19). Water was applied by farmers to one end of the plot, and allowed to sequentially flood the furrows between each crop row until the entire zone was flooded.

5.2.2.2 Sample Collection

Five grab samples of water were collected from the influent and effluent of the anaerobic ponds and from the final effluent from each system between 9am and 12pm in October and November of 2012 and in May, July, and October of 2013. A sample was also collected in July 2013 from the extraction point of the irrigation channel in Punata. Soil and crop samples were collected in October and November 2012, and in July 2013. Alfalfa was the only crop harvested from the Arani system irrigation field. Chard, beet greens, quinoa, and fava beans were harvested from the Punata system irrigation field. Leaves, stems, fruits, and seeds were harvested by aseptically clipping the plant at the root stem and placing it in a sterile container on ice.

Samples were processed and analyzed within 48 hours at the Centro de Aguas y Saneamiento Ambiental (CASA), Universidad Mayor de San Simon (UMSS), in Cochabamba, Bolivia. Water, soil, and crop samples were analyzed for the following microbiological parameters: *Escherichia coli*, coliphages (plaque-forming units on *E. coli* strain C-3000), *Giardia* cysts, *Cryptosporidium* oocysts, and helminth eggs. All water samples were analyzed for *E. coli*, coliphage, and helminth eggs and all crop and soil samples were analyzed for total coliforms, coliphage, and helminth eggs. All water samples, except for those collected in October 2012, were analyzed for *Giardia* and *Cryptosporidium*, and soil samples from the Arani system were analyzed for *Giardia* and *Cryptosporidium*, but not soil samples from the Punata system. No crop samples were analyzed for *Giardia* or *Cryptosporidium*. With the

exception of the samples collected in July 2013, all water, soil, and crop samples were analyzed at the *Centro de Aguas y Saneamiento Ambiental* in Cochabamba, Bolivia, for the following physical-chemical parameters, using the *Standard Methods* (APHA et al. 2012): total suspended solids (TSS), five-day biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), total and soluble phosphorus (TP and SP), nitrites (NO_2), nitrates (NO_3), ammonium nitrogen (NH_3), and total organic nitrogen (ON).

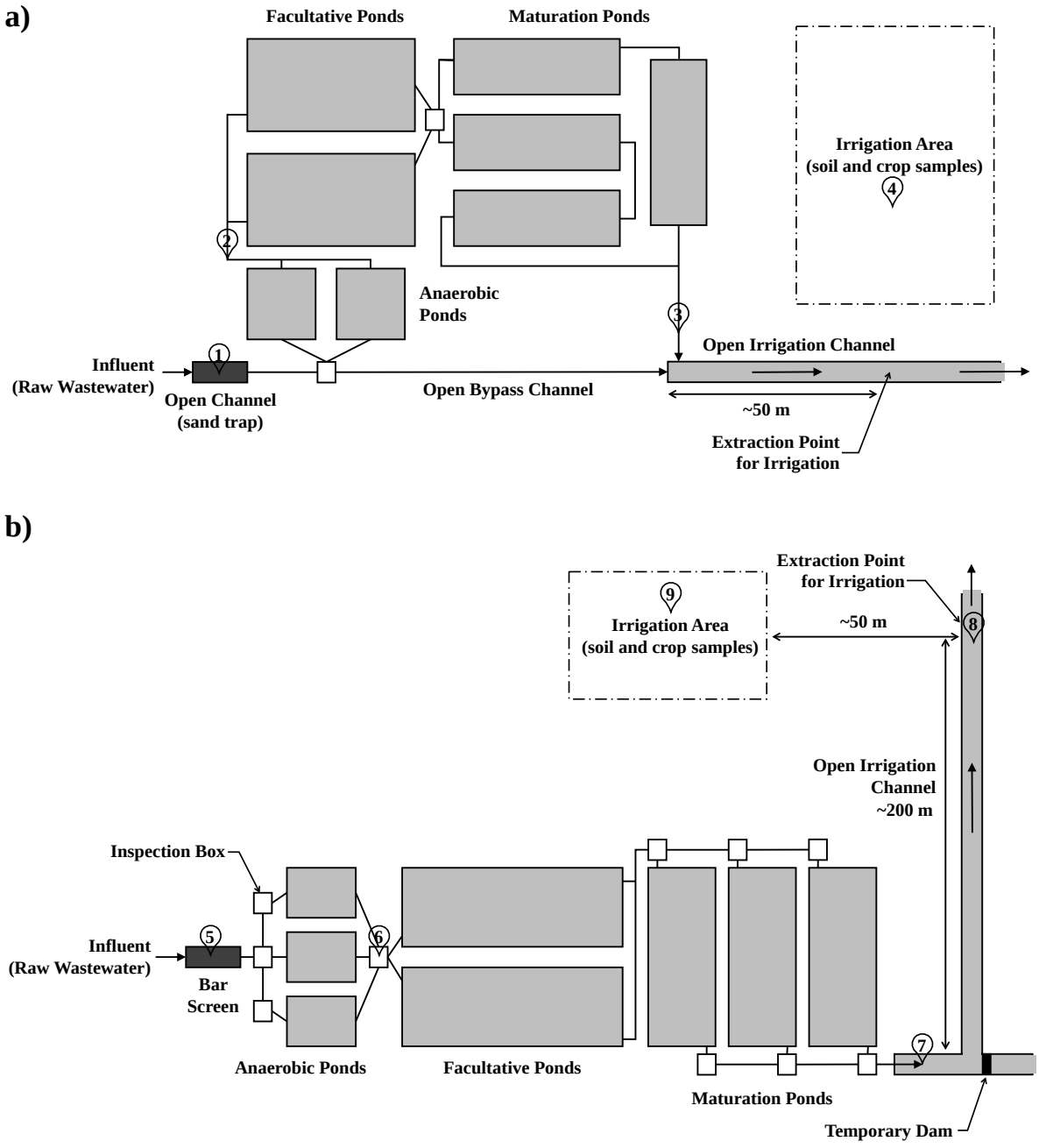


Figure 17. Schematic showing the nine sampling locations for the a) Arani and b) Punata waste stabilization pond and irrigation systems (not to scale).

Figure 18. Open channels used to transport treated wastewater from the stabilization pond systems in Arani (left) and Punata (right) to the farms where it is used for irrigation.

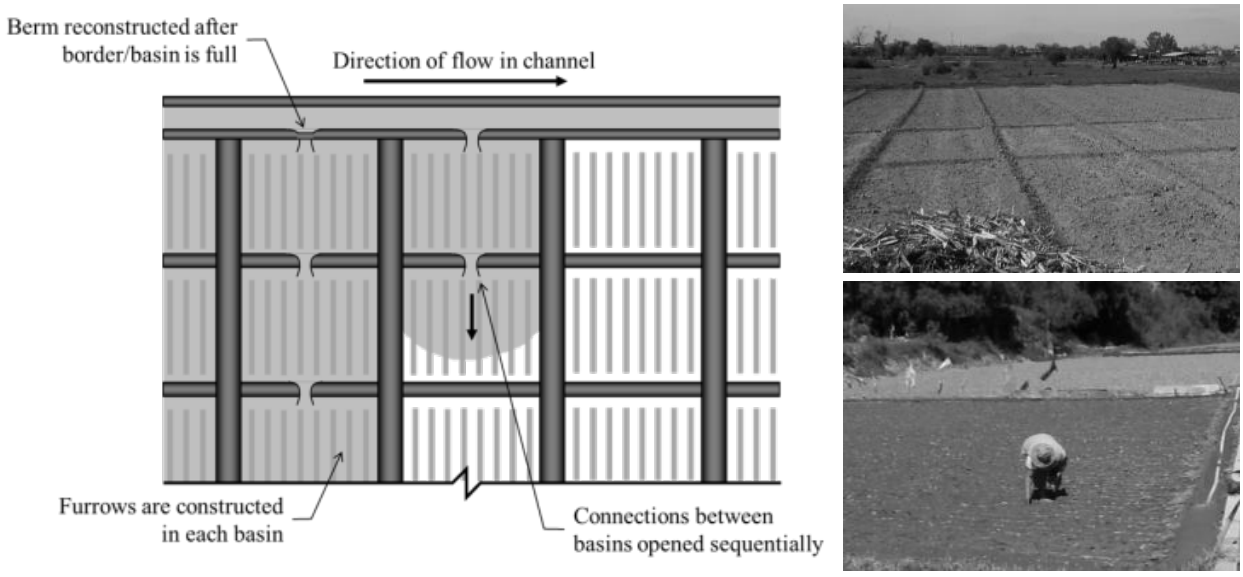


Figure 19. Traditional Bolivian border irrigation technique using *melgas* (left); construction of *melgas* on a new field in the Cochabamba Valley (right top); use of furrow irrigation with *melgas* on a field of lettuce in the Cochabamba Valley (right bottom).

5.2.2.3 Coliform Bacteria Analysis

MacConkey agar (BD Difco) was used to measure the concentration of lactose-fermenting bacterial colonies in soil and crop samples. For soil samples, 25 g of soil was diluted in 250 ml of sterile DI water and agitated manually by shaking vigorously for one minute. For crop samples, 25 g of the edible portion of the crop was blended in a food blender with 250 ml sterile DI water. Then, 0.2 ml of diluted soil or crop samples was poured onto a plate with MacConkey agar. After an incubation period of 24 hours at 37 °C, only bright pink colonies were counted. The presence of other non-lactose fermenting (atypical) colonies was noted as such. All samples (soil, water, and crop) were analyzed in duplicate and the average number of colonies was recorded and used to estimate the concentration. Colonies from at least one sample in each batch were transferred to a test tube with tryptic soy broth, incubated again, and evaluated for confirmation. The concentration of *E. coli* in water samples was measured using membrane filtration (US EPA 2006). Water samples or dilutions of 100 mL were passed through a 47-mm diameter filter with a pore size of 0.45 μm, and the filter was placed on a plate with modified mTEC agar, incubated at 35°C for 2 hours, and then at 44.5°C for 22 hours. Red- or magenta-colored bacteria colonies that formed after incubation were counted.

5.2.2.4 Coliphage Analysis

The concentration of coliphages was determined using a single agar layer method (APHA et al. 2012; US EPA 2001) with modifications as described by Grabow and Coubrough (1986) with host *E. coli* strain C-3000 (ATCC #15597, Manassas, VA, USA). For water samples, 5 mL of sample or sample dilutions were analyzed in duplicate. Plates were incubated overnight (8 – 12 h) at 35°C, and plaques forming on the lawn of the host bacteria were enumerated. The average number of plaques from duplicate samples was recorded and used to estimate the concentration. Soil and crop samples for coliphage enumeration were concentrated in the same manner as described above for coliform bacteria, using the same dilutions, except that a volume of 5 mL of samples (or sample dilutions) was analyzed for coliphage samples.

5.2.2.5 Analysis for Protozoan Parasites

Samples were analyzed for *Giardia* cysts and *Cryptosporidium* oocysts, using US EPA Method 1623 (US EPA 2005), with some modifications. For water samples, a sample volume of 5 to 10 L was concentrated to a

volume of 10 mL, using centrifugation at 1,100×*g* for 15 min. For soil samples, 25 g was added to a flask with 100 mL of Tris-Tween buffer solution (50 mM Tris with 0.5% v/v Tween 80), and mixed for 15 min. with a magnetic stir bar. The samples were then filtered using Whatman Grade 1 filter paper (Sigma Aldrich) with a filter funnel, a Buchner flask, and a vacuum pump. The filter was then washed three times with an additional 200 ml of Tris-Tween buffer. Next, the samples were placed in 50 mL tubes and centrifuged at 1100×*g* for 15 min, as was done for the water samples, to concentrate to a final volume of 10 mL. For crop samples, 200 mL of Tris-Tween buffer was added to a stainless steel blender with 25 g of the edible portion of the crop, and the mixture was blended at the low setting for 2 minutes. Then, the mixture was filtered over a stainless steel sieve with 150 μm pore size. An additional 200 ml of Tris-Tween buffer was washed over the sieve. The resulting filtrate was then distributed into 50 mL tubes and centrifuged at 1100×*g* for 15 min, as was done for the water and soil samples, to concentrate to a final volume of 10 mL. Cysts and oocysts in the concentrated 10-mL volumes from water, soil, and crop samples were further concentrated using immune-magnetic separation with the Applied Biosystems Dynabeads® G-C Combo Kit (IDEXX Laboratories, Inc., Maine, USA), in accordance with the manufacturer's instructions. Following this process, 50 μL of the concentrated cysts and oocysts were transferred to microscope slides, left overnight at room temperature to dry, and then stained using the Merifluor® *Cryptosporidium*/*Giardia* kit (Fisher Scientific, Pennsylvania, USA). Finally, cysts and oocysts were visualized using fluorescence microscopy, and enumerated based on their color under fluorescence (apple green) and the morphologies.

5.2.2.6 Helminth Egg Analysis

The concentration of helminth eggs in water samples was measured using CASA's standard protocol, which has also been described previously (Verbyla 2012; also see Chapter 2). Briefly, eggs were isolated from samples using sedimentation, flotation with magnesium sulfate, and biphasic separation using an ether-ethyl and sulfuric acid solution. Portions of the concentrated samples were added to Neubauer Improved Bright-Line counting chambers, which were examined under a microscope (Eclipse E600, Nikon) at 400× or 1000× magnification, and helminth eggs were identified and enumerated. For soil samples, helminth eggs were concentrated using the Tulane method, which has a rate of recovery of 75.5% with a precision (percent variation) of 32.5% for soil samples (Bowman et al. 2003). Briefly, 20 g of soil was mixed with 200 mL of sterile DI water and transferred to a stainless steel blender where it was agitated for 1 min. The mixture was then transferred to a beaker and allowed to settle

overnight (12 to 14 h), after which the supernatant was discarded, leaving the bottom 100 mL. The blending/settling process was then repeated once more. Each time samples were transferred, the sides and bottom of the containers were washed with sterile DI water at least twice, to recover any helminth eggs that may have attached to the containers. After this second round of blending/settling, 900 mL of 7× detergent solution (5%) (MP Biomedicals) was added to the sediment; the solution was mixed, allowed to settle for 2 hours, and then the top 90% was decanted. Then, 300 mL of detergent solution was added and mixed for five minutes. The entire mixture was passed through a #20 mesh sieve, then through a #50 mesh sieve. The tops of the sieves were brushed lightly with a hair brush, and sprayed with sterile DI water, to help separate any remaining eggs from soil particles retained on the sieves. All filtrate was collected in a large beaker in the bottom of the sieves. Once more, 900 ml of 7x detergent solution (5%) was added, mixed, and the samples were allowed to settle for 2 hours, after which time the supernatant was decanted, leaving only the bottom 10% of the sample, which was mixed and distributed into 50-ml centrifuge tubes. The contents of these tubes were then treated as water samples and analyzed as described above. Edible portions of crop samples were aseptically placed into sterile containers with Tris-Tween buffer, manually agitated for 5 minutes, and then the buffer was transferred to a beaker. This washing process was done three times for each sample, using a total buffer volume of 1 – 2 L, composited into a single beaker for each sample. The washing buffer was then processed like water samples.

5.2.2.7 Statistical Analyses

A probability plot correlation coefficient test (NIST/SEMATECH 2012) was used to assess conformity of concentrations to a lognormal distribution. The data sets for some of the samples were left-censored (the measured concentration in some sample replicates was below the method limit of detection). Therefore, summary statistics (mean, standard deviation) and interval estimates were computed with the 'robust' implementation of regression on order (ROS) statistics, which uses a least-squares regression on a probability plot of the data, assuming a log-normal distribution to impute values for the censored portion (Helsel 2012). For samples where microorganisms were only detected in one replicate, the most probable number equation (Jarvis et al. 2010) was used to estimate the concentration. A two-sample Wilcoxon rank sum test was used to test if the $\log_{10}$-transformed concentrations of microorganisms detected at two sample points were significantly different. A significance level of 0.05 was used for

all statistical tests. Calculations were performed using 'R' version 3.1.0 (The R-Project for Statistical Computing, Vienna, Austria) with the 'non-detects and data analysis' (NADA) package (Lee 2013).

5.2.3 Results and Discussion

5.2.3.1 Physical-Chemical Quality of Reclaimed Water

The pond systems in Arani and Punata, respectively, provided reductions of 74% and 86% for TSS, 69% and 54% for BOD_5, and 71% and 55% for COD. This treatment performance is lower than it should be for WSP systems with the sizes and flow rates of these two systems (Mara 2003). The poor performance is likely due to non-ideal flow conditions resulting from one or more of the following causes (Mercado et al. 2013): poor construction of diversion boxes used to split flow between ponds in parallel; changes in the water levels downstream from WSPs (caused by the construction of dams to divert treated water to farms); and/or excessive sludge accumulation causing the creation of preferential (short-circuiting) flow paths through the WSP systems. One interesting result is that the passage of wastewater through 200 m of an open irrigation channel in Punata, from the pond system outlet to the location where water is extracted for irrigation, resulted in additional reductions of 58%, 77%, and 25%, for TSS, BOD_5, and COD, respectively. This demonstrates that additional treatment is achieved between the wastewater treatment plant and the irrigation fields. The concentration of ammonia-nitrogen (NH_3-N) in the effluent of both systems was above 75 mg L^{-1}, which is well above most criteria for acute or chronic toxicity for aquatic organisms (National Research Council 2012). The concentration of total phosphorus was >15 mg L^{-1} in both systems. The nutrient levels in the treated effluent indicate the environmental benefits of land applying treated wastewater instead of discharging to surface water bodies.

5.2.3.2 Removal of Pathogens and Indicators in Stabilization Ponds

The $\log_{10}$ differences in the concentrations of *E. coli*, coliphage, *Giardia* cysts, *Cryptosporidium* oocysts, and helminth eggs between the overall influent and effluent points for the two WSP systems are shown in Table 15. With the exception of coliphage in the Arani WSP system, the $\log_{10}$ removal of all microorganisms was significantly greater than zero, at least at the 10% level. The $\log_{10}$ removal of *Giardia* was significantly greater in the Arani system than it was in the Punata system (p-value = 0.02), but there were no other statistically significant differences in the $\log_{10}$ removal of pathogens or fecal indicators in the two systems. One possible explanation for the difference

in the observed $\log_{10}$ removals of *Giardia* in the two systems is heterogeneity in the relatively small number of samples collected.

Table 15. Estimated $\log_{10}$ differences (and lower 95% confidence interval) in the concentration of pathogens and fecal indicator organisms in water samples collected at the overall influent and effluent points of the Arani and Punata waste stabilization pond systems.

	Arani WSP System	Punata WSP System
E. coli	1.1** (95% LCI: 0.4)	0.7* (95% LCI: -0.1)[a]
Coliphage	0.2 (95% LCI: -0.5)[a]	1.0** (95% LCI: 0.2)
Giardia	1.5*** (95% LCI: 1.0)	0.8* (95% LCI: -0.1)
Cryptosporidium	0.7*** (95% LCI: 0.4)	0.8*** (95% LCI: 0.4)
Helminth Eggs	0.6*** (95% LCI: 0.5)	0.5** (95% LCI: 0.1)

* Wilcoxon location shift ($\log_{10}$ removal) significantly greater than zero at the 10% level
** Wilcoxon location shift ($\log_{10}$ removal) significantly greater than zero at the 5% level
*** Wilcoxon location shift ($\log_{10}$ removal) significantly greater than zero at the 1% level
[a] Negative values would indicate a possible increase in the concentration

For both stabilization pond systems, the concentrations of *E. coli* in the final treated wastewater are two orders of magnitude above the previous limit recommended in the former WHO guidelines (WHO 1989) for wastewater reuse in agriculture (<1,000 MPN / 100 mL). The average *E. coli* removal in the Arani and Punata systems was approximately 1.0- and 0.8-$\log_{10}$ units, respectively. While this is less than ideal, it is not unexpected, given the dimensions of the ponds and the HRTs. The dispersed flow model, which accounts for axial dispersion in ponds with length-to-width ratios that are greater than one, has been proposed for coliform bacteria removal in WSP systems (von Sperling 1999; 2003; 2005), using the following equations:

$$C = C_o \frac{4a e^{1/2\delta}}{(1+a)^2 e^{a/2\delta} - (1-a)^2 e^{-a/2\delta}} \quad (7)$$

$$a = \sqrt{1 + 4K_b\theta\delta} \quad (8)$$

$$K_b = 0.682H^{-1.286}\theta^{(-0.103)} \quad (9)$$

where C is the predicted *E. coli* concentration exiting the pond, C_o is the *E. coli* concentration entering the pond, K_b is the decay rate, θ is the theoretical hydraulic retention time (volume divided by flow rate), and δ is the dispersion number, estimated as the pond width divided by the pond length (von Sperling 1999). According to this model, assuming no sludge accumulation, the Arani and Punata systems should both theoretically achieve 0.9-$\log_{10}$ removal of *E. coli*, which is similar to the $\log_{10}$ removals observed. However, the von Sperling (1999; 2003; 2005) model does not account for the effects of extreme sludge accumulation and short-circuiting. Assuming the same decay rates (K_b) but reduced volumes to account for observed sludge accumulation, the model under-predicts the *E. coli* removals observed in the Arani and Punata WSP systems. However, according to Equation 9, decay rates increase with shallower depths. If pond volume and depth reduction due to sludge accumulation are taken into consideration to estimate new decay rates, the model over-predicts the observed *E. coli* removals.

Coliphage removal in the Arani and Punata WSP systems was 1-$\log_{10}$ unit or less, and was not significant at the 5% level in either system. This was expected, given the low HRTs in these two systems. Results from the review of virus and bacteriophage removal in pond systems (see Chapter 3) indicated that pond systems require 14.5 to 20.9 days of retention on average to achieve each $\log_{10}$ removal of viruses or bacteriophage. The observed removals of protozoan parasites *Giardia* and *Cryptosporidium* in the Arani and Punata WSP systems were similar to values reported by others in previous studies. Reinoso et al. (2011) reported *Cryptosporidium* and *Giardia* removals of 1.6- and 2.0-$\log_{10}$ units in a pond system with a total HRT of 5.5 days, consisting of two anaerobic ponds in parallel, followed by a facultative pond and a maturation pond in series.

The most common helminth species detected in the water samples from Arani and Punata WSP systems were *Ascaris* spp. (41%), *Hymenolepis* spp. (27%), *Taenia* spp. (19%), *Fasciola* spp. (10%), and *Trichuris* spp. (3%). The helminth egg concentrations detected in the treated wastewater were between 31 and 130 eggs L^{-1} for the Arani system and between 69 and 352 eggs L^{-1} for the Punata system. These values exceed the maximum concentrations recommended by the WHO (2006), which are 1 egg L^{-1} if adults are working, and 0.1 eggs L^{-1} if children are present. The observed removals of helminth eggs in the Arani and Punata WSP systems were low compared to the removal predicted by the following two equations, which are based on the most widely-used model for helminth egg removal in WSP systems (Ayres et al. 1992; Mara 2003); the first equation is the line of best-fit,

while the second equation is the lower 95% confidence interval equation, recommended by Ayres et al. (1992) and Mara (2003) for design purposes:

$$C = 0.14C_o e^{-0.38\theta} \quad (10)$$

$$C = 0.41C_o e^{(-0.49\theta + 0.0085\theta^2)} \quad (11)$$

If the ponds were operating with their full volumes (i.e., zero sludge accumulation), then the Arani and Punata WSP systems should theoretically provide at least 3.6- and 3.1-$\log_{10}$ removal of helminth eggs, respectively, according to the lower 95% confidence interval of the Ayres et al. (1992) model (Equation 11). Even if accounting for the reduced HRTs due to sludge deposition (i.e., assuming 0.9 days overall HRT for Arani WSP system and 2.5 days overall HRT for Punata WSP system), the Ayres et al. (1992) model still predicts at least a 1.7-$\log_{10}$ removal of helminth eggs for both systems. The observed helminth egg removals in the Arani and Punata WSP systems were more than an order of magnitude lower than these predicted values. The likely explanation for the poor efficiency of helminth egg removal is the excessive build-up of sludge. Sludge build-up affects the hydraulics of ponds, creating short-circuits that may be capable of carrying helminth eggs through to the outlet, or re-suspending helminth eggs that have been deposited in the pond sediments (Verbyla et al. 2013). The low helminth egg removals observed here evidence the need for a new helminth egg removal model for WSP systems, in particular one that takes into account sludge accumulation.

5.2.3.3 Fate of Pathogens and Indicators in Irrigation Systems

The quantiles of the concentrations of microorganisms detected at each point in the two pond systems, as well as the concentrations in the samples of soil and crops irrigated with treated wastewater are presented in Figure 20. In the Punata system, the passage of water through the irrigation channels did not appear to result in any additional removal of the selected microorganisms. The concentrations of the selected microorganisms in soil and on crops are essentially the same in both systems, with the following exceptions: coliform bacteria, which were only detected in samples of beet greens and chard in Punata; and coliphage, which was only detected on fava beans in Punata.

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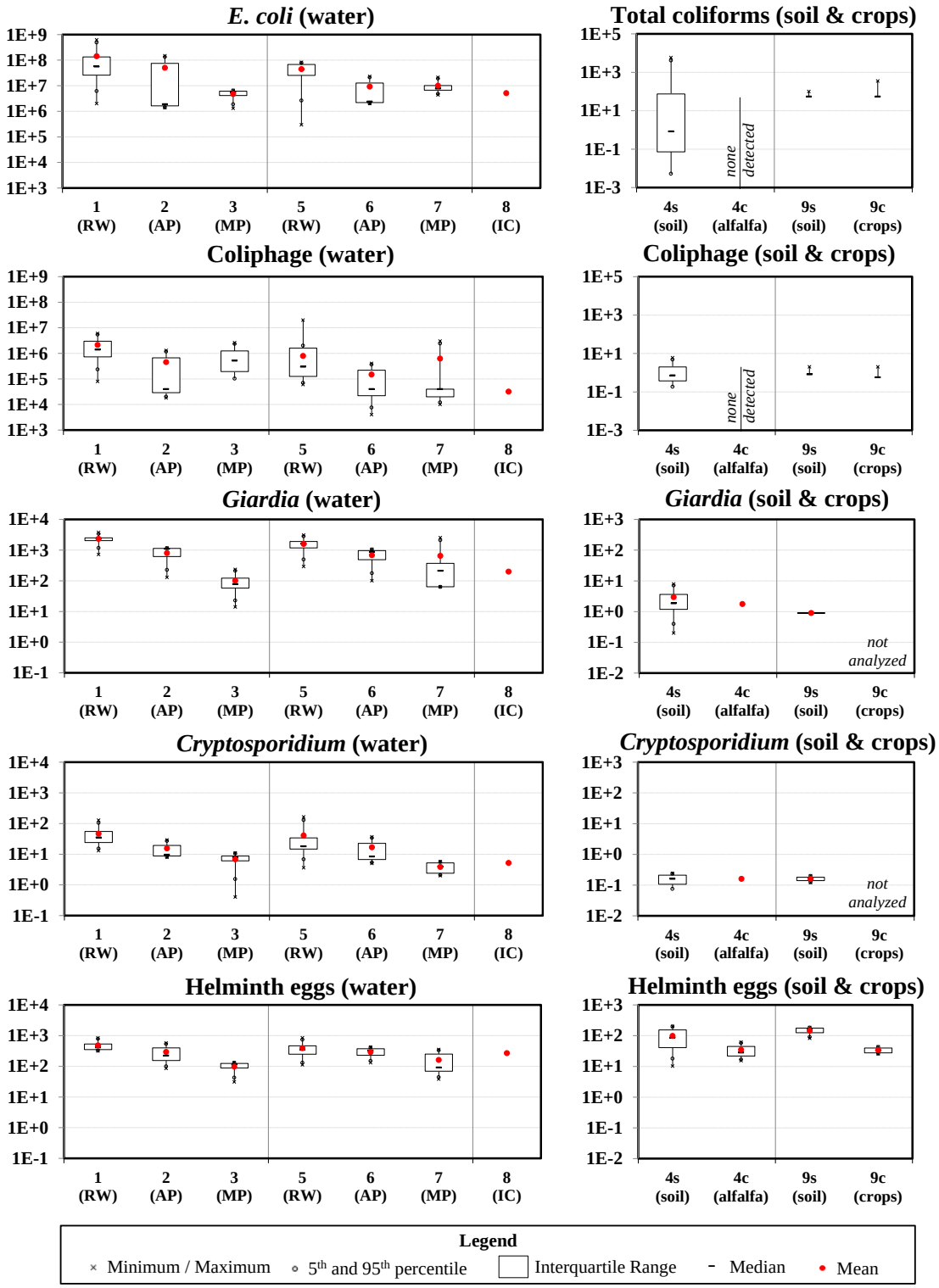

All concentrations are expressed per liter (water) or per gram (soil and crops). For samples in which no microorganisms were detected in any of the replicates, a black line is drawn between zero and the assay detection limit. RW = untreated sewage; AP = effluent of the anaerobic pond(s); MP = effluent of the final maturation pond; IC = irrigation channel; s = irrigated soil; c = irrigated crops.

Figure 20. Quantiles of the measured concentrations of *E. coli* (cfu), total coliforms (cfu), coliphage (pfu), *Giardia* (cysts), *Cryptosporidium* (oocysts), and helminth eggs (eggs) in water, soil, and crop samples from the waste stabilization pond and irrigation systems in Arani (samples 1 – 4) and Punata (samples 5 – 9).

To assess the transfer of the selected pathogens and fecal indicators from irrigation water to soil and crops, ratios of their concentrations in soil or crop samples divided by their respective concentrations in irrigation water (termed crop-water and soil-water ratios, with units mL g^{-1}) were calculated, using quantiles of the ROS concentration estimates to incorporate variability and uncertainty (Figure 21). A higher ratio equates to a higher concentration in soil or on crops relative to the concentration in irrigation water, which could indicate one or more of the following: more efficient transfer from irrigation water to soil or crops; longer survival in soil or on crops; or slower leaching or washing away from soil or crops. The crop-water and soil-water ratios calculated for coliphage were the lowest of all (generally lower than 0.1 mL g^{-1}); the ratios for *Giardia* and *Cryptosporidium* were similar to each other (generally between 1 and 100 mL g^{-1}) but were higher than the ratios for coliphage; the ratios for helminth eggs were the highest of all (generally between 100 and 1,000 mL g^{-1}). It is important to note that the differences in the methods used to detect the four different organisms are likely to have a great impact on the calculated ratios; for example, helminth eggs, *Giardia* cysts, and *Cryptosporidium* oocysts were all quantified by microscope examination based on morphology, without distinguishing between viable and non-viable (oo)cysts and eggs. In contrast, the assay used to quantify coliphages in water, soil, and crop samples measured only viable phages that were capable of forming plaques on the bacterial lawn formed by the host strain *E. coli* C-3000. Therefore, the lower soil-water and crop-water ratios may reflect the decay of coliphage in soil or on crops, since samples were generally collected a day or more after an irrigation event.

Helminth eggs, *Giardia* cysts, and *Cryptosporidium* oocysts are known to persist longer in the environment than most viruses (Feachem et al. 1983; Mara and Feachem 1999), meaning that their crop-water and soil-water ratios should be greater than the corresponding ratios for coliphage. However, since the methods used to measure helminth eggs, *Giardia* and *Cryptosporidium* did not measure viability, the reported ratios likely over-estimate the actual ratios of viable (oo)cysts and eggs. They could be used to estimate the concentration of pathogens in soil or on crops relative to a known concentration in irrigation water, and evaluate risks associated with established irrigation water quality standards. For example, for a treatment system that complies with the 2006 WHO Guidelines recommendation of <1 egg L^{-1} in irrigation water, given an estimated crop-water ratio of 300 mL g^{-1} for helminth eggs, the corresponding concentration of helminth eggs in the irrigated crop should not exceed 0.3 eggs g^{-1}, which would correspond with a dose of 30 eggs if 100 g raw produce were ingested by a consumer. In one study in Mexico, the median infective dose for *Ascaris* eggs was estimated to be between 35 and 859 (Navarro et al. 2009).

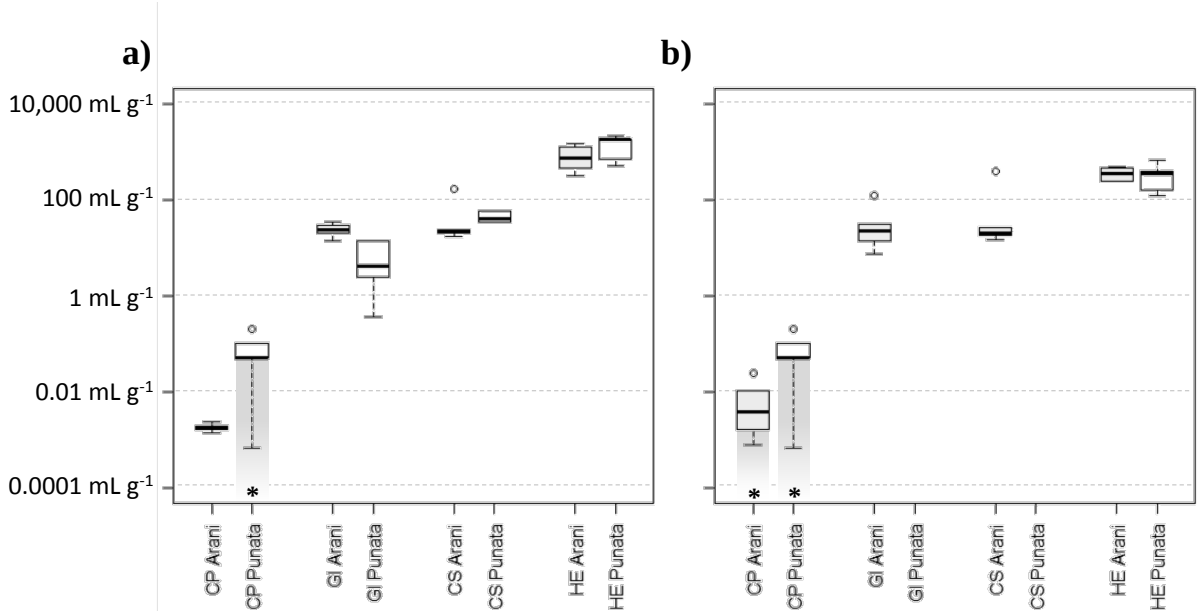

Figure 21. Ratios of the concentrations of coliphage (CP), *Giardia* (GI), *Cryptosporidium* (CS), and helminth eggs (HE) a) in soil relative to irrigation water; and b) in crops relative to irrigation water. Units are mL g^{-1}. A higher ratio could either indicate more efficient transfer from irrigation water to soil or crops, slower decay in soil or on crops, or slower leaching or washing away from soil or crops. Ratios for coliphage marked with an asterisk (*) were calculated using the assay limit of detection. Since coliphage were either not detected in these samples or were only detected in one sample replicate at the limit of detection, the actual ratios may be lower.

5.3 Managing Microbial Risks from Indirect Wastewater Reuse for Irrigation in Urbanizing Watersheds[7]

5.3.1 Introduction

With 33% of the world's population lacking basic sanitation (United Nations 2015b) and another 21% connected to sewerage systems without treatment (Baum et al. 2013), the contamination of surface waters in rapidly-urbanizing regions by fecal pathogens is widespread. Up to 18 million hectares of land are irrigated with untreated wastewater, either directly (irrigation with pure untreated wastewater) or indirectly (untreated wastewater mixed with another water source), causing health risks for farmers and consumers of irrigated produce (Drechsel et al. 2010). Target 6.3 of the Sustainable Development Goals, adopted recently by the United Nations, aims to reduce the proportion of untreated wastewater and substantially increase safe water reuse worldwide by 2030 (United Nations 2015c). Riverbank filtration (RBF) is a simple and practical technology used to treat surface water via multiple

[7] Section 5.3 was submitted on November 2, 2015 to *Environmental Science and Technology*: Verbyla, M.E., Symonds, E.M., Kafle, R.C., Cairns, M.R., Iriarte, M., Mercado, A., Coronado, O., Breitbart, M., Ledo, C., Mihelcic, J.R. "Managing Microbial Risks from Indirect Wastewater Reuse for Irrigation in Urbanizing Watersheds," *Environmental Science and Technology* (in review).

physicochemical and biological processes that naturally occur as water passes through riverbank soil (Tufenkji et al. 2002). Though RBF is widely used to treat surface water for irrigation, its effectiveness at removing pathogens has not been sufficiently assessed for rivers that are highly contaminated with untreated sewage, which is a common occurrence in the developing world (Keraita et al. 2014). Even if the ultimate environmental protection goal involves the installation of centralized wastewater treatment or resource recovery facilities, RBF may be an appropriate intermediary solution for growing urban areas in developing countries, especially since pathogen removal for safe water reuse is an important priority for these regions (see Section 2.1 of this dissertation).

The Rocha River in Cochabamba, Bolivia is a vital source of irrigation water that has become heavily impacted by untreated sewage, especially during the dry irrigation season (Ministerio de Medio Ambiente y Agua 2013; Zabalaga et al. 2007). Some farmers still irrigate with the polluted river water, but others use RBF to treat the river water. In the United States, *Cryptosporidium* removal credits are provided when RBF is used to extract surface water (US EPA 2010b). Credits for virus removal are not provided, despite the documented effectiveness of RBF at removing enteric viruses (e.g. human adenovirus (AdV), enterovirus, Aichi virus) and pepper mild mottle virus (PMMoV), a proposed viral surrogate (Betancourt et al. 2014; Weiss et al. 2005). In Bolivia (and in other countries where untreated wastewater is reused indirectly for irrigation), RBF is generally not a sanctioned method for treating irrigation water (e.g., pathogen removal credits are not provided). To our knowledge, the effectiveness of RBF for removing pathogens from sewage-contaminated river water has only been reported once, and only for enteric viruses (Sprenger et al. 2014).

The World Health Organization (WHO) recommends using quantitative microbial risk assessment (QMRA) to develop wastewater reuse policies (WHO 2006). However, QMRA is seldom used to evaluate the indirect reuse of untreated wastewater for irrigation in developing countries. Several limitations of previous QMRAs include using non-local exposure estimates (Diallo et al. 2008), assuming ratios between pathogens and fecal indicators instead of using pathogen measurements (Barker et al. 2014), and failing to incorporate variability and uncertainty from dose-response parameters (Sales-Ortells and Medema 2014). Ethnographic research methods can provide useful information for QMRAs, since behaviors linked to cultural practices affect pathogen exposure (Brown et al. 2011). Data for QMRAs in developing countries are often scarce, and if risks are modeled using simple point estimate assumptions, results can be misleading. The Bayesian approach enables the incorporation of variability and uncertainty in QMRAs (Schmidt et al. 2013), and therefore, can be valuable for determining water

reuse policies. This is particularly important because the United Nations reported that only 3% of countries in the world have water reuse plans, and 30% do not consider such a water management strategy to be relevant (UNEP 2012).

For this study, QMRA was used to evaluate an indirect water reuse scenario and determine how RBF affects the risk to consumers of irrigated produce, using a lettuce farm in Cochabamba as a model system. Microbial source tracking markers for PMMoV and the human-specific HF183 *Bacteroides* were measured to assess fecal contamination, and *Giardia*, *Cryptosporidium*, human rotavirus group A (RV), and AdV were measured for use in the QMRA. Additionally, *E. coli* and coliphage were measured for comparison purposes, given their common use as bacterial and viral fecal indicators, respectively. Culturally-relevant data regarding lettuce consumption and pathogen exposure were collected using ethnographic methods. A Bayesian approach was used to incorporate the uncertainty associated with dose-response data, ethnographic data, pathogen concentration estimates, and distributional assumptions for other model inputs. The impact of parameter variability and uncertainty on estimated health burdens was assessed using a sensitivity analysis. The results from this study provide governments, development organizations, farmers, and consumer groups with a decision-support framework to assess and manage health risks from indirect wastewater reuse in watersheds with extensive fecal contamination. The analysis of sensitivity between QMRA model inputs and health burden estimates can help decision-makers understand where interventions and data collection needs may be most beneficial. Although this study is focused on RBF and indirect reuse, the same approach can be applied for other water reuse scenarios.

5.3.2 Materials and Methods

5.3.2.1 Riverbank Filtration and Irrigation System

Indirect wastewater reuse for lettuce irrigation was compared at two sites located 1 km apart. At the first site, farmers irrigate with untreated Rocha River water; at the second site, farmers irrigate with water from an unlined RBF extraction well located 65 m from the river. The heterogeneous superficial soil in this region contains sand, silt, mud, and clay, originating from quaternary lake deposits and recent fluvial deposits; the recharge area consists of clays, sands, and igneous rock with low permeability and low recharge capacity (Renner and Velasco 2000). During the peak irrigation season (April – September), Cochabamba receives < 40 mm of rainfall, and farmers use the RBF well, which recharges at a rate of ~0.5 m h^{-1}, for several hours weekly.

5.3.2.2 Hazard Identification

RV, AdV, *Cryptosporidium*, and *Giardia* were selected as reference pathogens for this study. Samples were also analyzed for PMMoV, HF183, *E. coli*, and coliphage (using host strain *E. coli* C3000, which is susceptible to somatic and F-specific coliphages (Sobsey et al. 2004)). Though somatic coliphages have poor specificity for human fecal contamination (Harwood et al. 2014), coliphage assays are routinely used for research in Cochabamba. The HF183 *Bacteroides* marker has higher specificity for human feces than other bacterial microbial source tracking markers (Harwood et al. 2014). PMMoV is also specific for human feces (Harwood et al. 2014; Rosario et al. 2009), has been detected at high concentrations in wastewater in Bolivia (see Chapter 4), and may be a conservative surrogate for enteric viruses in RBF systems (Betancourt et al. 2014).

5.3.2.3 Exposure Assessment

QMRA model parameter assumptions are summarized in Table 16. Water, lettuce, and soil samples were collected throughout 2013 and 2014 to accommodate local crop rotation patterns. Grab samples from the river were collected and analyzed for RV (n=6), PMMoV (n=6), AdV (n=4), HF183 (n=2), *E. coli* (n=4), coliphage (n=4), *Giardia* (n=4), and *Cryptosporidium* (n=4). Grab samples of water treated by RBF were also analyzed for RV (n=10), PMMoV (n=10), AdV (n=2), HF183 (n=3), *E. coli* (n=3), coliphage (n=8), and *Giardia/Cryptosporidium* (n=3). Soil and lettuce samples irrigated with untreated river water (n=2), and irrigated with water treated by RBF (n=3), were analyzed for coliphage, *Giardia* and *Cryptosporidium*, but not AdV or HF183. Only lettuce samples irrigated with water from the RBF well (n=15) were analyzed for RV and PMMoV (i.e., RV and PMMoV were not measured in soil samples, nor were they measured in lettuce samples irrigated with untreated river water). Cross-contamination of lettuce and soil was not a concern because farmers irrigating with water from the RBF well did not irrigate with untreated river water, and *vice versa*.

A modified adsorption-elution method (see Section 4.2 of this dissertation) was used to concentrate water samples (≤1 L) for RV, PMMoV, AdV, and HF183 analyses. Following a standardized method (El-Senousy et al. 2013; ISO/TS15216-1 2013), viruses were eluted from lettuce samples (20 – 37 g) with Tris-glycine beef extract buffer, and concentrated using polyethylene glycol and NaCl. Sample filters and concentrates were preserved in 600 µL lysis buffer RLT Plus, and stored at -80°C until DNA/RNA purification with the AllPrep Mini Kit (Qiagen, Valencia, CA). RNA was immediately reverse-transcribed (RT) with the First Strand Synthesis Superscript III

Reverse Transcription Kit (Invitrogen, Carlsbad, CA) using random hexamer primers. Nucleic acid purification and RT positive and negative controls were also processed (see Appendix E). DNA and cDNA were stored at -20°C. Previously-published quantitative polymerase chain reaction (qPCR) and RT-qPCR assays for RV, AdV, PMMoV, HF183, and purification/RT controls (Appendix E, Tables E2 and E3) (Haugland et al. 2005; Jothikumar et al. 2005; Rosario et al. 2009; Staley et al. 2012a; b; Svraka et al. 2009; US EPA 2010c) were executed with the ABI7500 Real-Time PCR System (Life Technologies, Grand Island, NY) (Bustin et al. 2009). Coliphage concentrations were determined using a plaque assay (Grabow and Coubrough 1986) with host strain *E. coli* C3000 (ATCC #15597, Manassas, VA). Soil and lettuce samples (25 g) were added to 250 mL sterile DI water, and shaken vigorously (soil) or processed in a food blender (lettuce) for 1 min. Five mL of samples (or dilutions) were added to plates with the host culture, and plaques were enumerated after overnight incubation at 35°C. *Giardia* cysts and *Cryptosporidium* oocysts were isolated and quantified using filtration, immunomagnetic separation (IMS), fluorescent antibody (FA) staining and microscopic examination (US EPA 2005). *E. coli* was quantified using membrane filtration (US EPA 2006).

The theoretical (RT-)qPCR process limits of detection (pLOD) (defined as the number of copies corresponding with 95% probability of amplification (Bustin et al. 2009)) were 7.7×10^{2} PMMoV, 1.2×10^{3} RV, 1.1×10^{5} AdV, and 72 HF183 copies L^{-1}. The presence/absence of the AdV hexon gene was also determined in RBF well samples (n=5) using a nested PCR assay (Allard et al. 1992), which detects as few as 100 copies per reaction (Symonds et al. 2009) (2.7×10^{3} copies L^{-1} for 941-mL RBF well samples). The pLODs for other assays used in this study were 500 colony forming units (cfu) L^{-1} (*E. coli*), 20 plaque forming units (pfu) L^{-1} (coliphage), and 0.04 (oo)cysts L^{-1} (*Giardia/Cryptosporidium*).

For the QMRA, log-normal distributions were assumed for pathogen concentrations in irrigation water. For samples with <80% censored values, robust regression-on-order statistics (Helsel 2012) were used to estimate means and standard deviations of log-normal distributions. For samples with >80% censored values, the pLOD was assumed to correspond with the 95^{th} percentile of a log-normal distribution, and distribution parameters were estimated using categorical data about the presence/absence of the target in replicate samples (see Appendix E). Calculations were performed in Microsoft Excel and 'R' v3.1.0 (R-Project for Statistical Computing, Vienna, Austria) with the NADA package (Lee 2013).

Table 16. Quantitative microbial risk assessment parameters and assumptions.

Parameter	Units	Assumptions [a]	References
Concentrations in Irrigation Water			
Rotavirus	copies L^{-1}	Rocha River: dlnorm(15.24,3.69) Riverbank Filtrate: dlnorm(4.48,0.63)	Data collected for this study
Adenovirus	copies L^{-1}	Rocha River: dlnorm(19.51,1.22) Riverbank Filtrate: dlnorm(7.15,0.37)	
Giardia	cysts L^{-1}	Rocha River: dlnorm(5.78,0.52) Riverbank Filtrate: dlnorm(0.75,1.27)	
Cryptosporidium	oocysts L^{-1}	Rocha River: dlnorm(1.27,0.86) Riverbank Filtrate: dunif(0,1.0)	
Harmonization Factors			
Rotavirus	ffu $copies^{-1}$	dunif(5.26×10^{-4},1.0×10^{-3})	(McBride et al. 2013; Mok and Hamilton 2014)
Adenovirus	$TCID_{50}$ $copies^{-1}$	dunif(1.43×10^{-3},0.1)	(Flint et al. 2009; Heider and Metzner 2014; Kundu et al. 2013)
Volume of irrigation water captured by lettuce			
Lettuce	mL g^{-1}	dlnorm(-4.57,2.0,0.006)	(Mok and Hamilton 2014)
Post-Irrigation Decay in the Field [b]			
Rotavirus	$days^{-1}$	dnorm(1.07,14.29) [c]	(Mok and Hamilton 2014)
Adenovirus	log_{10} reduction	dunif(1,3) [d]	(Carratalà et al. 2013; Rzezutka and Cook 2004; Ward and Irving 1987)
Giardia	$days^{-1}$	dunif(0.06,0.46)	(Petterson and Ashbolt 2003)
Cryptosporidium	$days^{-1}$	dunif(0.01,0.03) [e]	(Davies et al. 2005; Petterson and Ashbolt 2003)
Conditional probability of illness (given infection)			
Rotavirus		$p_{ill\|inf}$ = Uniform(0.35,0.90)	(Havelaar and Melse 2003; Soller et al. 2010; Viau et al. 2011)
Adenovirus		$p_{ill\|inf}$ = Uniform(0.25,0.75)	(Havelaar and Melse 2003; Soller et al. 2010; Viau et al. 2011)
Giardia		$p_{ill\|inf}$ = Uniform(0.24,0.93)	(Barker et al. 2013b)
Cryptosporidium		$p_{ill\|inf}$ = 0.50	(McBride et al. 2013; Soller et al. 2010)
Disease burden per case of illness			
Rotavirus	DALYs $case^{-1}$	Uniform(0.015, 0.026)	Chapter 4 of this dissertation
Adenovirus	DALYs $case^{-1}$	Uniform(0.0004,0.0055)	(Gaunt et al. 2011; Harder et al. 2014; Katukiza et al. 2013)
Giardia	DALYs $case^{-1}$	Uniform(0.0021,0.00268)	(Barker et al. 2013b)
Cryptosporidium	DALYs $case^{-1}$	Uniform(0.00012, 0.0015)	(Gao et al. 2015)

[a] dlnorm = lognormal distribution(mean, precision, [shift parameter]);
dnorm = normal distribution (mean, precision);
dunif = uniform distribution(low, high)

[b] With the exception of adenovirus, it was assumed that all organisms exhibit monophasic exponential decay. Adenovirus on lettuce may exhibit a decay rate that conforms to linear regression (Carratalà et al. 2013)

[c] Truncated at 0 and 1000

[d] Uniform distribution of 1 to 3 log_{10} reduction achieved after 3 – 4 days in field after irrigation

[e] Range of rates reported for experiments performed at 20°C

Annually, 1.1 kg of lettuce is produced per person in Bolivia, with 96% consumed domestically (Appendix E, Table E6) (Instituto Nacional de Estadistica 2008). While previous studies assumed uniform lettuce consumption in a population (Mara and Sleigh 2010a; Seidu et al. 2008) some people likely consume more than average and others may not consume any. To better understand the distribution of lettuce consumption in Cochabamba, an ethnographic study, consisting of convenience-sample surveys and participant observations, was conducted. Surveys were administered to market-goers (n=42), and through participant observation, nearly 50 lettuce vendors informally discussed topics such as the origin of their lettuce and their perceptions of its quality. Methods were approved by the University of South Florida Institutional Review Board.

Survey data were used to estimate the average lettuce mass (m_j) consumed in households per person per year:

$$m_j = m_h \cdot \frac{h_j \cdot t_j}{N_j} \quad (12)$$

where h_j is the number of lettuce heads purchased per household, m_h is the average lettuce head mass, t_j is the number of times lettuce is reportedly purchased annually, and N_j is the number of people in the j^{th} household. The daily serving size (S_j, average mass of lettuce ingested in the j^{th} household) was estimated as:

$$S_j = \frac{m_j}{n_j} \quad (13)$$

where n_j is the number of days per year lettuce is reportedly served at home.

In Cochabamba, lettuce is harvested three or four days after irrigation. Crops are pulled from the soil and tossed onto a tarp, which is shipped directly to market. It was assumed that pathogen concentrations on lettuce did not change between harvest and consumption (Mok et al. 2014). The daily pathogen dose (d_j) ingested in the j^{th} household was determined as:

$$d_j = C \cdot S_j \cdot V \cdot e^{-k \cdot t} \quad (14)$$

where C is the modeled pathogen concentration in irrigation water, S_j is the lettuce serving size, V is the volume of irrigation water retained by lettuce (mL g^{-1}; log-normal distribution; mean = -4.57; SD = 0.5; shift = 0.006),(Mok and Hamilton 2014) k is the pre-harvest pathogen decay rate, and t is the time between irrigation and the time lettuce is harvested.

5.3.2.4 Dose-Response Assessment

Dose-response data from clinical studies were used for all reference pathogens (Appendix E, Table E5) (Couch et al. 1966, 1969; Okhuysen et al. 2002; Rendtorff 1954; Teunis et al. 2002; Ward et al. 1986). The number of volunteers infected in the i^{th} group (X_i) of N_i volunteers was assumed to be binomially-distributed with probability p_i, assuming volunteers received a Poisson-distributed dose (d_i). Dose-response models were selected based on previous recommendations (Couch et al. 1966, 1969; Okhuysen et al. 2002; Rendtorff 1954; Teunis et al. 2002; Ward et al. 1986). For RV, the beta-binomial conditional dose-response model (a decomposed version of the complete Beta-Poisson model) was used, where α and β are shape parameters (Schmidt et al. 2013):

$$p_i = 1 - \frac{\Gamma(\alpha+\beta)\Gamma(d_i+\beta)}{\Gamma(\beta)\Gamma(d_i+\alpha+\beta)}, i = 1,2, \ldots, \quad (15)$$

For AdV, *Cryptosporidium*, and *Giardia*, the exponential dose-response model was used with parameter r (Schmidt et al. 2013):

$$p_i = 1 - e^{-r \cdot d_i}, i = 1,2, \ldots, \quad (16)$$

5.3.2.5 Markov Chain Monte Carlo and Risk Characterization

OpenBUGS (v3.2.2, rev. 1063) was used with non-informative prior distributions for α, β, and r to generate initials and sample from the joint posterior density function using Markov Chain Monte Carlo (MCMC) with one chain (see Appendix E) (Schmidt et al. 2013). After a 10,000 iteration burn-in, 40,000 iterations were recorded. Posterior distributions of α, β, and r were used with Equations 4 and 5 (substituting subscript i with j) to estimate the daily probability of infection (p_j) for the j^{th} survey respondent, given the pathogen dose (d_j). The conditional probabilities of illness ($p_{ill|inf}$) were assumed to be uniformly-distributed ranges of previously-published values

(Table 16). The daily probability of illness ($p_{ill,j}$) was calculated as the product of p_j and $p_{ill|inf}$. Annual probabilities of illness ($P_{ill,j}$) were estimated with the following equation, where n_j is the number of days per year lettuce is served in the j^{th} household:

$$P_{ill,j} = 1 - (1 - p_{ill,j})^{n_j},\ j = 1,2,\ldots, \quad (17)$$

Risk was characterized as an annual health burden (B_j), expressed in disability-adjusted life years (DALYs), using Equation 7, where S_f is the susceptible fraction of the population and b_c is the health burden per case.

$$B_j = P_{ill,j} \cdot b_c \cdot S_f \quad (18)$$

It was assumed that everyone was susceptible to all pathogens, except for RV, where only children under 5 were considered. Since 88% of Bolivian children received the RV vaccine, which has an effectiveness rate of 54 to 79% (Patel et al. 2013; WHO 2014), the susceptible fraction of the population was estimated as:

$$S_f = 1 - (f_r + (1 - f_r) \cdot f_v \cdot e_v) \quad (19)$$

where the fraction with resistance (f_r) is 0.87 (children <5 years comprise 13% of the Cochabamba population), the fraction vaccinated (f_v) is 0.88, and the vaccine efficiency (e_v) ranges uniformly from 0.54 – 0.79. Using RV as an example, the sensitivity of the QMRA was analyzed by calculating Spearman rank order correlation coefficients between the MCMC-simulated model inputs and estimated annual disease burden. A significance level of 0.05 was used for all statistical tests.

5.3.3 Results and Discussion

5.3.3.1 Lettuce Consumption in Cochabamba

Survey respondents had a median age of 43 (SD = 11.5), and compared to census data, were over-represented by women and relatively representative of ethnic groups (based on reported languages spoken at home, see Appendix E). Using Equation 1, the median mass of lettuce purchased annually per household member was

estimated to be 3.4 kg (5^{th} percentile = 0.9 kg, 95^{th} percentile = 16 kg, SD=5.9). Survey respondents reported serving lettuce at home, with median serving sizes of 19 g (5^{th} percentile = 6.9 g, 95^{th} percentile = 130 g, SD=65), 169 times annually, on average (SD = 110, Appendix E, Figure E4). This estimated lettuce consumption is much lower than the WHO example of 100 g per person for 150 days per year (15 kg person^{-1} year^{-1}), and less than assumptions made previously for China, Australia and Spain (Appendix E, Table E7). In addition, since only 1.1 kg of lettuce is produced in Bolivia per capita for domestic consumption annually (Instituto Nacional de Estadistica 2008), the surveyed population likely consume three times more lettuce than average.

The informal conversations with lettuce vendors during participant observation revealed generally negative attitudes toward the sanitary quality of lettuce grown near the Rocha River. It was described as being irrigated with dirty water and was considered to contain "bichos" (Spanish word for 'bugs'). It was also reported that some farmers near the river had to sell their crops in other cities. Conversations with vendors overall supported our observations that river water irrigation (with or without on-farm treatment) is socially-stigmatized in Cochabamba as an unsanitary practice.

5.3.3.2 Irrigation Water Quality

Microbial pathogen and indicator concentrations in irrigation water are shown in Figure 22. Median concentrations in the Rocha River were: 3.2×10^{8} AdV copies L^{-1}, 6.4×10^{7} PMMoV copies L^{-1}, 1.8×10^{7} *E. coli* cfu L^{-1}, 1.4×10^{7} HF183 copies L^{-1}, 3.6×10^{6} RV copies L^{-1}, 1.1×10^{5} coliphage pfu L^{-1}, 530 *Giardia* cysts L^{-1}, and 4.0 *Cryptosporidium* oocysts L^{-1}. Lower concentrations were measured in RBF well water: 7.7×10^{4} PMMoV copies L^{-1}, 3.0×10^{3} *E. coli* cfu L^{-1}, 5.9×10^{2} coliphage pfu L^{-1}, 4.5×10^{1} HF183 copies L^{-1}, and 2.0 *Giardia* cysts L^{-1}. *Cryptosporidium* was detected at 0.04 oocysts L^{-1} in two of three sample replicates, but undetected in the other replicate. AdV was not detected in RBF well water via qPCR; however amplification was observed for two of five nested PCR sample replicates, indicating its presence at concentrations below the qPCR pLOD (1.1×10^{5} copies L^{-1}). For RV, amplification was also inconsistently observed in replicate RT-qPCR samples, corresponding to concentrations below the pLOD. Since DNA and cDNA isolation efficiencies (per positive controls) were 30 – 46% and 38 – 79%, respectively, measurements may underestimate actual concentrations.

The $\log_{10}$ differences between the concentrations of microbial pathogens and indicators in the river and the RBF well were estimated using the Wilcoxon rank-sum test (Table 17). The greatest $\log_{10}$ removals were for HF183

(5.5-$\log_{10}$), followed by *E. coli* (3.8-$\log_{10}$), RV (>3.5-$\log_{10}$), AdV (>3.5-$\log_{10}$), PMMoV (2.9-$\log_{10}$), *Giardia* (2.1-$\log_{10}$), coliphage (2.0-$\log_{10}$), and *Cryptosporidium* (1.7-$\log_{10}$). The RBF well has no casing and is not capped or sealed, leaving it vulnerable to contamination by non-point pollution sources. The well may be recharged to a certain extent by ambient ground water, excess irrigation water applied to nearby farm plots, or infiltrating precipitation. However, the recharge area located upstream has low permeability and low recharge capacity (Renner and Velasco 2000), and due to the lack of nearby onsite sanitation facilities, the presence of PMMoV and HF183 in the well strongly suggests human fecal contamination (Harwood et al. 2014) originating from the river. To prevent contamination by pathogens in animal waste, RBF wells should be capped and sealed.

The $\log_{10}$ virus removals in this RBF system are similar to other results reported in the literature. In RBF wells in the United States with 31 to 92 m setbacks from the river, 1.92- to 3.76-$\log_{10}$ removal was reported for PMMoV; AdV was only detected in one of 12 RBF well samples at 1.2 copies L^{-1}, despite being detected in river samples at 27 – 95,600 copies L^{-1} (Betancourt et al. 2014). In three other RBF wells in the United States with 24 to 177 m setback distances from the rivers, male-specific and somatic coliphage concentrations were respectively reduced by more than 2- and 4-$\log_{10}$ units (Weiss et al. 2005). In a heavily-contaminated river in India, AdV was detected at 3.6×10^5 copies L^{-1} in the river, but was not detected in a RBF well located 50 m away; >6-$\log_{10}$ removal of somatic coliphage was reported in the same system (Sprenger et al. 2014). Given their effectiveness and simplicity, RBF systems used to treat irrigation water should be sanctioned and regulated in developing countries, perhaps by offering pathogen removal credits and requiring sanitary inspections. Efforts should also be made to increase the public acceptance of this technology.

5.3.3.3 Contamination of Soil and Lettuce

In soil and lettuce samples irrigated with both water sources, coliphage and *Cryptosporidium* were detected intermittently at concentrations close to the detection limits (2 pfu g^{-1} and 0.04 oocysts g^{-1}, respectively). The average *Giardia* concentrations in soil samples irrigated with untreated and RBF-treated river water, respectively, were 1.6 cysts g^{-1} (SD=0.1) and 1.1 cysts g^{-1} (SD=0.1). *Giardia* was also detected at 0.3 cysts g^{-1} (SD=0.1) in lettuce samples irrigated with untreated river water, and at 0.2 cysts g^{-1} (SD=0.1) in lettuce samples irrigated with RBF-treated river water.

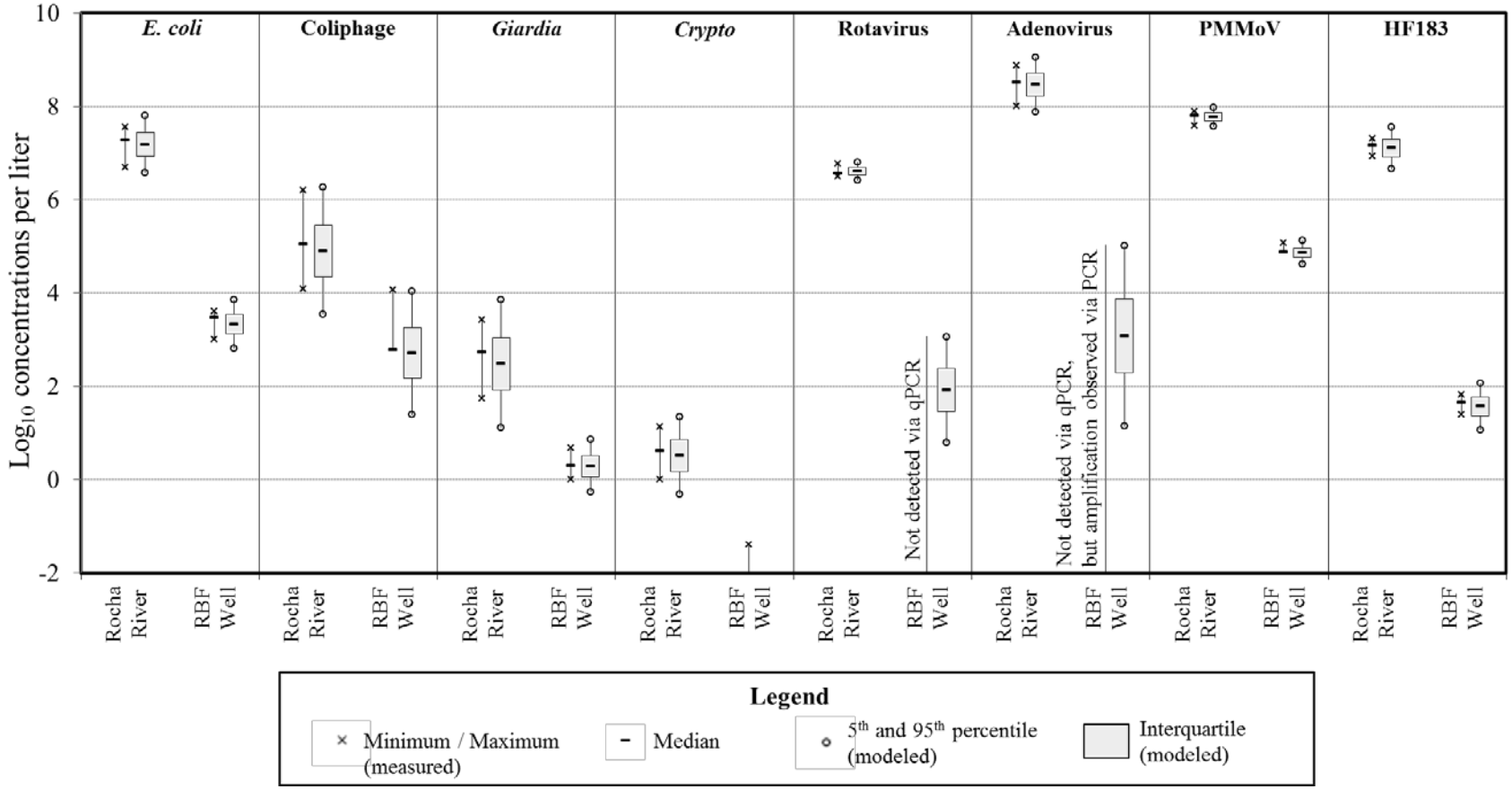

Figure 22. Measured and assumed distributions of concentrations for *E. coli*, coliphage, *Giardia*, *Cryptosporidium*, rotavirus (group A), adenovirus, pepper mild mottle virus (PMMoV), and the HF183 *Bacteroides* 16S rRNA genetic marker in irrigation water samples from the Rocha River and from the riverbank filtration well.

Table 17. Changes in the measured concentrations of microorganisms in the Rocha River and the riverbank filtration well, based on log_{10} location shifts determined by the Wilcoxon rank-sum test.

Microorganism	Method of Detection	Log_{10} Reduction (lower 95% C.I.)
Escherichia coli	Membrane filtration (mTEC agar)	3.8* (3.1)
Bacteroides (HF183 16S rRNA marker)	qPCR	5.5†
Coliphage (on *E. coli* C3000)	Single agar layer plaque assay	2.0** (1.4)
Pepper Mild Mottle Virus	RT-qPCR	2.9** (2.7)
Rotavirus Group A	RT-qPCR	> 3.5†
Human Adenovirus	qPCR	> 3.5†
Giardia	Filtration/IMS/FA Microscopic Examination	2.1* (1.1)
Cryptosporidium	Filtration/IMS/FA Microscopic Examination	1.7* (0.0)

* p-value < 0.05
** p-value < 0.01
† not enough data to determine p-value or confidence interval

AdV was not detected in lettuce samples by nested PCR; however, PMMoV was inconsistently detected via RT-qPCR in 53% of lettuce samples (n=15) irrigated with the RBF well at concentrations below the pLOD. The range of replicate sample masses (20.7 – 36.5 g) may have influenced these results, since lettuce samples where RT-qPCR amplification occurred had greater masses than the other samples (two-sample T-test, p-value = 0.069). Using the survival function with categorical data (see Appendix E), the PMMoV concentration on lettuce was estimated to be 4.1 copies g^{-1} (95% C.I.: 1.1, 18). The ratio of this concentration to the PMMoV concentration in the water used to irrigate the lettuce is 0.05 mL g^{-1} (95% C.I.: 0.0092, 0.35), which is similar to previously-reported values of irrigation water volume captured by lettuce (Mok and Hamilton 2014; Shuval et al. 1997). PMMoV has previously been proposed as a conservative tracer for enteric virus removal in RBF systems (Betancourt et al. 2014); results from the present study suggest that PMMoV may also be a useful enteric virus surrogate to identify crop contamination from indirect wastewater reuse for irrigation.

5.3.3.4 Microbial Risk of Consuming Irrigated Lettuce

QMRA results using data from the market surveys are shown side-by-side with results using the WHO scenario for lettuce consumption and the average mass of lettuce produced per capita in Bolivia for domestic consumption (Figure 23). Estimated disease burdens for consumers of irrigated lettuce were reduced significantly as a result of RBF. The following reductions were estimated (expressed as the range of median percent reductions of DALYs person^{-1} year^{-1}): from 99.9% to more than 99.999% for RV, from 94.2% to 99.9% for AdV, from 98.7% to 99.3% for *Giardia*, and a consistent reduction of 88.5% for *Cryptosporidium*. For the untreated river water irrigation site, estimated median disease burdens for RV, AdV, and *Giardia* were greater than the limit recommended in the 2006 WHO Guidelines (10^{-4} DALYs person^{-1} year^{-1}) (WHO 2006). However, for the RBF system case, the median estimates for disease burden were all below this threshold, thus satisfying WHO guidelines. Furthermore, the sum of median estimated health burdens from all reference pathogens for the untreated river water irrigation system represents 37% of the annual health burden per person from acute diarrhea in Bolivia (which is currently 0.015 DALYs (Prüss-Üstün et al. 2008)). The sum of median estimated health burdens for the RBF system case only represents 1.1% of this overall health burden. Such an increase is epidemiologically indistinguishable and would be very difficult to detect (Mara 2011). Thus, the implementation of RBF in Cochabamba effectively satisfies the WHO guidelines for safe wastewater reuse in agriculture. On-farm interventions, like RBF, can be effective, transitional solutions for communities currently lacking wastewater treatment, while they invest in long-term infrastructure.

The results of the ethnographic study used with this QMRA demonstrate the potential impact of culturally-specific diets on pathogen exposure. The irrigated lettuce exposure example used in the 2006 WHO Guidelines overestimates risk for most Bolivians (Figure 23), and leads to estimated disease burdens that align with the 94th percentile of the health burden distribution estimated in this study for Bolivian market-goers. The results from the sensitivity analysis (Figure 24) show that the variability and uncertainty associated with environmental and cultural factors affecting exposure (e.g., pathogen concentration, pre-harvest decay, transfer from water to lettuce, lettuce serving size, and lettuce consumption frequency) correlate more with estimated health outcomes than factors related to disease vulnerability (e.g., disease burden per case, the conditional probability of illness given infection, and parameters used to estimate the susceptible fraction of the population). This suggests the need for investing in the evaluation of interventions that reduce exposure as a means to satisfy WHO recommendations with respect to safe indirect wastewater reuse practices.

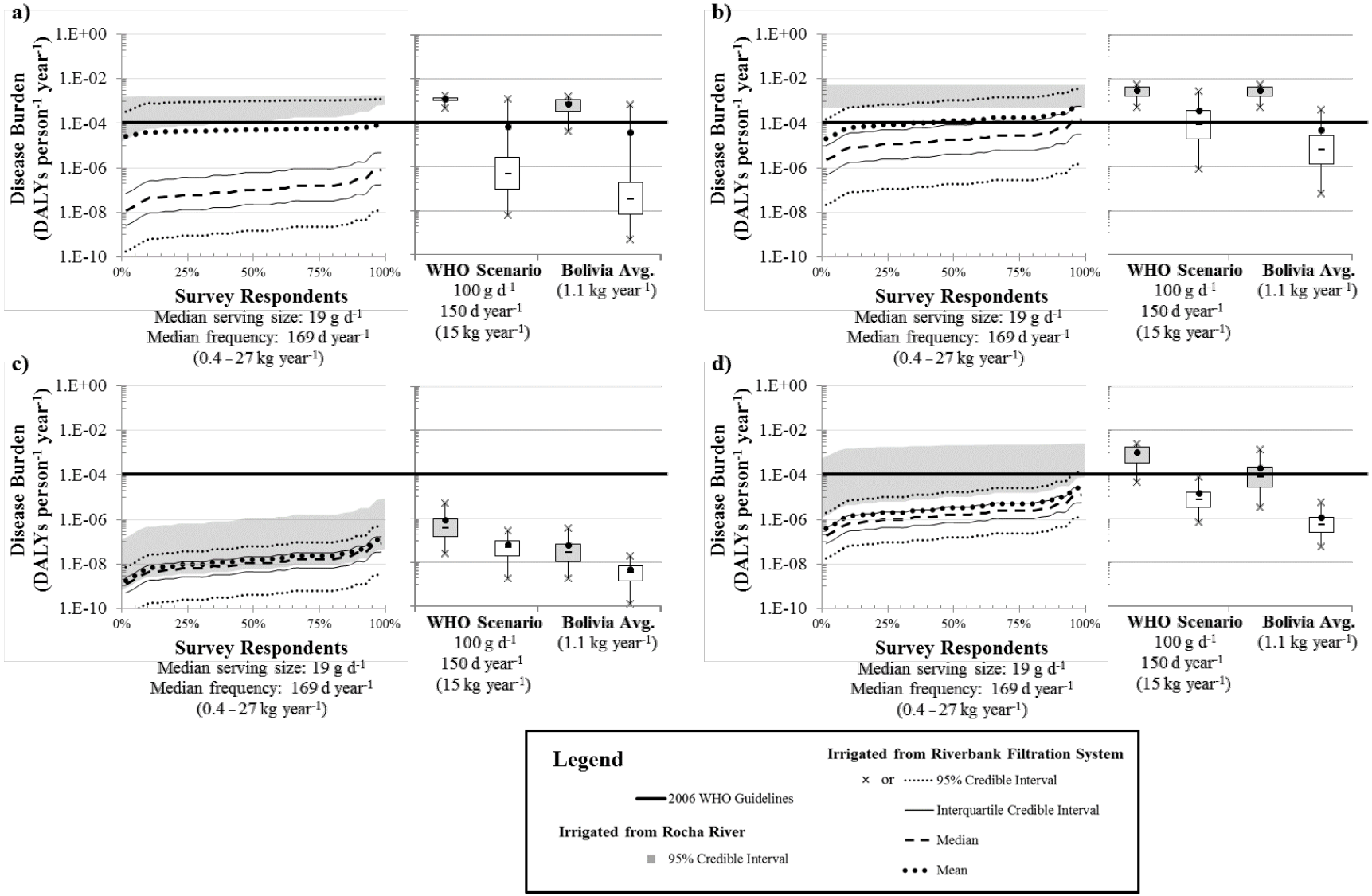

Figure 23. Credible intervals for the estimated disease burdens in DALYs resulting from the consumption of lettuce irrigated with water obtained directly from the Rocha River or with water from the riverbank filtration system, for (a) rotavirus, (b) adenovirus, (c) *Cryptosporidium*, and (d) *Giardia*.

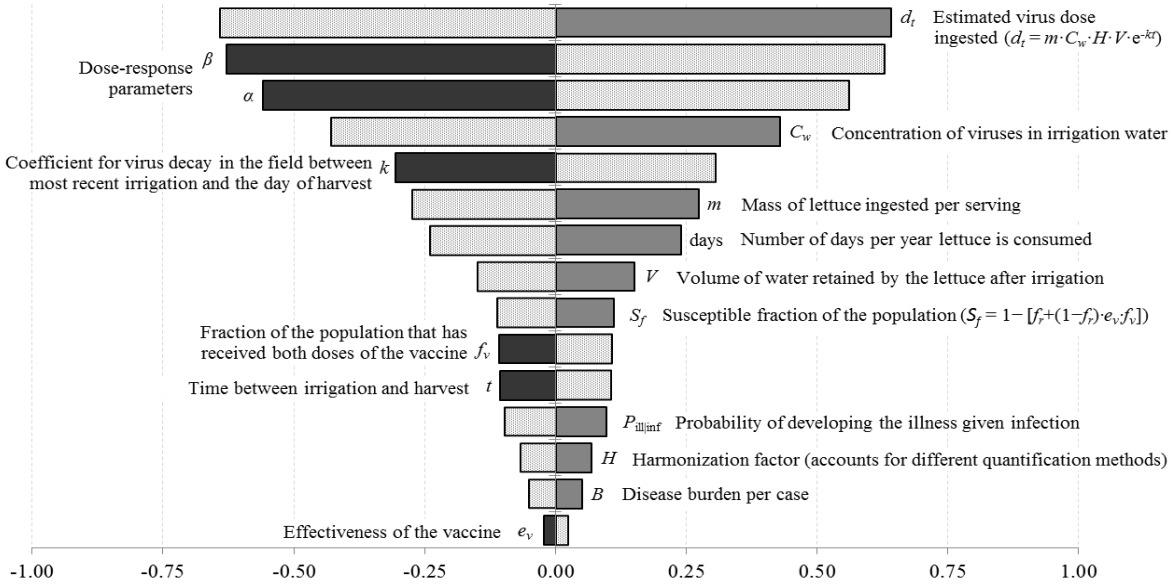

Figure 24. Tornado chart showing Spearman rank order correlation coefficients between QMRA model parameters and the predicted health burden caused by rotavirus illness resulting from the consumption of lettuce irrigated with water from the riverbank filtration system.

6 OVERALL CONCLUSIONS AND RECOMMENDATIONS

6.1 Selection of Appropriate Technologies based on Wastewater Management Priorities

The recent adoption of the SDGs by the United Nations will have a tremendous influence on the global development agenda during the next two to three decades. The inclusion of Target 6.2, which is to provide sanitation for all by 2030, and Target 6.3, which is to halve the proportion of untreated wastewater and increase safe water reuse globally by 2030, will lead to the mass construction of new wastewater treatment plants throughout the developing world. In order for these targets to be met with long-term success, governments and development organizations must utilize appropriate technologies that meet the needs of a wide range of community stakeholders (Wells et al. 2014), and can be adequately maintained by the community. The overwhelmingly predominant reason for the poor performance or failure of wastewater infrastructure in developing countries is the lack of maintenance (Libhaber and Orozco-Jaramillo 2012). The lack of maintenance was also the primary reason for the failure of approximately 50,000 water supply points that were recently installed in sub-Saharan Africa to support the MDGs, which has caused a loss of US$215 to $360 million, and has negatively impacted public health and the livelihoods of women in particular (Skinner 2009). Unless proper consideration is given to the operation and maintenance of the new wastewater treatment system infrastructure that will installed throughout the developing world in the years to come, these systems may encounter a similar fate.

Priorities for wastewater management (and for sanitation in general) tend to be very context-specific, and dependent on various socio-cultural and geographical particularities of a given location. Human waste can be returned to the environment, or reused for a variety of purposes. From a regional management standpoint, small cities should not expend resources to treat wastewater to levels suitable for discharge into surface waters, but should instead focus on removing pathogens to safely reuse the nutrient-rich water for irrigation (see Section 2.1). Wastewater reuse in agricultural activities can also help address the targets associated with SDG Goal 2, which is to end hunger, achieve food security and improved nutrition, and promote sustainable agriculture. The SDGs also contain targets related to the development of renewable energy, but energy recovery from wastewater may be a priority that is subservient to water and nutrient recovery, at least for small urbanizing cities in developing countries.

Originally developed in the Netherlands to treat industrial wastewater, UASB reactors were later tested in tropical environments. Successful demonstrations in Colombia during the 1980s led to the scaling up and replication of the technology throughout Colombia, all with support from the Dutch government (Lettinga 2010). Brazil quickly followed suit, adopting the widespread use of this technology to increase their national rate of wastewater treatment coverage. The momentum of the successes in Colombia and Brazil led to the popularity of the UASB reactor, which soon spread to other less economically-developed regions in South America, Central America, South Asia, and southern Africa. UASB reactors do not provide sufficient pathogen removal for safe water reuse or discharge to surface water bodies (Chernicharo 2007), and therefore must be used in combination with other treatment technologies. Today, they are often installed to replace overloaded, under-maintained, and failing WSP systems, or are used in combination with WSPs. Marketed as appropriate technologies for decentralized wastewater treatment, UASB reactors are frequently constructed in small cities or periurban communities, regions without the same human resources needed for operation and maintenance as larger, more established urban centers. They are sometimes managed by community water committees whose members may be volunteers or may have other full-time occupations.

One advantage of UASB reactors is that they are designed such that biogas, which contains up to 70% methane, can be easily harvested. Therefore, their use can support SDG Goal 7, which is to ensure access to affordable, reliable, sustainable and modern energy for all. However, just because UASB reactors can produce biogas does not mean that it will be used. In fact, very few communities in Central and South America with UASB reactors actually reuse the biogas that is produced. Biogas produced by the CePTS UASB reactor (see Section 4.3) is occasionally reused in a small cookstove for demonstration purposes (it is a pilot-scale system managed by a university research group to demonstrate the technology); biogas from the Minas Gerais UASB reactors (see Section 4.3) is flared; biogas from the Yungas UASB-Pond system (see Sections 2.1 and 4.2) is occasionally flared, but is often simply released into the atmosphere. The biogas from several other full-scale UASB reactors in Honduras and Bolivia is likewise either flared or released into the atmosphere (author's personal observations). It was recently demonstrated that the release of methane from small scale anaerobic reactors in developing countries could become a global "climate bomb," corresponding to more than 2% of global emissions by 2020 (Bruun et al. 2014). Some possible reasons for the lack of reusing biogas produced by UASB reactors in developing countries include long distances between the wastewater treatment plant and places of energy demand, low costs of alternative energy

sources, and lack of infrastructure to convert the energy into a more useable form (e.g., electricity). In order to adequately assess resource recovery priorities and choose the appropriate wastewater treatment technologies, *ex ante* analyses of energy, nutrient, and irrigation water demands should be used for all future projects. This will require collaboration from multiple sectors, including energy, environment, health, and agriculture.

6.2 Virus Removal in Waste Stabilization Ponds

Some small cities will choose to continue using WSPs to manage wastewater. Many of them either currently practice water reuse, or may be encouraged to practice water reuse, due to increasing water scarcity and pressure to address SDG Target 6.3. To protect public health, sufficient pathogen removal must be provided. As demonstrated in Chapter 3 of this dissertation, the following are knowledge gaps in the understanding of virus removal in WSP systems that still remain: lack of understanding about the extent of virus interactions with WSP particles (including other micro- and macro-organism); and the impact these interactions have on virus removal or inactivation (e.g., the effect of virus-particle associations on sunlight-mediated inactivation and effect of virus internalization by higher-trophic organisms on virus viability). There is also a lack of a model to predict virus removal in WSP systems. From a regional development perspective, this creates challenges for planning water reuse projects. Engineers and operators of WSP systems typically consider theoretical HRT to be a key design parameter. The removal of fecal indicator bacteria in WSPs can be well-predicted with the dispersed flow equation using theoretical HRT, pond dimensions, and ambient temperature (von Sperling 2005). However, this is not the case for viruses—there was only a weak to moderate correlation between theoretical HRT and virus removal in WSP systems (see Chapter 3), and 14.5 to 20.9 days of retention were required on average to achieve each $\log_{10}$ virus reduction (the 95th percentile was 54 days).

As described above, the use of the UASB reactor for domestic wastewater treatment in developing countries with tropical climates is becoming more common, especially in combination with WSPs. With respect to the water reuse implications of this trend, it appears that enteric viruses may become concentrated in the granular sludge formed in the sludge blanket. Viruses exiting the reactor also appear to be more associated with smaller, non-settleable particles. Virus-particle associations and virus removal in UASB reactors and WSPs appears to be different for different virus types (see Section 4.3). The results from the research presented in Chapter 4 of this dissertation generally do not support virus settling in WSPs (with the possible exception of AdV in WSPs following

UASB reactors). Because viruses were found to become volumetrically-concentrated in UASB sludge, some of these viruses may be removed from the reactor when sludge is evacuated. Because of this, several precautions should be taken by system operators to reduce risk during sludge disposal from these systems. For example, due to their small size, viruses in bioaerosols can travel orders of magnitude farther than other pathogens (Prussin et al. 2014); the flow of sludge removed from UASB reactors should remain laminar and quiescent, as turbulence can aerosolize viruses. Also, sludge removed from UASB reactors should be sufficiently stabilized prior to allowing close contact between sludge and people. Research may be needed to determine if viruses are as readily inactivated in UASB reactor sludge as they are in other types of wastewater sludge (e.g., activated sludge), for which more data exists (e.g., Dumontet et al. 1999; Sidhu and Toze 2009).

Viruses leaving UASB reactors were found to be associated with small, non-settleable particles (see Chapter 4). Most of these particles are likely too small to settle in WSPs. The inactivation of particle-associated viruses was not part of the scope of this dissertation, but should be examined in future research. Viruses in WSPs associated with particles that are too small to settle may decay at a faster rate, perhaps due to enzyme activity from other microbes in the particle or due to the closer proximity of the virus to reactive species created when sunlight is absorbed by organic material in the particle (indirect exogenous sunlight inactivation). Alternatively, these small particles may protect some viruses from direct sunlight damage, causing them to decay at a slower rate. The decay rates for these viruses is likely dependent on the characteristics of the virus and the particle (refer to Section 3.2.3 of this dissertation, Lytle and Sagripanti (2005), Mattle et al. (2015) for a more in-depth discussion about this topic).

With the exception of the culturable enterovirus results in the Yungas WSP systems (Section 4.2) and the F+ coliphage results from study of virus-particle associations in the Belding WSP system (Section 4.3.3.3), measurements of other viruses were made using molecular methods, which do not indicate the viability of the virus (only the presence of an intact portion of its DNA or RNA genome). Nevertheless, naked viral nucleic acids (especially RNA) will show measureable decay in water within several days (Walters et al. 2009). Also, virus genome damage resulting in the loss of viability can block enzymes used in RT-qPCR (e.g., Taq polymerase, reverse transcriptase), and cause the loss of a qPCR signal, especially if the damage occurs near the targeted region (Pecson et al. 2011). Still, virus measurements based on molecular analyses (e.g., qPCR or RT-qPCR) should not be directly compared to virus measurements made using culture methods (e.g., coliphage plaque assay or culturable enterovirus assay).

6.3 Water Reuse and Microbial Risk

Used alone, WSP systems may not always provide sufficient removal of all pathogen types for safe, unrestricted water reuse, especially in developing country settings if the systems are not adequately maintained. The two WSP systems in Cochabamba, Bolivia had very poor pathogen removal efficiencies, due to the lack of maintenance (see Section 5.3). Even the well-maintained WSP system in Yungas, Bolivia, with a theoretical HRT of more than 25 days, still required an additional 1- to 2-$\log_{10}$ removal of viruses in order for safe water reuse for irrigation (see Section 4.2). The UASB-Pond system, with a similar theoretical overall hydraulic retention time, and viruses that were more associated with particles, required an additional 2.5- to 4.5-$\log_{10}$ removal for safe reuse.

Farmers value the resources in wastewater, and water reuse for irrigation allows for synergy between multiple SDGs. Farmers in the Cochabamba Valley not only help maintain the grounds where WSPs are located, some of them also utilize on-farm treatment methods that reduce the overall health burden associated with water reuse (see Chapter 5). Still, without training, equipment, and other resources, most farmers will not be prepared to monitor pathogen removal. Pathogen removal is also not likely the farmer's top priority. Furthermore, if municipal or community wastewater authorities do not communicate and coordinate with farmers, the overall wastewater management system may not operate as it is intended. In the Cochabamba Valley, modifications made to the downstream irrigation canals by farmers may have altered flow conditions in WSPs, causing short-circuits and less-efficient pathogen removal. In order to avoid the stigmatization of wastewater reuse, farmers should be given credit for implementing on-farm interventions that reduce microbial risks.

QMRA is the approach recommended by the WHO for safe water reuse planning purposes, however its use may not always be practical for every individual water reuse project, due to the large amount of data needed to complete QMRAs. These data are often scarce in developing country settings (Howard et al. 2006), and scarce data leads to risk estimates with a greater uncertainty (see Section 5.3). QMRA can be useful, however, to characterize the effectiveness of a particular combination of wastewater treatment technologies, provided there is sufficient understanding about their effectiveness at removing pathogens. A sensitivity analysis of the QMRA developed for this dissertation (Section 5.3) revealed that the variability and uncertainty associated with cultural and environmental factors affecting exposure to pathogens may correlate more with health outcomes than the variability and uncertainty associated with factors related to disease vulnerability. This suggests the need to invest in the evaluation of interventions that reduce exposure to pathogens, particularly new technologies or novel treatment approaches. For

natural wastewater treatment systems, that would require developing models that accurately predict the removal of different types of pathogens based on environmental and operational parameters (as has been done for coliform bacteria, see von Sperling (2005)). For viruses, it may also be necessary to develop models based on virus characteristics (e.g., genome type, enveloped vs. non-enveloped).

6.4 Moving Forward: An Integrated Approach to Wastewater Treatment and Safe Water Reuse

With the adoption of the new SDGs, natural wastewater systems should and will remain an important part of the overall solution for small cities and towns in industrialized and developing countries. However, these natural systems are likely to be used more often in combination with other technologies that may be optimized for a variety of treatment objectives, including energy recovery. The simultaneous recovery of water, nutrients, and energy resources from wastewater could contribute to a variety of the SDGs, including targets associated with Goals 2, 6, and 7. In order to safely reuse water (i.e., SDG Target 6.3), pathogen removal is required.

In order for natural wastewater systems to be more resilient with respect to pathogen removal, they should be multifarious (incorporate a diverse array of technologies and with management input from various stakeholder groups) and redundant (provide multiple barriers for the removal of pathogens). Different types of pathogens are removed with varying efficiencies in different types of natural wastewater treatment systems. Multifarious systems that incorporate several different types of treatment units, including surface flow (e.g., WSPs) and subsurface flow units (e.g., subsurface wetlands, bank filtration systems), may provide better overall removal of all pathogen types (viruses, bacteria, protozoan, helminth eggs). Redundant systems will help improve resiliency in the event of technical or operational failures of one part of the system. For water reuse planning purposes, multiple barriers can be incorporated into the overall wastewater management plan using the sanitation chain approach (Figure 25), which is based on concepts presented by Stenström et al. (2011) and the findings presented in this dissertation. For example, water reuse systems could include onsite or decentralized collection and treatment of wastewater (to provide a preliminary barrier for pathogens), a centralized or semi-centralized wastewater treatment system (to provide a secondary barrier for pathogens), and an on-farm water treatment system (to provide a tertiary barrier for pathogens). Additional health protection measures, such as improved irrigation techniques or the washing of irrigated produce, can also be implemented to reduce the contamination of wastewater-irrigated produce.

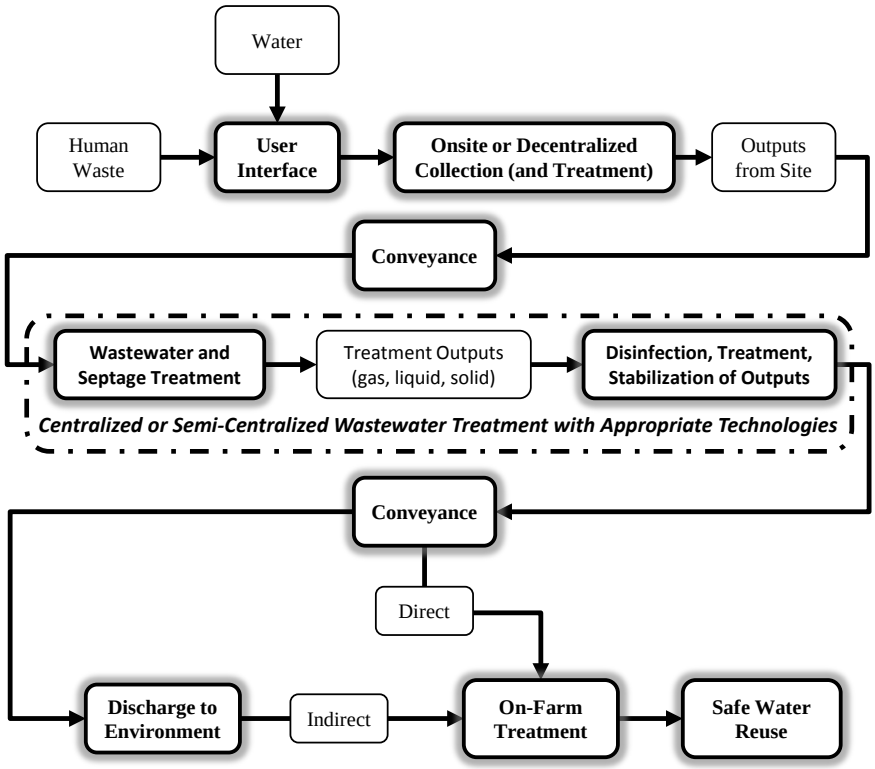

Figure 25. Proposed sanitation chain for sewerage systems that allows for safe water reuse by incorporating on-farm treatment in addition to centralized or semi-centralized wastewater treatment.

This sanitation chain approach can be integrated with traditional sewerage systems (which combine greywater, urine, and feces or blackwater into the same waste stream), or with segregated sewerage systems (which separate urine, greywater, or blackwater into different waste streams). Greywater and urine can be effectively managed and reused or returned to the environment at the decentralized level (onsite), since the concentration of pathogens in those waste streams are generally much lower than they are in feces or blackwater. It also applies to onsite sanitation systems, where the liquid fraction is discharged to a leach field and the solid portion (septage) is removed from the site and conveyed to a centralized treatment facility.

Instead of being managed solely by public sector or community authorities, new stakeholders, such as farmers, should be integrated into overall regional wastewater management and sanitation programs. They should be trained in the operation and maintenance of treatment technologies used in the overall Sanitation Chain and credited for contributions they make that improve the overall safety of water reuse practices. Simple, on-farm treatment methods, such as riverbank filtration, can result in significant reductions of health burdens associated with water reuse, and their use should be encouraged. Outputs from the centralized facility (whether liquid or solid) can still be treated on the farm prior to reuse, to provide an extra layer of redundancy in the overall sanitation chain.

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APPENDIX A: SUPPORTING INFORMATION FOR CHAPTER 3

A.1 Calculation of S_{90} Values for Virus Solar Disinfection

The S_{90} values for virus solar disinfection studies were estimated using the equation:

$$S_{90} = 0.0036 \cdot E_s \cdot \frac{t}{LRV} \quad (A1)$$

where E_s is the full spectrum solar irradiance (W/m^2), LRV is the $\log_{10}$ reduction value reported for the virus after time t, and 0.0036 is a conversion factor (W·h to MJ). If solar insolation was not reported, values from the U.S. National Aeronautics and Space Administration (NASA 2014) for average solar insolation at the particular geographic location of the experiment were used.

A.2 Calculation of Virus Concentrations from Presence-Absence Data

For studies in which authors only used semi-quantitative methods (i.e. reported only the presence or absence of viruses in replicate samples), the mean concentration ($\hat{u}$) of viruses was calculated using a maximum likelihood estimator (Jarvis et al. 2010), setting the derivative of the log-likelihood function equal to zero, and solving for $\hat{u}$. For experiments performed with a single dilution level, this equation simplifies to:

$$\hat{\mu} = \frac{\ln(1-\frac{x}{n})}{-dw} \quad (A2)$$

where n is the total number of replicates, x is the number of replicates with positive virus identification, d is the dilution level, and w is the volume of sample analyzed.

A.3 Methods for Systematic Literature Review of Virus Removal in Field Studies

A systematic review of studies on virus or bacteriophage removal in WSPs was carried out by searching for relevant keywords (virus, phage, coliphage, bacteriophage, pond, lagoon, stabilization, anaerobic, facultative,

maturation, polishing) in the following databases: ScienceDirect, Web of Science, ISI Web of Knowledge, PubMed, Academic Search Premier, JSTOR, and Google Scholar. Peer-reviewed journal articles, conference proceedings, theses and dissertations were all considered. In addition, a Google search was used to identify reports produced by government agencies. Similar searches were also performed on Google Scholar using keywords in Spanish (*laguna de estabilización*, *virus entérico*, *colifagos*) and in French (*bassin de stabilization*, *bassin de lagunage*, *lagune de stabilization*, *virus entérique*, *bactériophage*). Finally, the reference sections of all publications were reviewed to identify additional literature sources.

Systems with a greater number of ponds in series were more likely to include at least one maturation pond (Figure A1). Virus removal efficiency is expected to be highest for maturation ponds, so there is a possibility that the differences in the configurations of each pond system may bias some of the results of the statistical analyses.

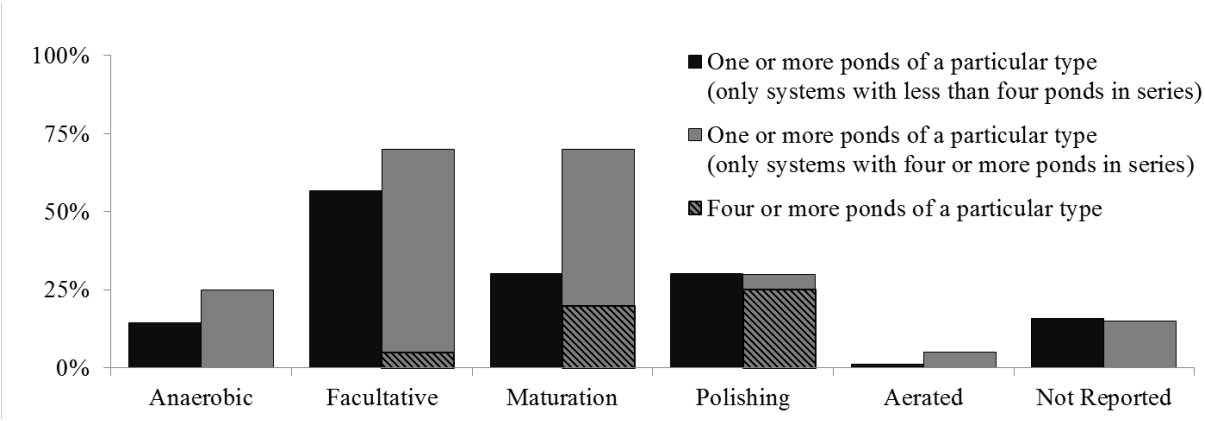

Figure A1. Percentage of systems with particular types of ponds (anaerobic, facultative, maturation, polishing, or aerated).

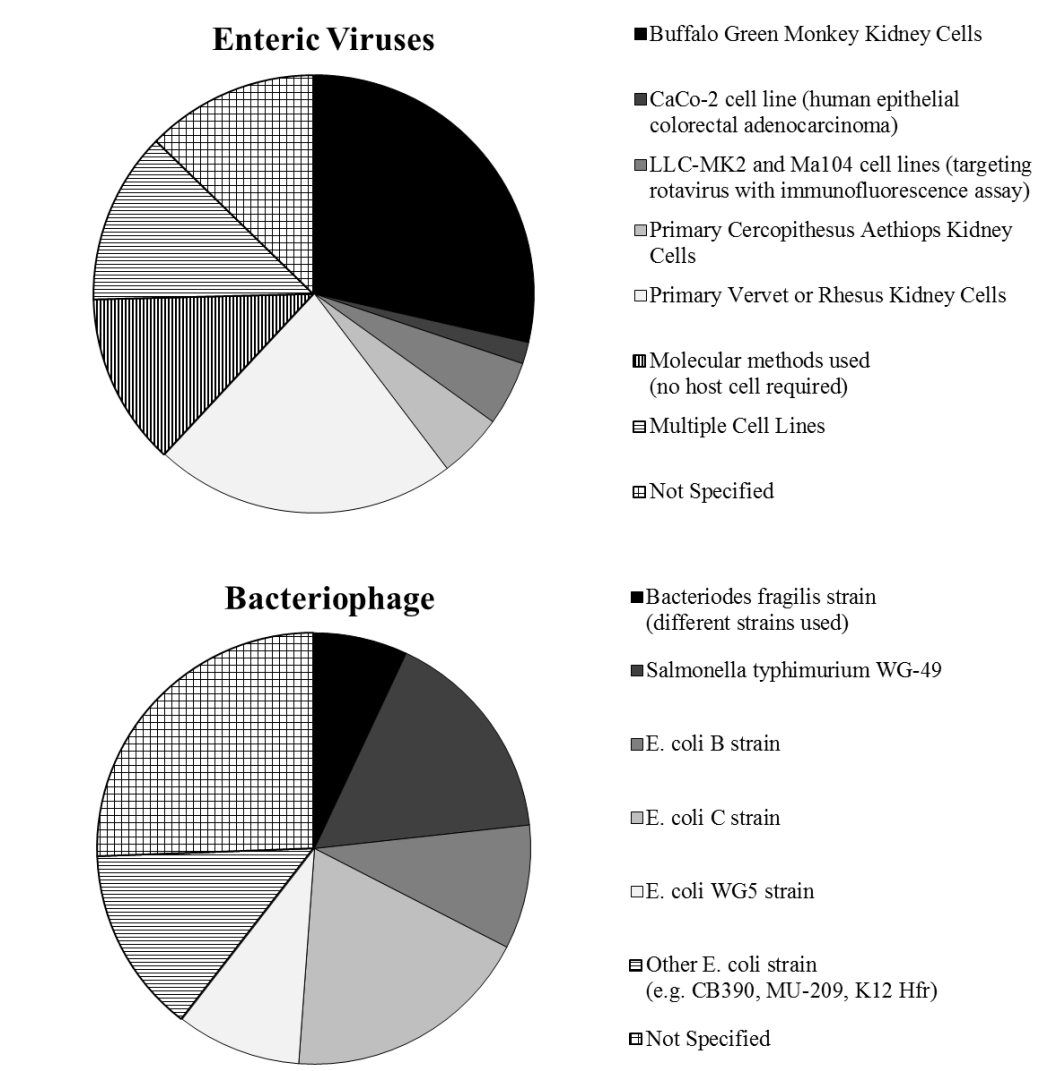

Figure A2. Different methods and host cell lines or bacteria strains used for enteric virus (top) and bacteriophage (bottom) assays.

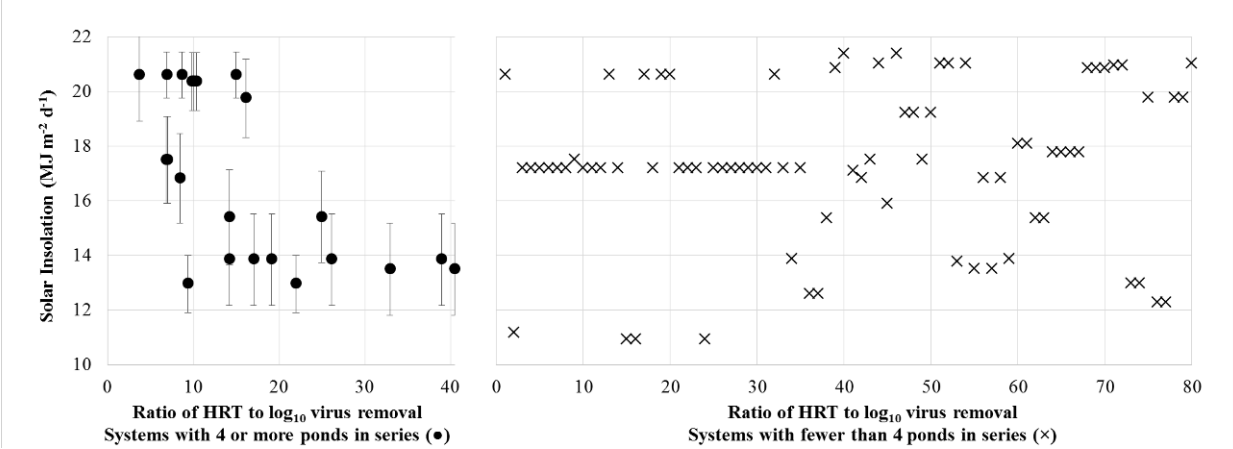

Figure A3. Average daily solar insolation at the latitude of the waste stabilization pond system vs. the ratio of the hydraulic retention time to the mean log_{10} virus removal, for systems with four or more ponds in series (●) and systems with fewer than four ponds in series (×). Error bars show the standard deviation (only for systems with four or more ponds in series, for clarity).

Table A1. Pearson's correlation coefficients for the log-transformed ratio of the theoretical hydraulic retention time to the mean observed log_{10} removal (LN(HRT-removal ratio)) and the average daily solar insolation at the latitude of each wastewater treatment pond system.

Number of Ponds in Series	Pearson's Correlation Coefficient (Pearson's *r*)	Number of Data Points	Probability (p-value)
1	-0.002	32	0.396
2	-0.149	20	0.318
3	-0.276	25	0.152
4	-0.213	10	0.314
More than 4	-0.602	13	0.026
All Data (any number of ponds)	-0.181	100	0.076

A.4 References for Appendix A

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APPENDIX B: SUPPORTING INFORMATION FOR SECTION 4.2

B.1 Synthesis of RNA Oligonucleotide for RNA Purification and Reverse Transcription Control

A 127 bp RNA oligonucleotide containing the amplicon of an existing Taqman qPCR assay for EAV (Svraka et al. 2009) was produced using previously described methods (Ulrich et al. 2010) with the following primers targeting the T7 promoter and a portion of the EAV replicase protein: (F) 5' – AAT TCT AAT ACG ACT CAC TAT AGG GTG TCG CTT GTG CTC AAT TTA CTG GGG CAG GTA GGC TAT GTA GCT CTG CAA AGA TCG AAC ACA GTG CAA GGA ACA TAA GCT GCA TCA AAG GTG GAA CGA GCT ACA TAG CC – 3' and (R) 5' – CGA TTG CGG CAG AGG TAC AGA ATA GCA AAG CTG CAA AGA TCG AAC ACA GTG CAA GGA ACA TAA GCT GCA TCA AAG GTG GAA CGA GCT ACA TAG CC– 3'. The resulting EAV RNA oligonucleotides were quantified using the NanoDrop Spectrophotometer ND-100 (Wilmington, DE), preserved with (1:1) RNA storage buffer (8 M guanidinium isothiocyanate, 80 mM Tris-HCl [pH 8.5], 24 mM $MgCl_2$, and 140 mM KCl), and stored at -80°C in single use volumes to prevent freezing and thawing.

B.2 Quantitative PCR Standard Curves

The standard curves for each of these assays were generated from purified plasmids manufactured by Integrated DNA Technologies (pIDTSMART with ampicillin resistance; Coralville, IA) containing the corresponding target sequence derived from GenBank sequences: NC_002532.2 (EAV), NC_001959.2 (NoVGI), AB550911.1 (PMMoV), and HM627561.1 (RV). The EAV, NoVG1, and RV standard curves were composed of a seven-point, 1:10 dilutions series ranging from 10 to 1×10^7 plasmids/reaction. The PMMoV standard curve was composed of a six-point, 1:10 dilution series ranging from 100 to 1×10^7 plasmids/reaction.

B.3 Molecular Quantification of EAV, NoVGI, RV, and PMMoV

Five 96-well plates were analyzed, each containing the standard dilution series in duplicate as well as no-template controls. Each 25 µl qPCR reaction targeting EAV, NoVGI, and RV contained 2 µl of sample cDNA and the following final concentrations: 1x TaqMan Environmental Mastermix 2.0 without Uracil N-Glycosylase (UNG)

(Life Technologies, Grand Island, NY), 500 nM of each primer, and 250 nM of probe (Table B1). For PMMoV, the qPCR reaction was slightly modified from Rosario et al. (2009) so each primer and probe had a final concentration of 400 nM and 125 nM, respectively (Table B1). Each sample was analyzed in duplicate and with an additional 1:10 dilution (to identify PCR inhibition), using an ABI7500 Real Time PCR system (Life Technologies). The following qPCR conditions, with data collection occurring at the elongation step, apply to the EAV and NoVGI analyses: denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 52°C for 20 s, and 60°C for 60 s. For the RV and PMMoV assays, the same conditions were used, except the annealing step was executed at 43°C for 20 s and 53°C for 60 s, respectively.

B.4 Molecular Quantification of EAV, NoVGI, RV, and PMMoV using the 'Pooled Approach'

Quantitative PCR (qPCR), using published primers and probes, was used to determine the concentrations of RV, NoVGI, and PMMoV as well as the EAV purification control in each sample (Table B1) (Svraka et al. 2009, Rosario et al. 2009). In order to account for the stochastic interplate variability inherent to qPCR analyses, the '*pooled approach*' was used to estimate the number of copies in each unknown sample from the corresponding mean Cq value for plasmid standards and unknown samples for each assay (Sivaganesan et al. 2010). Markov chain Monte Carlo simulations, using WinBUGS software V1.4.3 (Imperial College and Medical Research Council, UK) were carried out with 50,000 iterations and a burn-in period of 10,000. A modified version of the code provided by Mano Sivaganesan was used and is provided below. Slope and y-intercept parameters were assumed to be normally distributed for each run, and the gamma distribution (with shape and scale parameters equal to 0.01) was used as a prior distribution for the precision of the slope and y-intercept distributions, instead of the DuMouchel priors (Sivaganesan et al. 2010). The use of either of these two prior distributions tends to yield similar results (Sivaganesan et al. 2008).

B.5 WinBUGS Code (Modified from Sivaganesan et al. 2010)

```
model
	{

# DEFINE VARIABLES:
	# Y = Ct measurement of standards (plasmids)
	# x = copy numbers of standards (plasmids)
	# run = plate number
	# alpha = y intercept of the standard curve
	# beta = slope of standard curve
	# n = total number of data points in the standard curve
	# p = number of plates (aka number of runs)
```

```
        # s = number of different sample points (non-replicate unknowns)
        # r = number of replicates per (unknown) sample point

                for (i in 1 : n){
                        Y[i] ~ dnorm(mu[i], tau[run[i]])
                        mu[i] <- alpha[run[i]] + beta[run[i]] *(log(x[i]))/log(10)
                        }

                for (i in 1 : p){
                        alpha[i] ~ dnorm(alphab, tau.a)
                        beta[i] ~ dnorm(betab, tau.b)
                        }

                for (i in 1 : p){
                        tau[i] ~ dgamma(0.001, 0.001)
                        }

                alphab ~ dnorm(0.0,1000000)
                betab ~ dnorm(0.0,1000000)
                tau.a~ dgamma(0.01,0.01)
                tau.b~ dgamma(0.01,0.01)

# This part of the code predicts the concentration (copy numbers) in unknown samples
# It also takes the mean of any replicates

                for (i in 1 : s){
                        for (j in 1 : r){
                                copy[i,j]<-(ct[i,j]-alpha[1])/beta[1]
                                }
                        copymean[i]<-mean(copy[i,])
                        }
                }


# Here, list the data for the following variables:
        # n, the number of data points from the standard curves
        # e.g. if you run 5 plates, with 7-point standard curves in duplicate, n = 5 * 7 * 2 = 70
        # p, the number of plates that you ran
        # s, the number of non-replicate unknown sample points
        # r, the number of replicates per unknown sample point
list(n=70, p=5, s=32, r=3)

# Here, add the data from the standard curves from all runs (can copy & paste from Excel)
# If some of the standard curve points are not used or unknown, write NA for the Y[] value
run[]   x[]     Y[]
1       10000000        14.83086777
1       1000000 17.82779503
1       100000  21.80016327
1       10000   25.55435562
1       1000    28.44073677
1       100     31.74236107
1       10      35.72465515
1       10000000        14.91196918
1       1000000 17.11416817
1       100000  21.56788445
1       10000   24.45992279
1       1000    27.21128464
1       100     31.74817085
1       10      37.29232788
2       10000000        16.56287956
2       1000000 20.03527069
2       100000  22.42720604
2       10000   26.68701172
2       1000    30.17602921
2       100     33.48706818
2       10      38.01969528
2       10000000        15.01192474
2       1000000 19.6750927
2       100000  23.39949226
2       10000   27.08206177
2       1000    28.98237991
```

```
2       100     33.38588333
2       10      NA
3       10000000        15.28802204
3       1000000 20.29097366
3       100000  23.32222176
3       10000   26.97269249
3       1000    31.19098663
3       100     33.51592636
3       10      NA
3       10000000        15.20750141
3       1000000 20.05568886
3       100000  23.38633537
3       10000   23.36335182
3       1000    30.89651108
3       100     33.61697388
3       10      36.5364151
4       10000000        17.95369339
4       1000000 20.91512299
4       100000  24.97530174
4       10000   26.61340714
4       1000    32.10835648
4       100     35.57624054
4       10      40.97258377
4       10000000        17.97052383
4       1000000 20.90886307
4       100000  24.94770241
4       10000   28.26919174
4       1000    31.81770325
4       100     31.98748589
4       10      39.20402145
5       10000000        NA
5       1000000 19.62317467
5       100000  22.38277245
5       10000   25.89981651
5       1000    30.07412529
5       100     33.0720253
5       10      NA
5       10000000        16.38263321
5       1000000 19.39556313
5       100000  23.35000038
5       10000   26.49247742
5       1000    29.05232811
5       100     33.49391174
5       10      38.0681839
END;


# Here, list the data for the unknown samples, in the following order:
# 1st sample - replicate 1, replicate 2, replicate 3, etc. then 2nd sample - replicate 1, 2, 3,
etc.

list(ct = structure(.Data = c(27.72813797, 27.42074203, 27.68983841, 27.49964523, 27.48989868,
27.78788757, 30.5907135, 27.84589005, 27.85700989, 29.96239471, 28.98430252, 29.90208244,
36.87768555, 38.62608337, 36.61364746, 32.28911591, 33.10028076, 32.98776245, 30.69845581,
30.86000824, 31.17842484, 29.79073334, 30.05471039, 30.50596237, 32.21622467, 33.21928406,
31.26853561, 32.55735779, 33.80277252, 32.56066513, 33.81419754, 35.71244812, 34.47873306,
35.50968552, 36.70432663, 35.96899414, 35.23744202, 36.91765594, 34.75976944, 37.86174011,
36.44485474, 37.3629837, 37.20646286, 37.44822311, 36.30540466, 39.00939178, 38.7159729,
31.99326706, 39.37914658, 40.21757889, 37.76005936, 36.64976501, 39.22754669, 37.42768097,
38.54289246, 50, 38.98463058, 39.58310318, 37.52623367, 33.18672943, 31.402565, 33.59807587,
37.26535416, 33.87909698, 35.83087158, 38.61930847, 50, 39.93122101, 50, 38.06398773,
38.32701874, 50, 50, 39.03576279, 38.91719437, 50, 50, 39.22404099, 29.45872879, 31.00250626,
29.89751053, 30.20918465, 31.61616516, 30.94333267, 30.95830536, 32.55595016, 31.27261162,
41.92402267, 35.60179138, 34.66479492, 34.39841461, 33.24088287, 32.35334778, 33.12833405,
31.98050308, 32.73188019), .Dim = c(32, 3)))

# Note, for Dim above, enter total # of samples followed by # of replicates per sample

# These are initials for the parameters that define the standard curve slope and y-intercept
list(alpha=c(20, 31, 25 , 18, 19), beta= c(-3, -2, -2.5, -1, -0.7), tau =c(1, 1, 1, 1, 1), alphab
= 0.01, betab=0.05)
```

In addition to calculating the copy number measured from a Cq value, the 'pooled approach' also produces credible intervals associated with each calculation and these values are presented for both the analyses of virus concentration and virus-particle associations in the WSP systems (Tables B2 and B3, respectively).

B.6 Exposure via the Ingestion of Water vs. Soil

It has been commonly assumed that farmers irrigating with wastewater and children playing in fields accidentally ingest either a small volume of water (typically 1 ml) or a small volume of soil (typically 10 – 100 mg) per day of exposure. Agricultural soils in Bolivia have bulk densities (ρ) that typically range from 1.3 to 1.6 g/ml (Vargas Rojas 2009, Aguilera et al. 2012) and most agricultural soils have a moisture saturation point of ~15% (Rzezutka and Cook 2004). Assuming that up to 90% of viruses in irrigation water may adsorb to surface sites on the soils (Kimura et al. 2008), the dose of viruses that would correspond with the accidental ingestion of 100 mg of soil that has been irrigated once would be equal to the amount of viruses in 0.008 to 0.01 ml of irrigation water (VL = 0.90 * 0.15 * (100 g) / (1.6 g/ml) = 0.008; V_U = 0.90 * 0.15 * (100 g) / (1.3 g/ml) = 0.01). If the same ground gets irrigated 75 times in an irrigation season and new viruses are adsorbed every time, then the ingestion of 100 mg of soil would be nearly equivalent (slightly less than) to the ingestion of 1 ml of water.

B.7 Quantitative Microbial Risk Assessment (QMRA) Model

NoV has been recently recommended by the WHO as a suitable reference viral pathogen for QMRA models of wastewater irrigation (Mara et al. 2010). Given its high infectivity (Le Pendu et al. 2006) and its role in water- and food-related outbreaks (Goodgame 2007), it was used as a reference viral pathogen for adult farmers and children. While there is no available data for the prevalence of NoV in Bolivia, in Chile, NoV is almost as common as rotavirus and is a significant cause of moderate to severe and endemic acute diarrhea episodes in children (O'Ryan et al. 2010). NoV causes a disease burden that may range from 0.000371 to 0.00623 DALYs per case of illness (Mok et al. 2014). There is also currently no vaccine or evidence of acquired resistance to NoV in Bolivia. While a small percentage of people may have natural genetic resistance (Le Pendu et al. 2006), there are no available data for Bolivia. Therefore, it was assumed that the fraction of the population with genetic resistance to NoV infection (p_r) is uniformly distributed from 0 to 0.2 (Mok et al. 2014).

RV was only considered to be a hazard for children at play because adults typically acquire resistance (Chiba et al. 1986). Bolivia has historically experienced the highest annual rotavirus-related child mortality rate (2.95×10^{-3}) out of all Caribbean and Latin American countries (Linhares et al. 2011), which prompted the introduction of a RV vaccine in in Bolivia in 2008. However, 22% of children in Bolivia are unvaccinated (WHO 2014). Furthermore, the efficacy of the RV vaccine in Bolivia has been predicted to be only 69% (95% confidence interval of 54% to 79%) (Patel et al. 2013). It was assumed that the disease burden per case of RV is between 0.015 and 0.026 DALYs per case (Havelaar and Melse 2003, Prüss-Üstün et al. 2008)

EV comprise a diverse group of enteric viruses that are capable of causing a wide range of diseases, including meningitis, hepatitis, myocarditis, pneumonia, and the common cold (Melnick 1989). EV was also considered in the QMRA model for adults and children. Given the vast diversity of EV, it was assumed that every person may have susceptibility to infection from at least one of the types of viruses in this group (i.e. $S_f = 1$) and the disease burden per case of illness was between 0.0024 and 0.015 DALYs per case (Howard et al. 2007, Prüss-Üstün et al. 2008).

B.8 Dose-Response Models

The QMRA model was designed to predict a log reduction value, given a health target of $<10^{-4}$ disability-adjusted life years (DALYs) per person per year. Equation S1, which is the dose-response model for NoV, is the Pfaff transformation of a model from Teunis et al. (2008). This model was used with fit parameters from the combined inocula dataset (8fIIa + 8fIIb) reported by Teunis et al. (2008), because it makes no assumptions about the aggregation state of the viruses. The Pfaff transformation of the model was used as a close approximation here, assuming all doses $\leq$33,323, because the fit value for parameter a_{NV} provided by Teunis et al. (2008) exceeds a constraint of the Gauss hypergeometric function used in Equation S1 (Barker et al. 2013, Mok et al. 2014). The dose-response model for RV (Equation S2), is the exact Beta-Poisson model (Teunis and Havelaar 2000). It is used here instead of the "approximate" Beta-Poisson model, which has been widely used in the past with RV for QMRA studies (van Ginneken and Oron 2000, Mara et al. 2007, Seidu et al. 2008, Soller et al. 2010). This approximate model is only valid when $\beta \gg 1$ and $\alpha \ll \beta$ (Teunis and Havelaar 2000), conditions which are not satisfied for RV. The RV dose-response model was also used as a surrogate dose-response model for EV, as has been done by others (e.g. Schijven and de Roda Husman 2006), to provide a conservative estimate of infection probability for the wide

range of viruses that comprise the EV group. For comparison, an exponential dose-response model (Equation S3) was also used for EV, with fit parameter *k* determined using data from a study where pigs were challenged with porcine enterovirus type 7 (Cliver 1981, Huang 2013).

$$p_{\mathrm{inf_NV}} = 1 - \left({}_2F_1\left(\beta_{NV}, \frac{c_{NV}V(1-\alpha_{NV})}{\alpha_{NV}}, \alpha_{NV} - \beta_{NV}; \alpha_{NV}\right)\left(\frac{1}{1-\alpha_{NV}}\right)^{-\left(\frac{c_{NV}V(1-\alpha_{NV})}{\alpha_{NV}}\right)} \right) \quad (B1)$$

$$p_{\mathrm{inf_RV}} = 1 - {}_1F_1(\alpha_{RV}, \alpha_{RV} + \beta_{RV}, -c_{RV}V) \quad (B2)$$

$$p_{\mathrm{inf_EV}} = 1 - \exp(-k \cdot c_{EV} \cdot V) \quad (B3)$$

The dose-dependent conditional probability of illness for those developing a NoV infection ($p_{ill|inf\ NV}$) was modeled using Equation S4 (Teunis et al. 2008), where η_{NV} and r_{NV} are model parameters with best fit values reported by Teunis et al. (2008). For RV and EV, this probability was modeled using Equation S5 (Mok et al. 2014), where *I* is a parameter describing the proportion of infected people who develop an illness.

$$p_{ill|inf_{NV}} = 1 - (1 + \eta_{NV} c_{NV} V)^{-r_{NV}} \quad (B4)$$

$$p_{ill|inf_{RV}} = p_{inf_{RV}} \cdot I \quad (B5)$$

Given the convoluted nature of the system of equations described in Section 2.8 of the manuscript, the concentration (c) cannot be solved for analytically. 'R' Version 3.1.0 (The R Foundation for Statistical Computing 2014) was used to solve for c using an iterative approach. The maximum tolerable concentration was first set to a value of zero, then "stepped up" in increments of 0.1 (for NoV) or 0.00001 (for RV and EV), for a total of 10,000 iterations. Once the value of c produced an estimate of disease burden (DB) that exceeded a defined percentile of the maximum tolerable estimate of DB, the loop specifies that c stay at the same value (instead of continuing to be "stepped up"). At the end of the loop, the value of c was printed. A dummy variable "test" was also computed and printed to ensure that the loop did not run through all of the iterations without finding an appropriate solution for c.

B.9 'R' Code Used to Solve for LRV for NoV

```
library(hypergeo)
	DB_limit <- 0.0001 #Regulatory limits specified by WHO (2006)
	iter <- 10000
	step <- 0.1
	a <- 0.9997 #Point estimates for the dose-response model parameters (Teunis et al. 2008)
	alpha <- 0.04
	beta <- 0.055
	eta <- 0.00255
	r <- 0.086

#Assumptions about exposure, susceptible fraction of population, disease burden
	Sf <- runif(iter,0.8,1)
	B <- runif(iter,0.000371,0.00623)
	n <- 75
	V <- 1

#Pfaff Transformation of Eq 4 and Eq 5 (Teunis et al. 2008)
	a1 <- (1-a)/a
	pinf <- function(c){Re(1-(hypergeo(beta,c*V*a1,alpha+beta,a)*((1/a1)^(-c*V*a1))))}
	pillinf <- function(c){(1-(1+eta*c*V)^(-r))}

#Measured effluent concentrations
	logconc3P <- rnorm(iter,2.56,0.27)
	conc_2_3P <- quantile(10^logconc3P, c(0.025))
	conc_10_3P <- quantile(10^logconc3P, c(0.10))
	conc_25_3P <- quantile(10^logconc3P, c(0.25))
	conc_med_3P <- quantile(10^logconc3P, c(0.50))
	conc_75_3P <- quantile(10^logconc3P, c(0.75))
	conc_90_3P <- quantile(10^logconc3P, c(0.90))
	conc_98_3P <- quantile(10^logconc3P, c(0.975))

	logconcUB <- rnorm(iter,2.34,0.31)
	conc_2_UB <- quantile(10^logconcUB, c(0.025))
	conc_10_UB <- quantile(10^logconcUB, c(0.10))
	conc_25_UB <- quantile(10^logconcUB, c(0.25))
	conc_med_UB <- quantile(10^logconcUB, c(0.50))
	conc_75_UB <- quantile(10^logconcUB, c(0.75))
	conc_90_UB <- quantile(10^logconcUB, c(0.90))
	conc_98_UB <- quantile(10^logconcUB, c(0.975))

#Estimate required log reduction to meet WHO recommendations
#2.5th percentile
c <- 0
for(i in 1:iter){
	pill <- pinf(c) * pillinf(c)
	Pill_a <- 1 - (1 - pill)^n
	DB <- Pill_a * B * Sf
	ifelse(quantile(DB, c(0.025)) < DB_limit, c <- c + step, c <- c)
	}

	LRV_2_3P <- log10(conc_2_3P) - log10(c-step)
	LRV_2_UB <- log10(conc_2_UB) - log10(c-step)
	print(LRV_2_3P)
	print(LRV_2_UB)
	print(c-step)
	test <- ((iter * step) - c)/step
	print(test)

#10th percentile
c <- 0
for(i in 1:iter){
	pill <- pinf(c) * pillinf(c)
	Pill_a <- 1 - (1 - pill)^n
	DB <- Pill_a * B * Sf
	ifelse(quantile(DB, c(0.1)) < DB_limit, c <- c + step, c <- c)
	}

	LRV_10_3P <- log10(conc_10_3P) - log10(c-step)
	LRV_10_UB <- log10(conc_10_UB) - log10(c-step)
```

```
        print(LRV_10_3P)
        print(LRV_10_UB)
        print(c-step)
        test <- ((iter * step) - c)/step
        print(test)

#25th percentile
c <- 0
for(i in 1:iter){
        pill <- pinf(c) * pillinf(c)
        Pill_a <- 1 - (1 - pill)^n
        DB <- Pill_a * B * Sf
        ifelse(quantile(DB, c(0.25)) < DB_limit, c <- c + step, c <- c)
        }

        LRV_25_3P <- log10(conc_25_3P) - log10(c-step)
        LRV_25_UB <- log10(conc_25_UB) - log10(c-step)
        print(LRV_25_3P)
        print(LRV_25_UB)
        print(c-step)
        test <- ((iter * step) - c)/step
        print(test)

#Median
c <- 0
for(i in 1:iter){
        pill <- pinf(c) * pillinf(c)
        Pill_a <- 1 - (1 - pill)^n
        DB <- Pill_a * B * Sf
        ifelse(quantile(DB, c(0.5)) < DB_limit, c <- c + step, c <- c)
        }

        LRV_med_3P <- log10(conc_med_3P) - log10(c-step)
        LRV_med_UB <- log10(conc_med_UB) - log10(c-step)
        print(LRV_med_3P)
        print(LRV_med_UB)
        print(c-step)
        test <- ((iter * step) - c)/step
        print(test)

#75th percentile
c <- 0
for(i in 1:iter){
        pill <- pinf(c) * pillinf(c)
        Pill_a <- 1 - (1 - pill)^n
        DB <- Pill_a * B * Sf
        ifelse(quantile(DB, c(0.75)) < DB_limit, c <- c + step, c <- c)
        }

        LRV_75_3P <- log10(conc_75_3P) - log10(c-step)
        LRV_75_UB <- log10(conc_75_UB) - log10(c-step)
        print(LRV_75_3P)
        print(LRV_75_UB)
        print(c-step)
        test <- ((iter * step) - c)/step
        print(test)

#90th percentile
c <- 0
for(i in 1:iter){
        pill <- pinf(c) * pillinf(c)
        Pill_a <- 1 - (1 - pill)^n
        DB <- Pill_a * B * Sf
        ifelse(quantile(DB, c(0.9)) < DB_limit, c <- c + step, c <- c)
        }

        LRV_90_3P <- log10(conc_90_3P) - log10(c-step)
        LRV_90_UB <- log10(conc_90_UB) - log10(c-step)
        print(LRV_90_3P)
        print(LRV_90_UB)
        print(c-step)
```

```
        test <- ((iter * step) - c)/step
        print(test)

#97.5th percentile
c <- 0
for(i in 1:iter){
        pill <- pinf(c) * pillinf(c)
        Pill_a <- 1 - (1 - pill)^n
        DB <- Pill_a * B * Sf
        ifelse(quantile(DB, c(0.975)) < DB_limit, c <- c + step, c <- c)
        }

        LRV_98_3P <- log10(conc_98_3P) - log10(c-step)
        LRV_98_UB <- log10(conc_98_UB) - log10(c-step)
        print(LRV_98_3P)
        print(LRV_98_UB)
        print(c-step)
        test <- ((iter * step) - c)/step
        print(test)
```

B.10 Stokes' Equation and Settleable Particles in the WSP Systems

The terminal velocity of a spherical particle in a laminar fluid dynamics regime is described by Stokes' equation (Equation S6), where ρ_p and ρ_l are the respective densities of the particle and the liquid (assumed to be 1,038 kg/m^3 and 1,000 kg/m^3, respectively), g is the gravitational acceleration constant (9.81 m/s^2), d is the cross-sectional diameter of the particle (180 μm), and η_o is the liquid viscosity (assumed to be 0.001 kg/m·s). Making some assumptions about the viscosity and density of the wastewater treatment pond water and the density of wastewater flocs (Sears et al. 2006), the terminal settling velocity of a 180-μm floc particle would be approximately 2.4 m/hr. While flow conditions within a WSP may not be laminar, the Stokes' equation demonstrates that particles with average diameters >180 μm should be capable of settling in a pond with a depth of 1.5 m. This assumption is also supported by previous findings that report that wastewater flocs with cross-sectional diameters >180 μm would generally settle in a clarifier at a velocity of 0.5 mm/s or higher (Li and Ganczarczyk 1987).

$$v_s = \frac{(\rho_p - \rho_l) g d^2}{18 \eta_o} \qquad (B6)$$

B.11 Measurement of Total Solids, Volatile Solids, and Fixed Solids

Sludge core samples were collected in 2012, with a ½-inch PVC pipe, from the UASB reactor and from the facultative pond (near the influent pipe) after the second sampling event (same day as sludge samples that were analyzed for culturable enteroviruses). Sludge samples were sent in duplicate to the laboratory at the Centro de Aguas y Saneamiento Ambiental (CASA), at the Universidad Mayor de San Simón in Bolivia, to be analyzed for total solids, and volatile solids, using the *Standard Methods* (APHA et al. 2012). The results are in Table B4.

Table B1. RT-qPCR primers and probes utilized in this study.

Target Virus	Primer/Probe (5' to 3')	Amplicon size (bp)	Reference
Equine arteritis virus	CTTGTGCTCAATTTACTGG GGCAGAGGTACAGAATAG TxRed]TGCAGCTTATGTTCCTTGC[BHQ-2]	115	(Svraka et al. 2009)
Norovirus genotype I	CARGCCATGTTYCGYTGGATG CCTTAGACGCCATCATCATTTAC [FAM]TGGACAGGAGAYCGCRATCT[BHQ-1]	98	(Svraka et al. 2009)
Pepper mild mottle virus	GAGTGGTTTGACCTTAACGTTTGA TTGTCGGTTGCAATGCAAGT [FAM]CCTACCGAAGCAAATG[TAMRA]	68	(Rosario et al. 2009)
Rotavirus group A	ACCCTCTATGAGCACAATA GGTCACATAACGCCCCTA [FAM]CTAACACTGTCAAAAACCTAA[TAMRA]	73	(Svraka et al. 2009)

Table B2. The mean (n=3) and standard deviation virus concentration (copies/ml) with the minimum 2.5% credible interval (CI) and maximum 97.5% CI for a given triplicate sample collected throughout the three-pond (sites A-D) and UASB-pond (sites F-I) systems. +BLOQ represent samples that were positive but below the limit of quantification.

	Rotavirus group A (copies/ml)				Norovirus genotype I (copies/ml)				Pepper mild mottle virus (copies/ml)			
Site	**Mean**	**Standard Deviation**	**2.5% CI**	**97.5% CI**	**Mean**	**Standard Deviation**	**2.5% CI**	**97.5% CI**	**Mean**	**Standard Deviation**	**2.5% CI**	**97.5% CI**
A	1,260	407	760	1,932	2,039	229	1,386	3,076	96,684	2,562	79,601	117,738
B	4,535	1,115	3,097	6,515	2,064	219	1,333	2,944	405,227	112,982	255,222	633,750
C	1,925	829	847	2,890	1258	834	203	2,349	431,462	271,083	100,674	727,643
D	741	417	273	1,327	579	221	311	1,140	296,738	119,557	184,467	521,096
F	211	18	161	265	+BLOQ	n/a	n/a	n/a	52,387	7,407	37,577	70,294
G	204	36	153	289	73	22	37	144	144,369	59,396	64,109	214,743
H	+BLOQ	n/a	n/a	n/a	241	37	137	389	323,444	128,660	153,080	517,509
I	+BLOQ	n/a	n/a	n/a	407	92	215	687	375,331	65,393	280,490	539,409

Table B3. Mean virus concentration (copies/ml) with the minimum 2.5% credible interval (CI) and maximum 97.5% CI for a given triplicate sample collected for particle analysis. +BLOQ represents samples that were positive but below the limit of quantification. <LOD represents samples that were negative, whose concentration is likely less than that of the detection limit of the assay.

		Rotavirus group A (copies/ml)				Norovirus genotype I (copies/ml)				Pepper mild mottle virus (copies/ml)			
Site	Filter	Mean	Standard Deviation	2.5% CI	97.5% CI	Mean	Standard Deviation	2.5% CI	97.5% CI	Mean	Standard Deviation	2.5% CI	97.5% CI
B	0.45	777	696	226	1,807	182	114	54	441	22,196	12,213	6,642	35,999
B	180	197	185	28	468	+BLOQ	n/a	n/a	n/a	2,399	418	1,557	3,482
B	0.45+	1,647	2,296	19	4,874	111	133	4	376	84,379	77,718	1,992	186,331
B	0.45T	18,426	12,596	5,598	35,751	666	316	231	1,337	638,551	102,732	445,950	880,780
C	0.45	505	524	97	1,274	104	43	34	198	24,810	8,279	13,191	38,662
C	180	241	25	187	306	61	101	1	260	2,240	911	1,250	3,581
C	0.45+	659	238	418	961	23	25	1	81	29,101	24,945	1,247	59,705
C	0.45T	13,238	10,238	1,928	25,485	395	182	160	834	555,564	263,632	215,779	903,669
D	0.45	111	52	44	186	37	22	9	91	19,899	5,006	13,062	30,480
D	180	127	70	37	206	+BLOQ	n/a	n/a	n/a	1,449	843	471	2,871
D	0.45+	<LOD	n/a	n/a	n/a	+BLOQ	n/a	n/a	n/a	199	82	103	340
D	0.45T	5,594	4,519	1,199	11,776	259	114	85	497	509,185	77,710	356,459	687,076
G	0.45	30	24	12	70	14	5	5	31	226,854	75,022	139,594	368,865
G	180	<LOD	n/a	n/a	n/a	+BLOQ	n/a	n/a	n/a	9,201	4,189	5,494	16,916
G	0.45+	<LOD	n/a	n/a	n/a	+BLOQ	n/a	n/a	n/a	84,211	100,259	5,912	234,297
G	0.45T	43	n/a	35	53	26	12	10	55	318,742	68,488	228,490	437,389
H	0.45	28	n/a	22	34	20	12	4	50	40,638	21,448	20,715	77,319
H	180	<LOD	n/a	n/a	n/a	+BLOQ	n/a	n/a	n/a	1,739	1,145	794	3,800
H	0.45+	54	n/a	44	66	+BLOQ	n/a	n/a	n/a	807	857	148	1,782
H	0.45T	24	12	12	41	87	50	25	208	251,697	107,825	147,914	446,693
I	0.45	19	n/a	15	24	+BLOQ	n/a	n/a	n/a	20,148	14,315	3,873	39,447
I	180	<LOD	n/a	n/a	n/a	30	45	0	124	20,281	32,849	951	69,185
I	0.45+	<LOD	n/a	n/a	n/a	+BLOQ	n/a	n/a	n/a	1,294	n/a	1,012	1,633
I	0.45T	30	n/a	24	37	127	51	56	269	394,741	115,162	228,039	592,938

Table B4. Total solids, volatile solids, fixed solids, % volatile solids, and % fixed solids (mean +/- standard deviation) of the UASB reactor and the facultative pond sludge (n=2) from the UASB-pond and three-pond systems, respectively.

Site	Total Solids (g/L)	Volatile Solids (g/L)	Fixed Solids (g/L)	% Volatile Solids	% Fixed Solids
UASB reactor	285.2 +/- 7.3	210.2 +/- 5.7	75.1 +/- 1.7	73.7 +/- 0.1	26.3 +/- 0.1
Facultative pond	157.7 +/- 12.8	126.3 +/- 9.2	31.4 +/- 3.5	80.1 +/- 0.6	19.9 +/- 0.6

Figure B1. Modified can crusher used with sterile syringe and filter holder. Craig Carlson is acknowledged for the idea and design. Guy Grant is acknowledged for the modification of this can crusher.

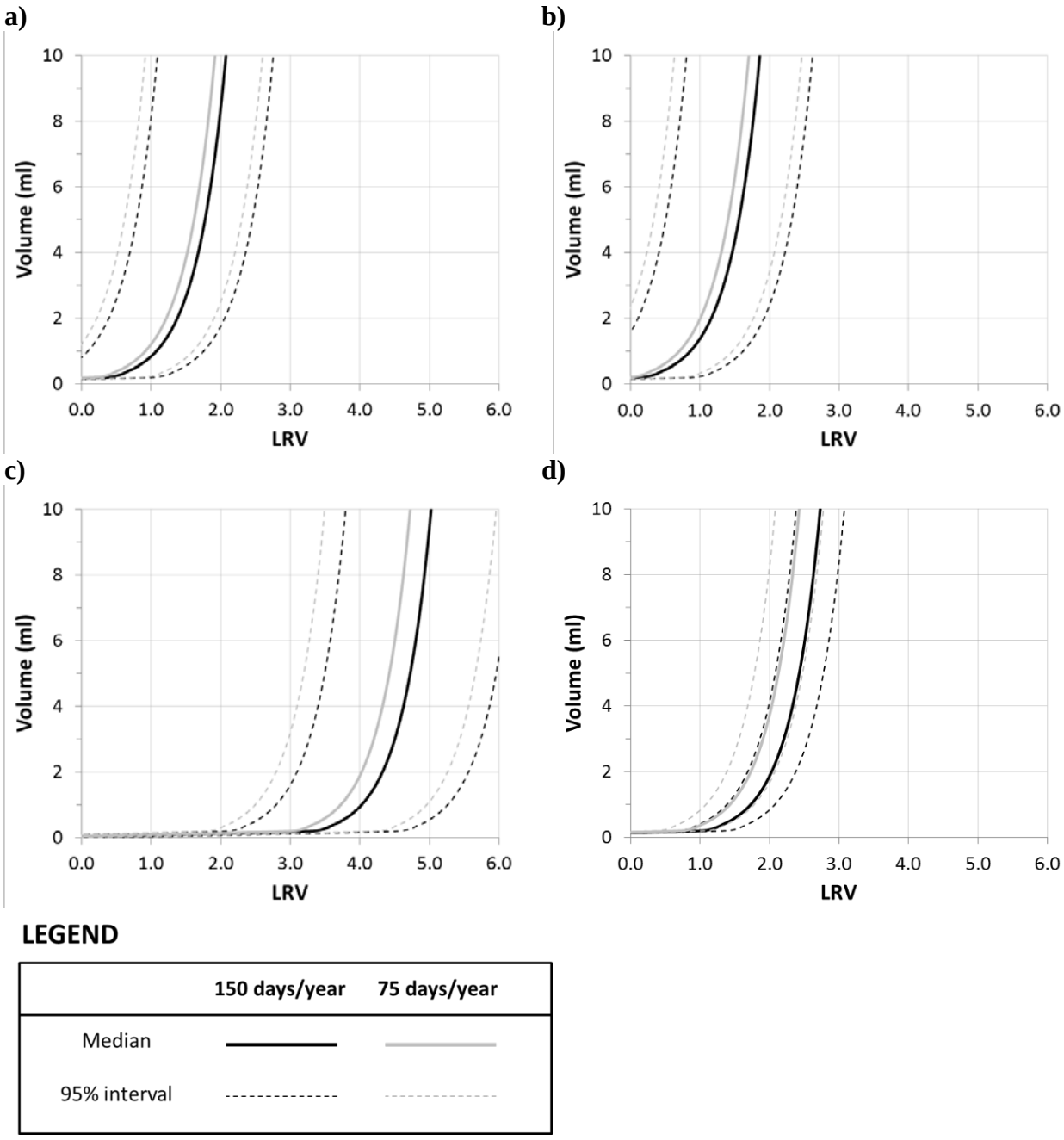

Figure B2. Analysis of the sensitivity of the QMRA model to the assumed volume of wastewater accidentally ingested (*V*) per day of exposure and the number of days of exposure per year (*n*), for norovirus in a) the three-pond system and b) the UASB-pond system, as well as rotavirus in c) the three-pond system and d) the UASB-pond system.

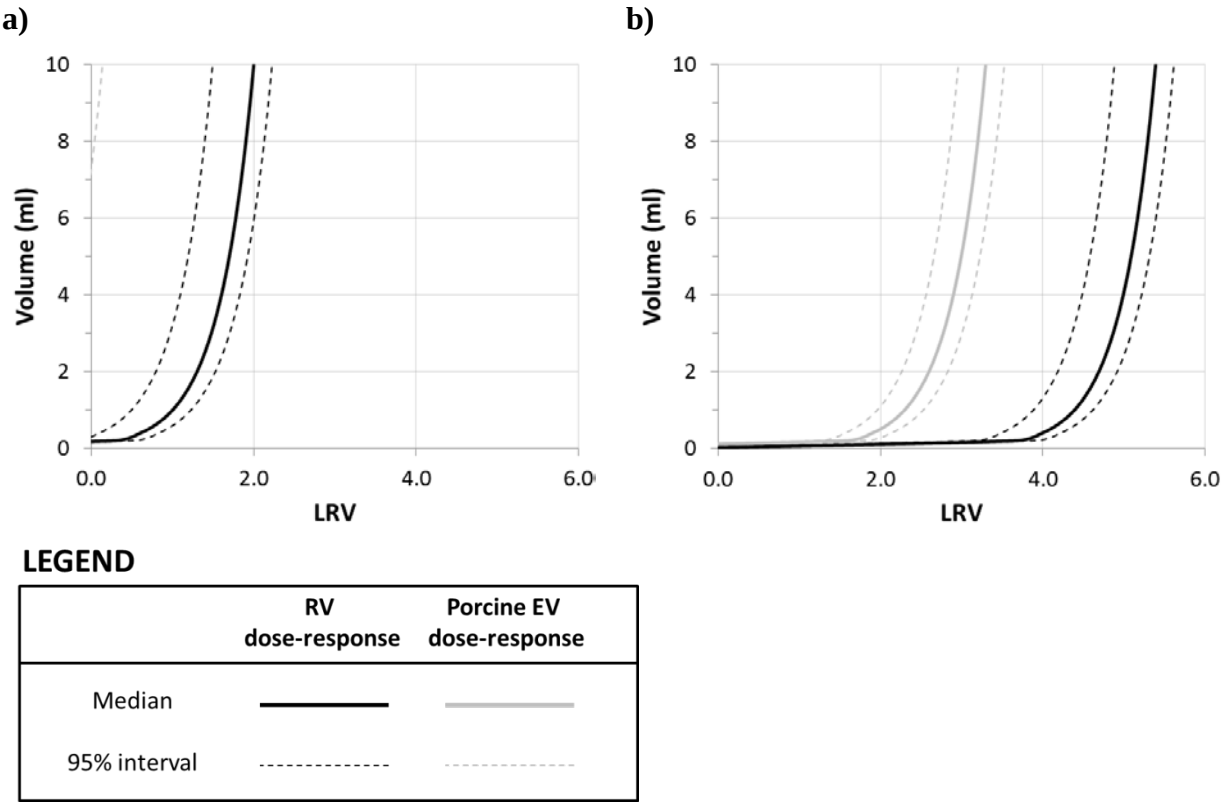

Figure B3. Analysis of the predicted log reduction value for culturable enteroviruses using the rotavirus exact Beta-Poisson dose-response model vs. the porcine enterovirus exponential dose-response model, for a) the three-pond system and b) the UASB-pond system.

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APPENDIX C: SUPPORTING INFORMATION FOR SECTION 4.3

C.1 Survival Function Used to Determine (RT-)qPCR Process Limits of Detection

The survival model is described by the following equation, which specifies the probability of amplification ($P_1(d)$ for a given mean number of target genome copies added to a reaction well (d) and assuming a "survival" probability r:

$$P_1(d) = 1 - e^{-rd} \quad (C1)$$

Using categorical data for each of the standard curve dilution series (the number of times amplification occurred ($X_{amp,q}$) in the q^{th} standard dilution series), and the total number of trials for the q^{th} standard dilution (N_s)), the Generalized Reduced Gradient (GRG2) Algorithm was used in Microsoft Excel to produce a locally-optimal solution for r that minimized the deviance Y (Haas et al. 2014), defined as

$$Y = -2\sum_{q=1}^{m} X_{amp,q} \ln\left(\frac{f_{ant,q}}{f_{obs,q}}\right) + \left(N_{s,q} - X_{amp,q}\right) \ln\left(\frac{1-f_{ant,q}}{1-f_{obs,q}}\right) \quad (C2)$$

where $f_{ant,q}$ is the anticipated probability of amplification ($f_{ant,q} = P_1(q)$), and f_{obs} is the observed fraction of amplification ($f_{obs,q} = X_{amp,q} / N_{s,q}$) for the q^{th} standard dilution.

C.2 Salmon Sperm DNA Extraction and Purification Control

140 ng of salmon sperm DNA was added to the samples from the Belding WSP system as a DNA extraction and purification control. Refer to Appendix E, Tables E2 and E3 for more information about the qPCR primers and probes used to detect the salmon sperm DNA as well as the qPCR chemistry and cycling conditions.

APPENDIX D: SUPPORTING INFORMATION FOR SECTION 5.1

D.1 Probability Plot Correlation Coefficient Test to Assess Conformity to Lognormal Distribution

Using the method specified by NIST/SEMATECH (2012), the log-transformed concentrations were plotted with the Normalized Z score corresponding with the probability of measuring a given concentration based on its rank within the data set, using the following equation:

$$Prob = (Rank - 0.375) / (N + 0.25) \quad (D1)$$

Data coming from a log-normal distribution should fall along a straight line (Figure D1). Pearson's correlation coefficients were calculated from the vectors of log-transformed concentrations and Z scores, and compared to a table of critical values for different-sized data sets (Table D1), using $\alpha = 0.05$.

Table D1. Critical values of Pearson's correlation coefficients based on the probability plot correlation coefficient test for normality (NIST/SEMATECH 2012)

N = number of data points	α = 0.01	α = 0.05
3	0.8687	0.879
4	0.8234	0.8666
5	0.824	0.8786
6	0.8351	0.888
7	0.8474	0.897
8	0.859	0.9043
9	0.8689	0.9115
10	0.8765	0.9173

D.2 References for Appendix D

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	E. coli			Coliphage			Giardia			Cryptosporidium			Helminth Eggs		
	Data	Z score (observed)	Expected	Data	Z score (observed)	Expected	Data	Z score (observed)	Expected	Data	Z score (observed)	Expected	Data	Z score (observed)	Expected
1	6.3	-1.36	6.6	4.9	-1.36	5.2	2.9	-1.43	3.0	1.1	-1.43	1.1	2.5	-1.43	2.4
2	7.2	-0.76	7.1	5.8	-0.76	5.6	3.3	-0.85	3.2	1.3	-0.85	1.3	2.5	-0.85	2.5
3	7.6	-0.35	7.4	5.9	-0.35	5.8	3.3	-0.47	3.2	1.4	-0.47	1.4	2.6	-0.47	2.6
4	7.7	0.00	7.7	6.1	0.00	6.1	3.4	-0.15	3.3	1.5	-0.15	1.5	2.6	-0.15	2.6
5	7.9	0.35	8.0	6.3	0.35	6.3	3.4	0.15	3.4	1.6	0.15	1.6	2.6	0.15	2.7
6	8.3	0.76	8.3	6.6	0.76	6.5	3.4	0.47	3.4	1.7	0.47	1.7	2.7	0.47	2.7
7	8.8	1.36	8.8	6.8	1.36	6.9	3.4	0.85	3.5	1.8	0.85	1.8	2.8	0.85	2.8
8							3.6	1.43	3.6	2.1	1.43	2.0	2.9	1.43	2.9
N	**7**			**7**			**8**			**8**			**8**		
Mean	**7.7**			**6.1**			**3.3**			**1.6**			**2.7**		
St Dev	**0.8**			**0.6**			**0.2**			**0.3**			**0.2**		
Pearson	**0.984**			**0.963**			**0.862**			**0.992**			**0.947**		
Signif.	**YES**			**YES**			**NO**			**YES**			**YES**		

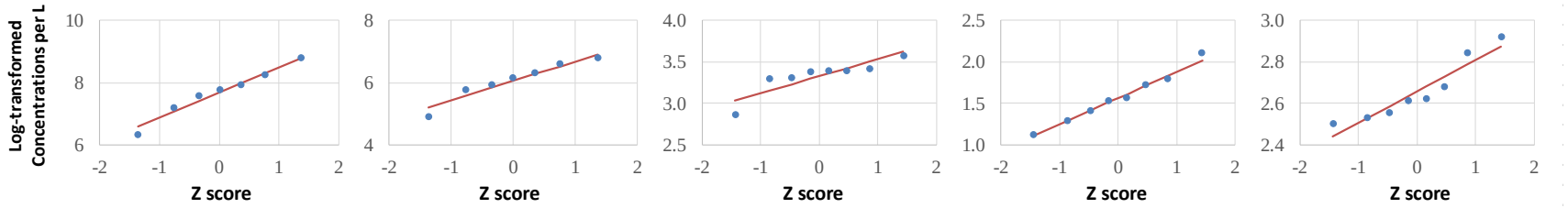


Figure D1. Probability plot correlation coefficient tests of pathogen and fecal indicator concentrations in wastewater from the Arani WSP system.

APPENDIX E: SUPPORTING INFORMATION FOR SECTION 5.2

E.1 Description of the Research Site

The Cochabamba Valley, located in the Andean mountain range, is the third most-populated urban region in Bolivia. The department of Cochabamba has the largest area of irrigated agriculture in Bolivia, and also leads the country in production of vegetable crops (119,000 metric tons per year), with 86% of this production resulting from irrigated agriculture.(Instituto Nacional de Estadistica 2008) However, Cochabamba only receives an average of 482 mm of rainfall per year, and is one of the most water-scarce urban regions in South America.(Instituto Nacional de Estadistica 2011) The Rocha River runs directly through the center of the lower Cochabamba valley, passing within 10 kilometers of more than one million people who live in this sprawling urban region. The flow rate in this river typically ranges from 0.03 to 0.12 $m^3\ s^{-1}$ during the month of June (which is the peak of the dry season and the irrigation season).(Medrano and Derpic 2006)

The Rocha River sediment contains 3% gravel, 3% clay, 16% silt, and 81% sand, with only 0.4% (±0.9%) organic matter; it has a conductivity of 889.5 $\mu S\ cm^{-1}$, a cationic exchange capacity of 5.64 ±0.52 cmol kg^{-1}, and a chloride concentration of 72.5 mg L^{-1}, and a pH of 8.0.(Tapia Vidal and Oporto Pereyra 2012) The well is recharged by the river and may also be recharged to a certain extent by ambient ground water, which can originate from bank infiltration at upstream sections of the river, excess irrigation water applied to nearby farm plots, or infiltrating precipitation. However, the recharge area south of the river consists of clays, sands, and igneous rock with low permeability and low recharge capacity.(Renner and Velasco 2000) Lettuce farmers in this region construct small rectangular plots (known locally as *melgas*) separated from each other by small earthen berms. They irrigate using border or basin irrigation, adding water to a trench running along one side of the plots, allowing furrows between crop rows to fill up sequentially with water (Figure E1).

E.2 Sampling Schedule

Samples were collected to accommodate local crop rotation patterns. Not all samples were analyzed for all parameters on each sampling occasion. Table E1 contains a summary of the complete sampling schedule. Water

samples from the Rocha River and the riverbank filtration (RBF) well were analyzed for *E. coli*, *Giardia*, and *Cryptosporidium* on four of the nine sampling occasions; and analyzed for coliphage on eight of the nine sampling occasions. All qPCR and RT-qPCR assays (for RV, AdV, PMMoV, and HF183) were performed on samples collected in replicate (5 to 10 replicates per sample) on a single sampling occasion in June 2013; additional samples were collected in November 2014, but not in replicate. Soil samples were only analyzed for coliphage, *Giardia*, and *Cryptosporidium* (qPCR and RT-qPCR methods were not used for soil samples). Lettuce samples were analyzed for coliphage, *Giardia*, and *Cryptosporidium* (two to three sampling occasions), and also for PMMoV and AdV (one sampling occasion with 15 replicates).

Figure E1. Typical rectangular plots separated by earthen berms and used for border/basin irrigation of crops in the Cochabamba Valley of Bolivia.

E.3 Modified Adsorption-Elution Method

The modified adsorption-elution method used to concentrate water samples for qPCR and RT-qPCR analysis of enteric viruses RV and AdV, and microbial source tracking targets PMMoV and HF183was the same one described in Section 4.2 of this dissertation. Briefly, water samples (50 mL for untreated river water, 1,000 mL for RBF system water), adjusted to pH 3.0 – 3.5 with 1 M acetic acid, were filtered onto 0.45-µm, 47-mm mixed cellulose ester filters (HAWP type, Millipore, Billerica, MA, USA). Filters were placed into bead-beating tubes (lysis matrix E, MP Biomedical, Solon, OH, USA). Unlike the adsorption-elution method described in Section 4.2, water samples for this study were filtered using a vacuum pump and filter manifold set-up. Stainless steel filter holders were rinsed with autoclaved distilled water between samples and disinfected via flaming with ethanol. RBF well samples were processed prior to the Rocha River samples, to minimize cross-contamination. Process blanks,

using autoclaved distilled water, were collected from each filter holder after all samples had been processed. Filters were mechanically disrupted with a Pewee Boxer Model 3115RS-12T-B20 bead beater (Biospec Products) for three min during the lysis step. Supernatants from the bead-beating tubes were transferred to clean tubes, then DNA and RNA were extracted and purified using the AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA, USA), eluting off the membranes with 50 µL of molecular grade water; RNA was immediately reverse-transcribed using the Superscript III First Strand Synthesis Kit with random hexamers (Invitrogen, Carlsbad, CA, USA).

E.4 Extraction, Purification and Reverse Transcription Controls

A synthetic equine arteritis virus (EAV) RNA oligonucleotide was used as an RNA purification and reverse-transcription control, as described in Section 4.2 of this dissertation. Salmon sperm DNA (Sigma-Aldrich, St. Louis, MO, USA) was used as a DNA extraction and purification control (US EPA 2010c). Each sample, as well as a calibrator sample containing molecular grade water, was spiked with 5×10^9 copies of a synthetic EAV RNA oligonucleotide and 140 ng of salmon sperm DNA, prior to bead-beating. Based on the recovery of these controls, water samples had RNA purification and reverse-transcription efficiencies of 28 – 79%, and DNA extraction and purification efficiencies of 30 – 46%. Based on these recoveries, it is likely that the reported virus concentrations under-represent actual concentrations.

DNA/RNA purification blanks and (RT-)qPCR no-template controls were all negative, and process controls showed negligible contamination. None of the extraction blank samples exhibited amplification with (RT-)qPCR, but the process control samples tested positive for RV and PMMoV, with concentrations below the pLOD of the assays. Fortunately, the samples were processed in order from lowest to highest virus concentrations to minimize cross-contamination (well water samples were filtered before Rocha River samples), and the process blanks were filtered at the end; thus, the observed contamination represents a worst-case contamination scenario.

Table E1. Water, soil, and lettuce sample collection dates for microbiological analyses.

Site	Parameter	Sample Collection Dates (total number of sample replicates)		
		Water Samples	**Soil Samples**	**Lettuce Samples**
Rocha River	*E. coli*	Mar., Oct. 2013 (4)	(0)	(0)
	Coliphage	Mar., Oct. 2013; Sep., Oct., Nov., Dec., 2014 (5)	Mar., Oct. 2013 (2)	Mar., Oct. 2013 (2)
	Giardia & Cryptosporidium	Mar., Oct. 2013 (4)	Mar., Oct. 2013 (2)	Mar., Oct. 2013 (2)
	Helminth eggs	Mar., Oct. 2013 (4)	Mar., Oct. 2013 (2)	Mar., Oct. 2013 (2)
	(RT-)qPCR targets[a]	June 2013 (2 – 5)	(0)	(0)
RBF system	*E. coli*	Mar., Oct. 2013 (3)	Mar., Oct. 2013 (3)	Mar., Oct. 2013 (3)
	Coliphage	Mar., Oct. 2013; Sep., Oct., Nov., Dec., 2014 (8)	Mar., Oct. 2013 (3)	Mar., Oct. 2013 (3)
	Giardia & Cryptosporidium	Mar., Oct. 2013 (3)	Mar., Oct. 2013 (3)	Mar., Oct. 2013 (3)
	Helminth eggs	Mar., Oct. 2013 (3)	Mar., Oct. 2013 (3)	Mar., Oct. 2013 (3)
	PMMoV (RT-qPCR); AdV (regular PCR)	June 2013 (10)	(0)	June 2013 (15)
	All other (RT-)qPCR targets[b]	June 2013 (5 – 10)	(0)	(0)

[a] RV, AdV (qPCR only), PMMoV, and HF183
[b] RV, AdV (qPCR only), and HF183 only

E.5 Quantitative Polymerase Chain Reaction (with Reverse Transcription) ((RT-)qPCR)

The DNA or cDNA samples were analyzed in duplicate. For EAV, PMMoV, AdV, and RV assays, a third replicate was either diluted 2:20 or spiked with 10^5 copies of custom-made pIDTSMART purified plasmids with ampicillin resistance (Integrated DNA Technologies, Coralville, IA, USA), to test for qPCR inhibition. All pIDTSMART purified plasmids contained the target sequence of the assay, which was identified from the following GenBank sequences: NC_002532.2 (EAV), AB550911.1 (PMMoV), L19443.1 (AdV), and HM627561.1 (RV). All samples were run alongside a six- or seven-point standard curve of plasmids, ranging from 10 - 10^7 plasmids per reaction for RV and EAV, from 100 – 10^8 plasmids per reaction for PMMoV, from 100 – 10^7 plasmids per reaction for AdV, and from 1 – 10^6 plasmids per reaction for HF183. Standard curve dilutions were run in duplicate or triplicate during each run and all of the data were pooled together at the end to produce a single standard curve for each assay. All no-template controls, which contained molecular grade water, were also analyzed in duplicate for each (RT-)qPCR analysis to ensure no cross-contamination. All standard curves met the recommended standards for qPCR, having R^2 values >0.97 and qPCR efficiencies between 90% and 110% (Bustin et al. 2009).

Up to four plates were analyzed per assay. Each 25 µL qPCR reaction (20 µL for the AdV assay) contained 2 µL or 5 µL volumes of sample DNA or cDNA, 1x Taqman Environmental Master Mix 2.0 (Life Technologies, Grand Island, NY), as well as primers, probes, and other reagents with the concentrations shown in Tables E2 and E3. The ABI7500 Real Time PCR system (Life Technologies, Grand Island, NY) was used with the cycling temperatures and conditions shown in Table E3. The 7500 software v2.0.6 (Life Technologies) was used, with the default settings, to determine the quantification cycle (Cq) values for each qPCR well. The results for the standard curves for each of the assays are presented in Table E4. The number of virus copies in unknown samples was determined by comparing the quantification cycle (Cq) value of the sample to the log-linear relationship between the Cq values and copy numbers of the standard dilution curve.

E.6 Determination of Process Limit of Detection (pLOD) for (RT-)qPCR

The pLOD for a (RT-)qPCR assay is defined as the lowest concentration at which amplification occurs before 40 cycles for 95% of positive sample replicates (i.e., the concentration for which <5% failed reactions occur). A very large number of sample replicates would be required to determine the concentration with this probability of amplification. Therefore, an exponential survival model was applied to the standard dilution series data, to

determine a theoretical pLOD that corresponds with 95% probability. The methods used for this model follow those described for QMRA dose-response models (Haas et al. 2014), as the two situations can be viewed as analogous. The process of qPCR amplification requires that a reaction must contain at least one copy (and not less) of the target genome sequence, and these genome targets may undergo decay, or a number of other factors may otherwise inhibit the qPCR; thus, only a fraction of the genome targets may successfully undergo the reaction and exhibit amplification. The survival model was constructed based on the following three assumptions: 1) DNA/cDNA/plasmids in samples are randomly distributed into reaction wells (i.e. the Poisson distribution can be assumed); 2) each DNA/cDNA/plasmid copy has an independent and identical probability of "surviving" the conditions to successfully complete the qPCR (i.e. the binomial distribution can be assumed); 3) at least one DNA/cDNA/plasmid copy has to be physically present in the qPCR in order to cause amplification within 40 cycles. Given these three assumptions, the probability of amplification is described by the following equation, where *j* represents the number of target genome copies actually present in a qPCR well, *d* represents the mean number of target genome copies added to each qPCR well (mean concentration multiplied by volume pipetted into the PCR well), *k* represents the number of genome copies that successfully "survive" to complete the qPCR, and *r* represents the probability of "survival":

$$P_a(d) = \sum_{k=k_{min}}^{\infty} \sum_{j=k}^{\infty} P_1(j|d) P_2(k|j) \qquad (E1)$$

$$P_1(j|d) = \frac{d^j}{j!} e^{-d} \qquad (E2)$$

$$P_2(k|j) = \frac{j!}{k!(j-k)!} (1-r)^{j-k} r^k \qquad (E3)$$

As demonstrated previously (Haas et al. 2014), assuming that k_{min} = 1, Equations E1, E2, and E3 simplify to give the following exponential model, which specifies the probability of amplification, given the mean number of target genome copies added to a reaction well and assuming a "survival" probability *r* (which is assumed to be unique for each (RT-)qPCR assay):

$$P_a(d) = 1 - e^{-rd} \quad (E4)$$

The pLOD is the number of target genome copies (d) that correspond with a 95% probability of amplification ($P_a(d)$). Using categorical data for each of the standard curve dilution series (the number of times amplification occurred ($X_{amp,q}$) in the q^{th} standard dilution series), and the total number of trials for the q^{th} standard dilution (N_s)), the r value producing the minimum deviance (Y) is found using a log-likelihood ratio (Haas et al. 2014), where $f_{ant,q}$ is the anticipated probability of amplification ($f_{ant,q} = P_1(q)$), and f_{obs} is the observed fraction of amplification ($f_{obs,q} = X_{amp,q} / N_{s,q}$) for the q^{th} standard dilution:

$$Y = -2\sum_{q=1}^{m} X_{amp,q} \ln\left(\frac{f_{ant,q}}{f_{obs,q}}\right) + \left(N_{s,q} - X_{amp,q}\right) \ln\left(\frac{1-f_{ant,q}}{1-f_{obs,q}}\right) \quad (E5)$$

If the measured fluorescence for a sample did not pass the (RT-)qPCR threshold value before 40 cycles for any of the sample replicates, the sample was labeled as "no amplification (NA)", and the concentration was considered to be below the limit of detection (<LOD).

E.7 Estimation of Concentration Distribution for Samples with >80% Censored Values

Log-normal distributions were assumed for the concentration of all pathogens for use in the QMRA. With the exception of RV and AdV, samples contained <80% censored values, and the robust regression-on-order statistics (ROS) method was used to specify the mean and standard deviations and define the log-normal distributions (Helsel 2012). However, for RV, >80% of the sample replicates were censored. It was assumed that the 95th percentile of the log-normal distribution was equal to the pLOD, and the survival function (Equation E4) was used to determine the median value of the log-normal distribution of the RV concentration in the RBF well (i.e., the proportion of positive samples to total samples was substituted in place of $P_a(d)$, and the equation was solved for d). With those two assumptions of the median and 95th percentile, the log-normal distribution was completely defined.

AdV was not detected at all in water from the RBF well using qPCR, but it was detected in 2 out of 5 sample replicates using the regular nested PCR assay. The most probable number method was used to estimate the median value of the log-normal distribution for AdV:

$$MPN = \frac{\ln\left(1 - \frac{X_{amp}}{N}\right)}{-V} \quad (E6)$$

where X_{amp} is the number of sample replicates detected positive via nested PCR, N is the total number of sample replicates analyzed using the nested PCR assay, and V is the effective volume of the sample analyzed (calculated as the inverse of the nested PCR limit of detection). It was assumed that the mean of the log-normal distribution was equal to the natural logarithm of *MPN*, and that the 95th percentile of the log-normal distribution was equal to the AdV qPCR pLOD. With those assumptions, the distribution was completely defined.

E.8 *Giardia* and *Cryptosporidium* Analysis

Giardia cysts and *Cryptosporidium* oocysts were quantified in water, soil, and crop samples using a standard method (US EPA 2005), with the following modifications. For water samples, 5 to 10 L were concentrated to 10 mL by centrifugation in 50 mL centrifuge tubes at 1,100×*g* for 15 min. The supernatant was discarded and the pellet was resuspended in 10 mL of sterile DI water. Resuspended pellets from multiple centrifuge tubes were combined into a single centrifuge tube and the process was repeated (centrifugation, discarding of supernatant, resuspension of pellet), until only 10 mL remained. For soil samples, 25 g of soil were mixed with 100 mL of Tris-Tween buffer (TTB; 50 mM Tris with 0.5% v/v Tween 80) for 15 min, then passed through Whatman Grade 1 filter paper (Oberon SRL, La Paz, Bolivia). The filter was washed three times with 200 mL of TTB. For lettuce samples, 25 g were mixed with 200 mL of TTB in a blender (low setting) for 2 min. This mixture was filtered over a 100-mesh sieve (149 µm pore size) and washed with 200 mL of TTB. TTB filtrate from lettuce and soil samples was centrifuged at 1,100×g for 15 min to concentrate to 10 mL. (Oo)cysts were then further concentrated using immunomagnetic separation, and stained with fluorescent antibodies for subsequent microscopic examination.

Table E2. (RT-)qPCR primers and probes.

Assay	Primers and Probes (5'-3')*	Amplicon size (bp)	Reference
Equine arteritis virus (EAV)	F: CTTGTGCTCAATTTACTGG R: GGCAGAGGTACAGAATAG [TxRed]TGCAGCTTATGTTCCTTGC[BHQ-2]	115	(Svraka et al. 2009)
Pepper mild mottle virus (PMMoV)	F: GAGTGGTTTGACCTTAACGTTTGA R: TTGTCGGTTGCAATGCAAGT [FAM]CCTACCGAAGCAAATG[TAMRA]	68	(Rosario et al. 2009)
Human rotavirus group A (RV)	F: ACCCTCTATGAGCACAATA R: GGTCACATAACGCCCCTA [FAM]CTAACACTGTCAAAAACCTAA[TAMRA]	73	(Svraka et al. 2009)
Human adenovirus (AdV)	F: GGACGCCTCGGAGTACCTGAG R: ACIGTGGGGTTTCTGAACTTGTT [FAM]CTGGTGCAGTTCGCCCGTGCCA[MGBNFQ]	96	(Jothikumar et al. 2005; Staley et al. 2012b)
Salmon sperm DNA	F: GGTTTCCGCAGCTGGG R: CCGAGCCGTCCTGGTC [FAM]AGTCGCAGGCGGCCACCGT[TAMRA]	77	(Haugland et al. 2005; US EPA 2010c)
HF183 *Bacteroides* 16S rRNA genetic marker	F: ATCATGAGTTCACATGTCCG R: TACCCCGCCTACTATCTAATG [FAM]TTAAAGGTATTTTCCGGTAGACGATGG[TAMRA]	84	(Staley et al. 2012a)

* F = forward; R = reverse;
TxRed = texas red; BHQ = black hole quencher; FAM = 6-Carboxyfluorescein; TAMRA = tetramethylrhodamine;
MGBNFQ = minor groove binder non-fluorescent quencher (Life Technologies, Grand Island, NY, USA)

Table E3. Quantitative polymerase chain reaction chemistry[a] and cycling conditions.

Assay	Primer Concentrations	Probe Concentrations	Cycling Conditions
Equine arteritis virus (EAV)	500 nM each	250 nM	95°C for 10 min, then 40X: (95°C for 10 s, 52°C for 20 s, 60°C for 1 min)
Pepper mild mottle virus (PMMoV)	400 nM each	125 nM	95°C for 10 min, then 40X: (95°C for 30 s, 53°C for 1 min, 60°C for 1 min)
Human rotavirus group A (RV)	500 nM each	250 nM	95°C for 10 min, then 40X: (95°C for 10 s, 43°C for 20 s, 60°C for 1 min)
Human adenovirus (AdV) [b]	250 nM each	150 nM	95°C for 10 min, then 40X: (95°C for 15 s, 55°C for 20 s, 60°C for 1 min)
Salmon sperm DNA (Sketa22) [c]	1,000 nM each	80 nM	95°C for 10 min, then 40X: (95°C for 15 s, 60°C for 2 min)
HF183 *Bacteroides* 16S rRNA genetic marker [c]	1,000 nM each	80 nM	50°C for 2 min, 95°C for 10 min, then 40X: (95°C for 15 s, 60°C for 1 min)

[a] Taqman Environmental Master Mix 2.0 (Life Technologies, Grand Island, NY, USA) was added to each reaction at a final concentration of 1x. The total reaction volume was 25 µL, unless otherwise noted. The reaction chemistry consisted of Taqman Environmental Master Mix 2.0, primers and probe (at concentrations specified above), template DNA/cDNA (2 µL or 5 µL), and adjusted to the final volume with molecular grade water.

[b] Total reaction volume for this assay was 20 µL.

[c] Bovine serum albumin (BSA) was added to the reaction mix to a final concentration of 0.2 mg mL^{-1} for these assays

Table E4. (RT-)qPCR standard curve summary data.

Assay	Copies	Average Cq Value (*Stand. Dev.*)	Number of Replicates that Amplified	Total Number of Replicates
Pepper mild mottle virus (PMMoV)	10,000,000	16.9 (*0.9*)	7	7
	1,000,000	20.1 (*0.9*)	7	7
	100,000	23.7 (*1.0*)	7	7
	10,000*	26.8 (*0.9*)	7	7
	1,000	30.6 (*1.5*)	7	7
	100	34.0 (*1.2*)	7	7
	10	38.3 (*0.2*)	2	3
Human rotavirus group A (RV)	10,000,000	17.0 (*0.4*)	8	8
	1,000,000	20.7 (*0.3*)	8	8
	100,000	24.2 (*0.4*)	8	8
	10,000	27.4 (*0.5*)	7	7
	1,000*	31.6 (*1.0*)	8	8
	100	35.2 (*1.1*)	8	8
	10	37.7 (*1.0*)	4	8
Human adenovirus (AdV)	10,000,000	22.4 (*0.3*)	4	4
	1,000,000	25.9 (*0.5*)	6	6
	100,000	30.1 (*0.3*)	6	6
	10,000*	33.7 (*0.3*)	5	5
	1,000	36.5 (*1.1*)	2	6
	100	38.4 (*1.2*)	2	6
HF183 *Bacteroides* 16S rRNA genetic marker	1,000,000	17.3 (*0.1*)	3	3
	100,000	21.6 (*0.1*)	2	2
	10,000	24.6 (*0.1*)	3	3
	1,000	28.1 (*0.0*)	3	3
	100	29.8 (*0.1*)	3	3
	10*	34.1 (*0.1*)	3	3
	1	38.2 (*0.0*)	2	3

* Limits of quantification (LOQ)

E.9 Markov Chain Monte Carlo

OpenBUGS (v3.2.2, rev. 1063) was used to generate initial values and sample from the joint posterior density function using Markov Chain Monte Carlo (MCMC) with one chain. The OpenBUGS software conducts the Bayesian analysis using the adaptive Metropolis block updating algorithm for estimating dose-response model parameters (Schmidt et al. 2013). The OpenBUGS code used for *Rotavirus* is provided below. The box plots of the dose-response model parameters and history plots from MCMC simulations are shown in Figures E4 and E5.

E.10 Sample OpenBUGS Code for *Rotavirus* QMRA Model

```
model
       {
### Calculate a and b using the dose response data
       # p[i] = probability of infection due to integer dose d[i].
       # Beta-binomial probability of infection
       # a = alpha (shape) parameter of beta distribution describing variation in host-pathogen
       # b = beta (shape) parameter of beta distribution describing variation in host-pathogen
       # lambda = mean # of pathogens (mean dose) consumed by ith subject in Ward et al (1986)
       # X = infection indicator (Ward et al 1986)
              loga ~ dunif(-6, 6)
              logb ~ dunif(-6,6)
              a<-pow(10,loga)
              b<-pow(10,logb)
                     for(i in 1 : n) {
                            d[i]~dpois(lambda[i])
                            p[i]<- 1-exp(loggam(a+b)+loggam(d[i]+b)-loggam(b)-loggam(d[i]+a+b))
                            X[i]~dbin(p[i],N[i])
                            }

### Obtain the actual dose(dt) ingested by an individual, and calculate the probability of
infection/illness
       # dt[i] = dose for the ith subject consuming lettuce in Cochabamba
       # W = weight of lettuce consumed by Bolivians per person per day (grams) (from surveys)
       # C = concentration of pathogens in irrigation water (organisms / L)
       # H = harmonization factor
       # V = volume of irrigated water caught by lettuce (mL/gram); 0.001 is to convert mL to L
       # k = in-field decay rate (1/days)
       # t = time between last irrigation and lettuce harvested (days)
       # pinf = daily probability of infection
       # Xinf = infection indicator variable for QMRA
       # pill = daily probability of illness
       # pillinf = conditional probability of illness given infection

          for(i in 1:m){
              dtw[i] <-W[i]*Cw*H*V*0.001*exp(-k*t)
              pinfw[i] <- 1-exp(loggam(a+b)+loggam(dtw[i]+b)-loggam(b)-loggam(dtw[i]+a+b))
              Xinfw[i] ~ dbern(pinfw[i])
              pillw[i] <- pillinf*pinfw[i]

              dtr[i] <-W[i]*Cr*H*V*0.001*exp(-k*t)
              pinfr[i] <- 1-exp(loggam(a+b)+loggam(dtr[i]+b)-loggam(b)-loggam(dtr[i]+a+b))
              Xinfr[i] ~ dbern(pinfr[i])
              pillr[i] <- pillinf*pinfr[i]
              }
                     pillinf ~ dunif(0.35,0.9)
                     Cw ~ dlnorm(4.484,0.63)
                     Cr ~ dlnorm(15.24,3.69)
                     H ~ dunif(0.000526, 0.001)
                     V1 ~ dlnorm(-4.57,2)
                     V <- V1 + 0.006
                     k ~ dnorm(1.07, 14.29)T(0, 1000)
```

```
                        t ~ dunif(3, 4)

                        mean.pinfw <- mean(pinfw[])
                        mean.pinfr <- mean(pinfr[])

### Obtain the annual probability of illness and estimate the annual disease burden
        # annual.pill = annual probability of illness
        # days = number of days per year the subjects consume lettuce
        # annual.db = annual disease burden
        # B = disease burden per case of illness
        # Sf = susceptible fraction of the population
        # pr = percentage of the population that has natural resistence (over age of 5)
        # e = effectiveness of the vaccine
        # pv = percentage of the population that have received the vaccine
                for(g in 1:m){
                        annual.pillw[g] <- 1-pow((1-pillw[g]), days[g])
                        annual.dbw[g] <- annual.pillw[g]*B*Sf

                        annual.pillr[g] <- 1-pow((1-pillr[g]), days[g])
                        annual.dbr[g] <- annual.pillr[g]*B*Sf

                        cred.diff[g] <- annual.dbr[g] - annual.dbw[g]
                        percent.change[g] <- cred.diff[g] / annual.dbr[g]
                }

                Sf <- 1-(pr+(1-pr)*e*pv)
                B ~ dunif(0.015,0.026)
                pr <- 0.87
                e ~ dunif(0.54,0.79)
                pv <- 0.78

                mean.annual.pillw<-mean(annual.pillw[])
                mean.annual.dbw<-mean(annual.dbw[])
                mean.annual.pillr<-mean(annual.pillr[])
                mean.annual.dbr<-mean(annual.dbr[])

### Obtain the annual probability of illness and estimate the annual disease burden using WHO
assumptions and Bolivia data
                dtwho <- 100*Cw*H*V*0.001*exp(-k*t)
                pinfw.who <- 1-exp(loggam(a+b)+loggam(dtwho+b)-loggam(b)-loggam(dtwho+a+b))
                Xinfwho ~ dbern(pinfw.who)
                pillw.who <- pillinf*pinfw.who
                annual.pillw.who <- 1-pow((1-pillw.who), 150)
                annual.dbw.who <- annual.pillw.who*B*Sf

                dtbol <-  34*Cw*H*V*0.001*exp(-k*t)
                pinfw.bol <- 1-exp(loggam(a+b)+loggam(dtbol+b)-loggam(b)-loggam(dtbol+a+b))
                Xinfbol ~ dbern(pinfw.bol)
                pillw.bol <- pillinf*pinfw.bol
                annual.pillw.bol <- 1-pow((1-pillw.bol), 32)
                annual.dbw.bol <- annual.pillw.bol*B*Sf

                dtwhor <- 100*Cr*H*V*0.001*exp(-k*t)
                pinfr.who <- 1-exp(loggam(a+b)+loggam(dtwhor+b)-loggam(b)-loggam(dtwhor+a+b))
                Xinfwhor ~ dbern(pinfr.who)
                pillr.who <- pillinf*pinfr.who
                annual.pillr.who <- 1-pow((1-pillr.who), 150)
                annual.dbr.who <- annual.pillr.who*B*Sf

                dtbolr <-  34*Cr*H*V*0.001*exp(-k*t)
                pinfr.bol <- 1-exp(loggam(a+b)+loggam(dtbolr+b)-loggam(b)-loggam(dtbolr+a+b))
                Xinfbolr ~ dbern(pinfr.bol)
                pillr.bol <- pillinf*pinfr.bol
                annual.pillr.bol <- 1-pow((1-pillr.bol), 32)
                annual.dbr.bol <- annual.pillr.bol*B*Sf
        }
Data
list(n=8, m=33)

#Dose response data
lambda[ ]      N[ ]    X[ ]
```

```
0.009   7       0
0.09    7       0
0.9     7       1
9       11      8
90      7       6
900     8       7
9000    7       5
90000   3       3
END;


#Lettuce consumption data
W[ ]    days[ ]
5.1     78
7.7     78
13.3    78
19.0    78
124.4   12
9.5     182
22.1    78
11.4    182
26.5    78
5.7     365
11.4    182
14.2    182
9.0     286
14.2    182
37.9    78
19.0    182
19.0    182
44.2    78
44.2    78
22.7    182
11.3    365
14.2    365
14.2    365
28.4    182
28.4    182
66.3    78
66.3    78
79.6    78
18.9    365
28.4    365
132.7   78
133.0   182
349.2   78
END;
```

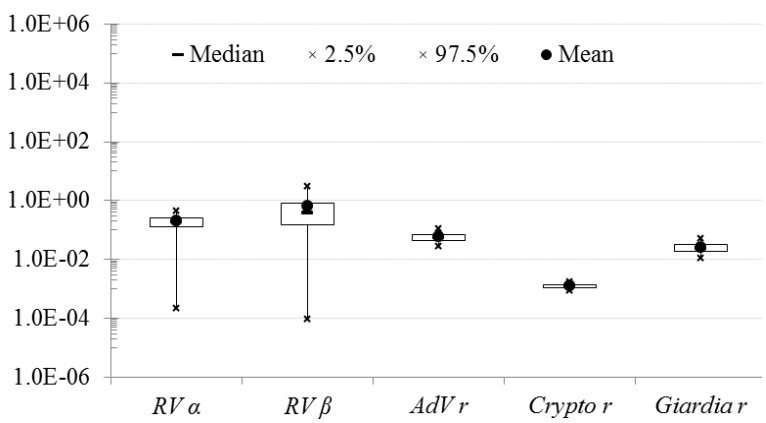
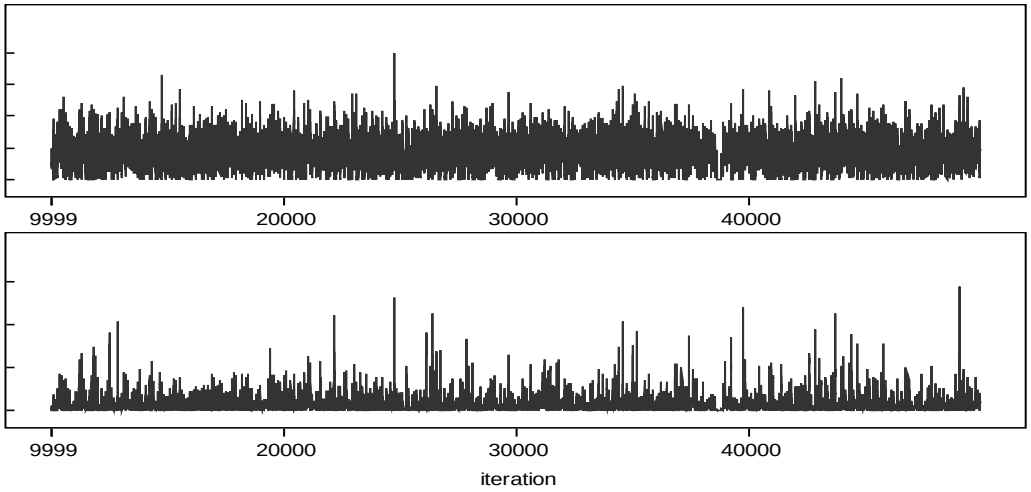
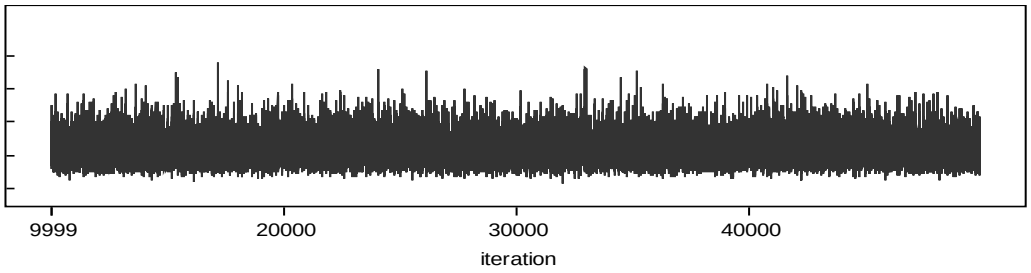


Figure E2. Credible intervals from MCMC for QMRA dose-response parameter estimates. (RV = rotavirus; AdV = adenovirus; Crypto = *Cryptosporidium*)

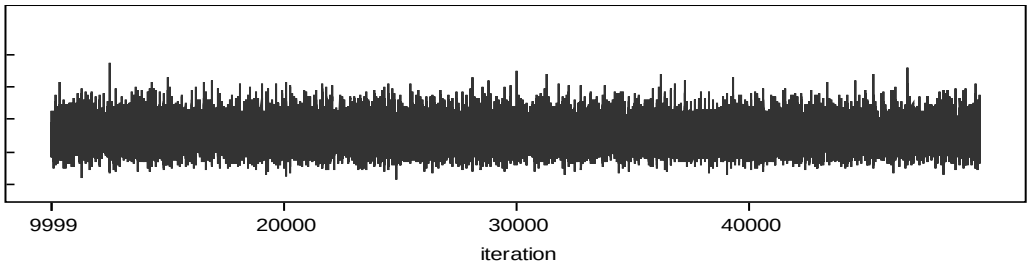
a)



b)



c)



d)

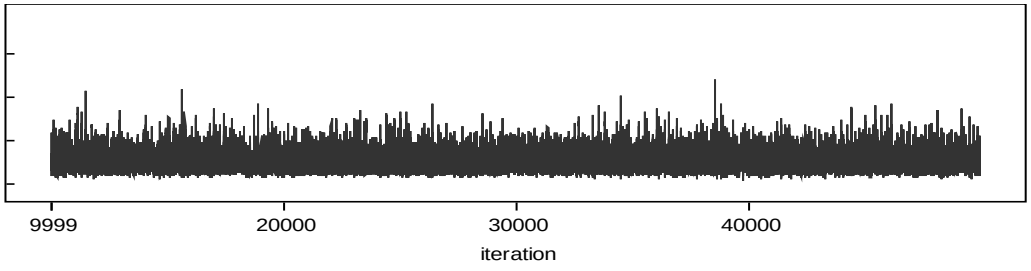


Figure E3. History for MCMC estimates of dose-response parameters for a) rotavirus, b) adenovirus, c) *Cryptosporidium*, and d) *Giardia* (a = alpha, b = beta)

E.11 Dose-Response Data

The dose-response data from clinical studies using human volunteers are presented in Table E5. A mixed set of dose-response data were used for AdV and *Cryptosporidium*. Currently, the only dose-response data available for AdV (Couch et al. 1966, 1969) are for respiratory infections caused by serotype 4, not for gastrointestinal illness caused by other serotypes of AdV transmitted via ingestion. Nevertheless, these data have been used by others to model the risk of gastrointestinal illness resulting from the accidental ingestion of adenoviruses in water or food (Chigor et al. 2014; Crabtree et al. 1997; Soller et al. 2010; Viau et al. 2011). However, AdV 4 may only represent fraction of the adenoviruses in water samples, and using dose-response data from AdV to model the probability of gastrointestinal infection via the oral route may overestimate the actual risks (Kundu et al. 2013). Some previous QMRA studies with AdV (Chen et al. 2012; Chigor et al. 2014; Crabtree et al. 1997; National Research Council 2012; Soller et al. 2010; US EPA 2010a; Viau et al. 2011) and *Cryptosporidium* (de Man et al. 2014; Ottoson and Stenström 2003; Teunis et al. 1996) have used the exponential dose-response model, with simple point estimates of ~0.4172 and ~0.004, respectively, for the parameter *r*. These are the best-fit values for only one set of dose-response data for each pathogen: AdV 4 (aerosolized) (Couch et al. 1966) and an Iowa strain of *Cryptosporidium parvum* (DuPont et al. 1995). In the present study, dose-response data from several different studies were used. There are advantages to using data from a combination of dose-response studies (Kundu et al. 2013), especially since dose-response relationships may be different for different isolates of the same pathogen (Teunis et al. 2002).

Table E5. Dose-response data used in QMRA model.

Pathogen	Assumptions	References
Rotavirus	λ = c(0.009,0.09,0.9,9,90,900,9000,90000) N = c(7,7,7,11,7,8,7,3); X = c(0,0,1,8,6,7,5,3)	(Ward et al. 1986)
Adenovirus	lambda = c(3,10,14,79,400,1,5,11,1000) N = c(6,2,2,3,3,3,3,3,6); X = c(0,0,1,2,3,1,3,3,6)	(Couch et al. 1966, 1969)
Giardia	lambda = c(1,10,25,100,10000,100000,300000,1000000) N = c(5,2,20,2,3,3,3,2); X = c(0,2,6,2,3,3,3,2)	(Rendtorff 1954)
Cryptosporidium	λ = c(10,30,100,500,30,100,300,500,1000,10000,100000, 1000000,100,300,1000,3000,500,1000,5000,10000) N = c(3,3,3,5,5,8,3,6,2,3,1,1,4,5,3,4,5,3,5,4); X = c(2,2,3,5,2,4,2,5,2,3,1,1,2,2,1,3,3,2,2,4)	(DuPont et al. 1995; Messner et al. 2001; Okhuysen et al. 2002)

E.12 Convenience-Sample Market Surveys

Convenience-sample surveys were administered by Maryann Cairns (Michigan Technological University) to 42 market-goers in August 2013. The following information was collected: gender, age, language(s) spoken at home, number of people living in the household, quantity of lettuce purchased, and frequency with which lettuce is consumed at home. Of the 42 market-goers surveyed, nine were omitted from further analysis due to lack of survey completion or because they reported commercial use of the lettuce purchased. Data from the remaining 33 surveys were used to estimate the average lettuce mass consumed in the household per person per year.

Half of the respondents reported using Spanish exclusively in the household; 38% reported speaking a mixture of Spanish and indigenous languages; the remaining 12% reported speaking foreign languages; this was not significantly different from census data ($\alpha = 0.05$, p-value = 0.281).

E.13 Mass of Lettuce Consumed in Bolivia

Because lettuce is often sold by the head, five heads of lettuce were weighed, and the average value was used in calculations to estimate lettuce consumption rates by market-goers. These heads of lettuce were chosen at random from several different market stalls and purchased during the market surveys. In the QMRA, lettuce consumption estimated for surveyed market-goers (Figure E4) was compared with average lettuce production rates per capital in Bolivia (Table E6). It can also be compared with lettuce consumption reported for other regions in the world (Table E7).

Table E6. Lettuce production and use in Bolivia (Instituto Nacional de Estadistica 2008).

Department	Population (thousands)	Annual Lettuce Production		Percent sold in the domestic market or consumed by farmers
		Total (metric tons)	Per Capita (kg)	
Chuquisaca	576	294	0.5	100%
La Paz	2,706	1,506	0.6	91%
Cochabamba	1,758	4,505	2.6	100%
Oruro	494	113	0.2	100%
Potosí	824	359	0.4	100%
Tarija	482	654	1.4	99%
Santa Cruz	2,655	3,368	1.3	92%
TOTAL	**9,495**	**10,799**	**1.1**	**96%**

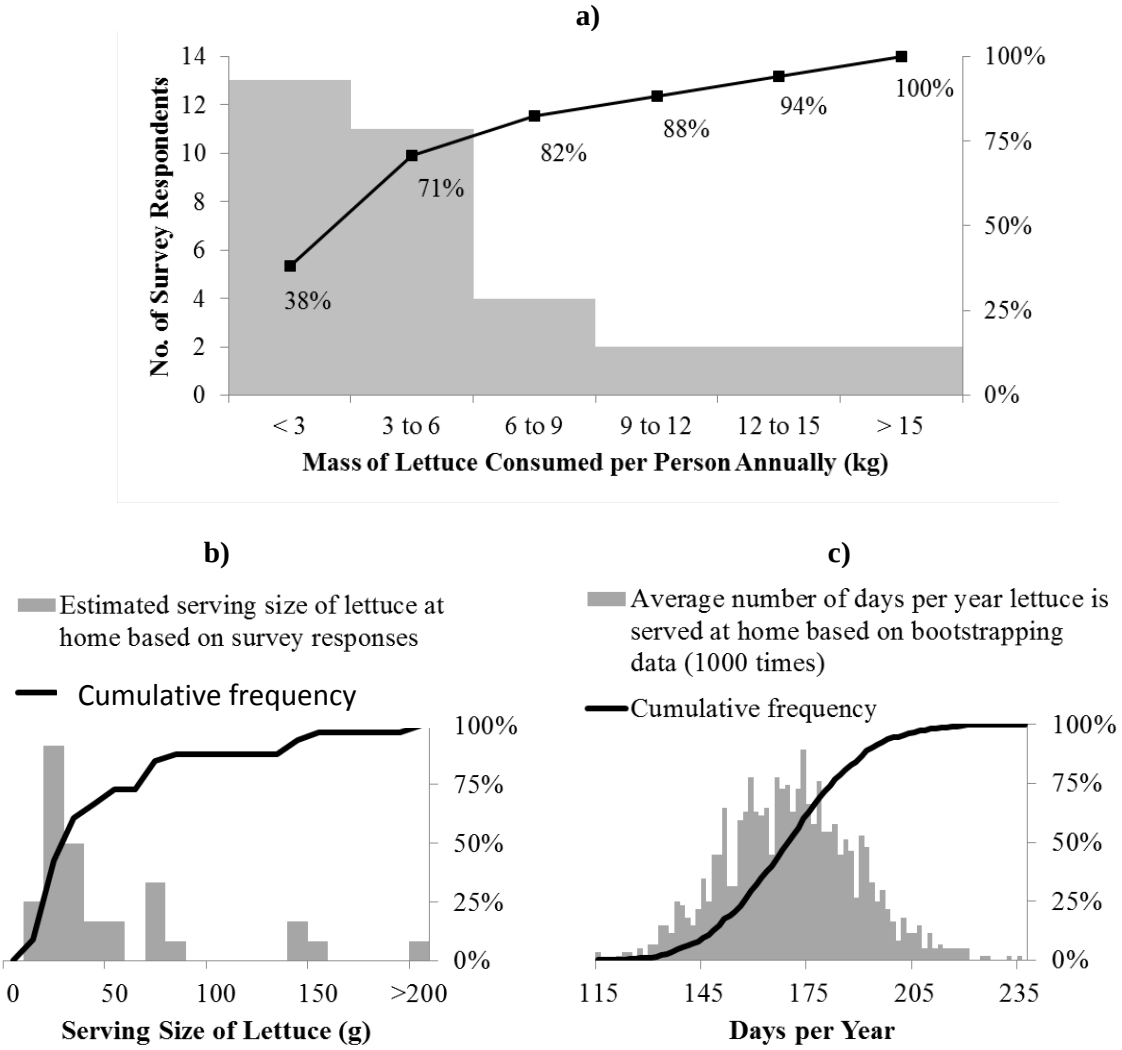


Figure E4. Distributions of a) the estimated amount of lettuce consumed per person per year in Cochabamba, b) the estimated serving size of lettuce at home based on survey responses, and c) the mean annual frequency with which lettuce is served at home, based on bootstrapping survey response data (1,000 times).

E.14 Quantitative Microbial Risk Assessment Sensitivity Analysis

To assess model sensitivity to input parameters, Spearman rank order correlation coefficients were calculated between model inputs and the model output (estimated disease burden in DALYs per person per year), using the 40,000 values simulated and recorded by MCMC. To evaluate the sensitivity of lettuce serving size, lettuce consumption frequency, and vaccine coverage rate, the former two parameters were input as uniform distributions using the minimum and maximum values estimated from the market survey data; and the vaccine coverage rate (f_v) was input as a uniform distribution between 0 and 1 (solely for the purposes of the sensitivity analysis).

Table E7. Assumptions about the mass of lettuce consumed and frequency of consumption from different geographic regions.

Average mass per person per year (kg)	Lettuce consumed (g person^{-1} day^{-1})	Frequency (days year^{-1})	Population	References
28.0	Lognormal(4.35,1.27) (median = 76.6)	365	China	(Mok and Hamilton 2014)
15.0	100	150	n/a (example from 2006 WHO Guidelines)	(Shuval et al. 1997; WHO 2006)
6.0	Mixture (mean = 23.8)	251	Melbourne, Australia	(Barker et al. 2013a)
4.4	Average = 20.7 ±26.4 (95% UCL = 74.2)	214	Representative sample of 3000 people in Spain	(Sales-Ortells et al. 2015)
3.4[a]	19[a] (sd = 65)	169 (sd = 110)	Market-goers in Cochabamba, Bolivia	data from this study
3.4	65 (overall)	52	People in the Mezquital Valley of Mexico	(Navarro and Jiménez 2011; Navarro et al. 2009)
2.5	6.8	365	Brazil	(Pavione et al. 2013)
2.3	Uniform(10, 12)	208	Accra, Ghana	(Seidu et al. 2008)
2.2	30 – 54	52	Children in the Mezquital Valley of Mexico	(Navarro and Jiménez 2011; Navarro et al. 2009)
1.1[b]	-	-	Bolivia (overall)	(Instituto Nacional de Estadistica 2008)

[a] median value; distribution appeared to be non-normal
[b] represents the mass of lettuce produced annually per capita in Bolivia for domestic consumption

E.15 References for Appendix E

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Chen, Z., Ngo, H. H., and Guo, W. S. (2012). "Risk Control in Recycled Water Schemes." *Critical Reviews in Environmental Science and Technology*, 43(22), 2439–2510.

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ABOUT THE AUTHOR

Matthew Verbyla's work addresses global sustainable development challenges at the intersection of food-energy-water systems and public health. He is interested in health-related water microbiology, wastewater resource recovery, and microbial risk assessment. Matthew has a Bachelor of Science in Civil Engineering from Lafayette College (Easton, PA, USA) and a Master of Science in Environmental Engineering from the University of South Florida (Tampa, FL, USA). He was awarded a Fulbright Fellowship in 2007 to study the sustainability of rural water service in Honduras under decentralization policies, and in 2012, he received a U.S. National Science Foundation Graduate Research Fellowship to pursue his Ph.D. in Environmental Engineering at the University of South Florida. In 2015, Matthew spent six months in Brazil with a USAID Research and Innovations Fellowship to develop a solar-powered sludge-drying system and assess the microbial risk of wastewater resource recovery in small cities. He passed the Fundamentals of Engineering (FE) examination in 2005, and has maintained LEED Green Associate credentials from the U.S. Green Building Council since 2011.