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Assessment of a Modified Double Agar Layer Method to Detect Bacteriophage for Assessing the Potential of Wastewater Reuse in Rural Bolivia

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Assessment of a Modified Double Agar Layer Method to Detect Bacteriophage for Assessing the Potential of Wastewater Reuse in Rural Bolivia

by

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DEDICATION

To God, the Creator of nature and its amazing wonders, for directing my steps toward His promises. To my mother for her love and support, my brother for being my motivation, to my family and friends for their encouragement, and to all the future generations who will someday inherit the responsibility of continuing the efforts to sustain our global environment.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Water scarcity is a global concern that impacts many developing countries, forcing people to depend on unclean water sources for domestic, agricultural, and industrial needs. Wastewater is an alternative water source that contains nutrients needed for crop growth. Wastewater reuse for agriculture can cause public health problems because of human exposure to pathogens. Pathogen monitoring is essential to evaluate the compliance of wastewater with established World Health Organization (WHO) and U.S. Environmental Protection Agency (EPA) wastewater reuse guidelines. Indicator organisms are commonly used to detect pathogens in water and wastewater because they are quick and easy to measure, non-pathogenic, and have simple and inexpensive methods of detection.

The objective of this research was to develop a modified double agar layer assay method that can be conducted in the field to quantify bacteriophage to assess the quality of wastewater for agricultural reuse. Results from the modified double agar layer assay were used to investigate the potential of somatic coliphage as an indicator organism for assessing the potential presence of enteric viruses in developing world treated wastewater, and to use the criteria of a good indicator organism to compare the potential of two commonly used

indicator organisms, somatic coliphage and fecal coliforms, as an indicator of enteric viruses in wastewater.

A modified EPA double agar layer method was developed and deployed in a developing world rural community to effectively quantify the concentration of somatic coliphage in a community managed wastewater treatment system composed of a Upflow Anaerobic Sludge Blanket (UASB) reactor followed by two maturation lagoons. The modified method served as a good indicator of enteric viruses in the water. Somatic coliphages were easily detected and quantified in the field setting using a modified double agar layer method. Somatic coliphage was found to be a potential indicator for enteric viruses rather than fecal coliforms because of their similarity in characteristics and resistances to wastewater treatment. The concentration of somatic coliphage was only reduced by 1.05 log units across the two series maturation lagoon system. Previous literature suggested removal would range from 2.1 to 4.6 log units. Influent wastewater (previously treated by an UASB reactor) had a concentration of 4.38 E+06 PFU/ 100 mL (standard deviation = ±3.7E+06, n = 9) and the treated effluent contained 3.90 E+05 PFU/100 mL (standard deviation = ± 4.5E+05, n = 8) of somatic coliphages. Results suggest that somatic coliphage is a good potential indicator for enteric viruses in wastewater but further research needs to be done.

CHAPTER 1: INTRODUCTION

Supporting a population of approximately 7 billion people, our global environment is under stress from improper disposal of wastes, urbanization and population growth, loss of ecological services, and rapid consumption of natural resources. Global leaders have come to realize the seriousness of the environmental crisis. For example, in efforts to meet eight identified goals directed towards combating global poverty, health concerns, and environmental degradation, the United Nations established the Millennium Development Goals (MGDs) in September 2000 (United Nations, 2012). Goal 7 of the MGDs is to ensure environmental sustainability. Target “c” of Goal 7 relates to improving access to safe drinking-water and sanitation. Collaboration between UNICEF and the World Health Organization (WHO) to establish the Joint Monitoring Program (JMP) has provided a way to monitor and report information on the global progress towards meeting the MGD related to provision of drinking -water and sanitation (UNICEF and World Health Organization, 2012).

According to the 2012 JMP Report, the goal for water provision has been met but the MDG for sanitation is unlikely to be met by the 2015 deadline (UNICEF and World Health Organization, 2012). Although it appears that the sanitation MDG will not be met, there were positive strides made in efforts to meet the goal because 1.8 billion people have gained access to improved

sanitation since the signing of the MGDs (UNICEF and World Health Organization, 2012). Despite the worldwide progress that has been made over the past decade, the global community still has a significant amount of work to do. This is because there are still 2.5 billion people lacking access to adequate sanitation facilities (UNICEF and World Health Organization, 2012).

Freshwater is one of the most valuable natural resources that is rapidly becoming scarce due to its limited availability and high demand. 3,800 km^3 of water are now being withdrawn every year in the world to meet domestic, industrial, and agricultural needs (Mihelcic and Zimmerman, 2010). The percentage of freshwater consumed by each sector is shown in Figure 1. Driven by increase in economic growth and population, freshwater consumption rates have more than doubled in the last century (UNWater, 2013). Freshwater scarcity is a reality that affects nearly 780 million people who are living without access to safe freshwater supplies (UNICEF and World Health Organization, 2012).

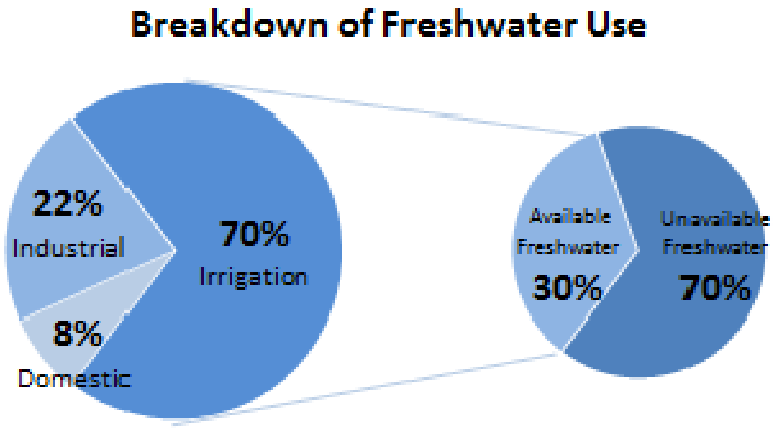

Figure 1: Percentage of the world's supply of freshwater (available and unavailable) used for domestic, industrial, and agricultural use
Source: UN Water (2012)

As shown in Figure 1, the agricultural sector is the leading consumer of water, which accounts for 70 % of total freshwater use. Food production is highly dependent upon water availability for crop irrigation, especially in arid or semi-arid regions where water supplies are limited. Forty percent of the world's food production comes from irrigated agriculture (UN Water, 2013). Irrigation provides farmers with higher crop yields thus increasing economic gain. While irrigation offers advantages for farmers, it may lead to negative environmental impacts, such as diminished soil quality, reduced biodiversity, and strained freshwater supplies (Jimenez, 2006). Freshwater scarcity and food security concerns have forced many farmers to explore alternative water sources for agricultural irrigation.

1.1 Wastewater as a Resource

Domestic wastewater is commonly used as a source of water for agricultural irrigation, particularly in developing countries where freshwater is scarce. Domestic wastewater contains mostly water but also human excreta which contains organic matter and nutrients (especially nitrogen and phosphorous). The water and nutrients in wastewater are two resources that are necessary for plant growth. For this reason, wastewater reuse is a possible resource for agricultural irrigation. Wastewater reuse has numerous advantages and disadvantages for agricultural irrigation, as described in Table 1. Note in the table that the benefits to water reuse outweigh the drawbacks.

Table 1: Advantages and disadvantages of wastewater as a source for agricultural irrigation

Advantages	Disadvantages
• Provides nutrients and organic matter to soil for needed for plant growth. • Increases crop yields. • Available year-round. • Decreases the cost and need for fertilizers. • Reduces the use of freshwater supplies. • Reduces cost of pumping (wastewater pumping is less expensive than groundwater pumping). • Can recharge aquifers through soil infiltration • Diverts pollutants from being discharged into surface water bodies.	• Increases concentration of salts and metals in the soil • Wastewater contains pathogenic organisms which increase human health risks in water reuse • Runoff of excess nutrients (nitrogen and phosphorus) into environment • Potential groundwater contamination • Leaches endocrine-disrupting chemicals into the environment • Pharmaceuticals infiltrate the soil and enter groundwater supplies

Source: Jimenez (2006), Salgot et al. (2006)

Reclaimed (treated) and raw (untreated) water are two forms of wastewater that are applied to crops through irrigation in developing world settings. The type of wastewater used depends on the economic state of the country and their ability to supply treatment facilities to the communities. Developed countries have regulatory policies in place and adequate funds to support construction and operation of wastewater treatment facilities which can provide some farmers access to efficiently treated wastewater for irrigation. On the other hand, only a small portion of the wastewater produced by developing countries is treated; thus untreated wastewater is the most accessible and widely used form of water used in irrigation in these nations (Mara, 2004). Globally, an

estimated 3 to 20 million hectares of land are currently irrigated with untreated wastewater (Dreschsel, 2002).

In general, wastewater has several advantages and disadvantages as a water source for crop irrigation; however, the type of wastewater used (treated vs. untreated) has different effects on the environment and human health. Untreated wastewater has a higher concentration of organic carbon and nutrients and thus has a higher strength than treated wastewater. As a result, irrigating with untreated wastewater may overload the environment with excess nutrients, such as phosphorus and nitrogen, which may cause eutrophication in surface water bodies. Also, untreated wastewater contains high quantities of various pathogens, such as bacteria, viruses, helminth eggs, and parasites, which cause disease and in some cases can be fatal. Nearly two million people die each year from waterborne diseases, the majority of whom are children under the age of five (Fry et al., 2005). To minimize environmental and human health risks, wastewater should be adequately treated and strict water quality standards should be established for wastewater reuse in crop irrigation.

In agriculture, there are two components of domestic wastewater that can be applied to crops, reclaimed water (for irrigation) and biosolids/sludge (as fertilizer) (Mara, 2004). Both components contain pathogenic microorganisms that present potential public health risks. The presence of pathogens in both treated and untreated wastewater may thus have serious health implications. Human exposure to pathogens by means of wastewater reuse in agricultural practice (e.g. irrigation and land application of biosolids) can lead to the

transmission of infectious diseases (Mara, 2004). Agricultural workers have the highest exposure risk, along with individuals who consume raw crops that are irrigated with wastewater (Jimenez, 2006). In developing countries, limited water resources and inadequate wastewater management systems can lead to increases in waterborne diseases due to the presence of disease-causing pathogens in the water and treated wastewater (Gantzer, 2001). Table 2 lists the most prevalent pathogenic microorganisms in wastewater and the diseases associated with them.

Table 2: Examples of pathogens associated with wastewater including their concentrations in wastewater and the disease associated with the pathogen

Class of Pathogens	Pathogenic Microorganism	Numbers in Wastewater (per liter)	Disease
Bacteria	*Salmonella typhi*	No data	Typhoid fever
	Samonella sp.	$1 - 10^5$	Gastroenteritis
	Shigella sp.	$10 - 10^4$	Shingellosis, bacillary, dysentery
	Vibrio cholera	$10^2 - 10^5$	Cholera
	Escherichia coli	No data	Gastroenteritis
	Campylobacter jejuni	$10 - 10^4$	Gastroenteritis
Viruses	Hepatitis A	No data	Infectious hepatitis
	Poliovirus	No data	Poliomyelitis
	Echovirus	$10^5 – 10^6$	Enteric infection
	Coxsackievirus	$10^5 – 10^6$	Enteric infection
	Adenovirus	No data	Conjunctivitis
	Rotavirus	$10^2 - 10^5$	Gastroenteritis
	Norwalk virus	No data	Gastroenteritis
Helminth Eggs	*Entamoeba hystolytica*	No data	Amoebic dysentery
	Giardia lamblia	No data	Giardiasis
	Cryptosporidium parvum	No data	Cryptosporidiosis
	Tanaea sp.	No data	Tape worm infection
	Ascaris lumbercoides	$1 - 10^3$	Round worm infection
	Trichuris trichuria	$1 - 10^2$	Trichuriasis
	Necator americanus	$1 - 10^3$	Hookworm infection

Source: World Health Organization (2006)

The presence of pathogenic organisms in wastewater and the public health risks they present have led to the establishment of wastewater reuse guidelines. The guidelines serve as regulatory “limits related to specific practices which will minimize detrimental effects without affecting the benefits” (Hespanhol and Prost, 1993). In efforts to minimize health risks associated with wastewater reuse, the World Health Organization (WHO) developed international water reuse guidelines with a health based target of 10^{-6} DALYs per person per year (World Health Organization, 2006). Disability Adjusted Life Years (DALYs) is the measure of years lost by a population of people due to the burden of disease or a specific risk factor (WHO, 2006). The WHO guidelines are meant to be a baseline standard that can be adapted the country’s national priorities based on the country’s socio-economic, cultural, geographical, and technological aspects (Hespanhol and Prost, 1993). National guidelines can be implemented and enforced by the national government to ensure that companies comply with the set removal standards to protect public health with wastewater reuse. In the United States, the Environmental Protection Agency (EPA) set specific standards for agricultural wastewater reuse according to the WHO international guidelines. The EPA wastewater reuse requirements are different for each state. Table 3 shows the WHO international guidelines and EPA national guidelines for wastewater reuse pertaining to irrigation practices. It is important to note the differences between the two guidelines: WHO guidelines are indicated by log unit reduction while EPA guidelines are specified by fecal coliform counts. Fecal coliforms are bacterial organisms, such as *E. coli*, that are commonly used as

indicators of the presence of fecal contamination in water quality monitoring (Salgot et al., 2006). The guidelines become more specific when adapting the international guidelines to create national and state standards.

Target levels for pathogen reduction established by wastewater reuse guidelines can be achieved through wastewater treatment. The efficiency of pathogen removal during wastewater treatment can be measured by comparing the pathogen concentrations in pre-treatment (influent wastewater) and post-treatment (effluent wastewater). Understanding the fate of pathogens in wastewater treatment is thus important if we are to minimize the health risks associated with using reclaimed wastewater as a resource. The detection of pathogens in water is important for assessing water quality, especially in developing countries where water quality is low.

Table 3: EPA and WHO agricultural wastewater reuse guidelines

WHO Guidelines	EPA Guidelines
Unrestricted Irrigation (Food Crops): 6 – 7 log unit reduction **Restricted Irrigation:** 3 – 4 log unit reduction **Localized (Drip) Irrigation:** 2 – 4 log unit reduction	**Urban Reuse (irrigation of landscape):** No detectable fecal coliform/ 100mL **Agricultural Reuse for Non-Food Crops:** $\leq$ 200 fecal coliforms/ 100mL **Agricultural Reuse for Food Crops:** $\leq$ 200 fecal coliforms/ 100mL

Sources: EPA (2004), EPA (2004), WHO (2006)

The health risks associated with the use of reclaimed water can be quantified by analyzing the organisms present in the water; however some pathogens are currently more difficult to measure in wastewater. The methods for

analyzing microbial concentrations can be costly and require a high level of expertise. Accordingly, indicator organisms have been identified, that have similar characteristics to specific pathogens of concern but are easier to quantify. A good indicator organism is qualified as having a high concentration in fecal waste, easy to detect, and easy to identify. The presence of indicator organisms in the water supply thus suggests the existence of pathogens and a higher level of risk if humans are exposed to the indicator organism. Figure 2 lists the attributes of good indicator organisms.

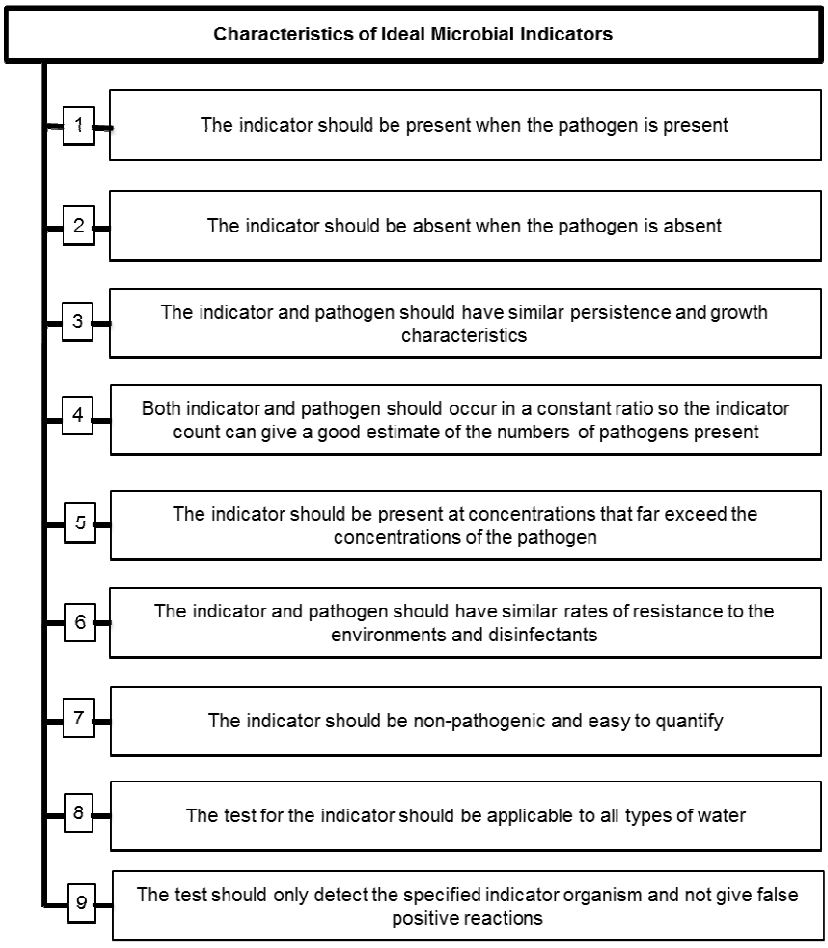

Figure 2: Characteristics of good indicator organism
Sources: Stetler (1984)

Numerous indicator organisms have been identified that exist in wastewater but the detection of a particular indicator organism does not suggest the presence of all pathogens. That is, as shown in Table 4, certain indicators are associated with specific pathogens based on their similar characteristics and resistances to treatment. The most commonly used indicator organisms used in assessing microbial water quality have been bacteria, such as *E.coli* or fecal coliforms. While very useful in assessing water quality, their large size (0.1 to 1 µm) compared to viruses (20 to 300 nm) suggest they may not be appropriate indicators for the health risks associated with the many viruses that are found in wastewater (Gerardi and Zimmerman, 2005).

Table 4: Wastewater pathogens and their associated indicator organisms

Pathogen Classification	Common Wastewater Pathogens	Indicator Organisms
Bacteria	Shigella Enterotoxigenic *E. coli* *Campylobacter* *Vibrio cholerae* (cholera)	Intestinal enterococci Fecal coliforms (e.g. *E. coli* and *Klebsieala pneumonae*)
Viruses	Adenovirus Rotavirus Enteric viruses Hepititus A virus Norovirus	Bacteriophages: Somatic coliphages F-RNA coliphages
Protozoa	Cryptosporidium oocytes *Giardia* cytes	*Clostridium perfringens*
Helminth Eggs	*Ascaris lumbricoides* *Trichuris trichiura* ova	*Ascaris* ova

Sources: Food and Agricultural Organization (2012), World Health Organization (2006), Bitton (2005)

The difficulty of microbial analysis in a developing world setting poses additional problems for detection of pathogens such as viruses. This is because of the lack of access to laboratory facilities for efficient water quality analysis and

associated monitoring to access the risk of wastewater reuse. There is thus a need for the development of an affordable and fairly simple method for quantifying pathogens in wastewater. Bacteriophages are non-pathogenic viruses that only infect bacteria. Research shows that bacteriophages may serve as a good indicator organism for the presence of pathogenic viruses because they share common characteristics, have similar resistances to wastewater treatment, and are easy and quick to detect (Simkova and Cervenka, 1981; Grabow, 2001).

1.2 Research Objectives and Hypothesis

Reclaimed water is a possible source of water and nutrients that can be utilized in developing countries for the irrigation of agriculture. In the particular location of Bolivia in which this research was performed, community managed wastewater system that utilizes an upflow sludge anaerobic blanket digester followed by two maturation lagoons can efficiently remove traditional water quality constituents and some pathogenic organisms and may have potential to produce quality reclaimed water that can be used in agriculture (Verbyla, 2012; Lizima, 2012). Accordingly, the hypothesis for this research is: a modified EPA double agar layer method can be developed and deployed in a developing world rural community to effectively quantify the concentration of somatic coliphage in a community managed wastewater treatment system. Additionally, somatic coliphages serve as a potential indicator of pathogenic enteric viruses in the treated wastewater.

The three specific research objectives to address the hypothesis are:

1. Modify an established double agar layer (DAL) assay method for the detection of bacteriophage that can be conducted under developing world field conditions.
2. Measure the concentration and removal of somatic coliphage at two points of a wastewater stabilization pond treatment system (effluent from an upflow anaerobic sludge blanket (UASB) reactor and effluent from the second maturation lagoon).
3. Compare the concentrations and removal of somatic coliphage and fecal coliform indicators to enteric virus pathogens in order to investigate whether somatic coliphage is a potential alternative indicator for assessing the potential presence of pathogenic enteric viruses.

This thesis will discuss research that was conducted on the wastewater treatment system in the small community of Sapecho, Bolivia. Chapter 2 provides detailed background information and a literature review that discusses previous research on the topics presented in this thesis. The methods section (Chapter 3) provides details of the research site in Bolivia and the methods used to collect and analyze water samples. Results and a detailed discussion are presented in Chapter 4. Chapter 5 provides overall conclusions and recommendations for future research.

CHAPTER 2: BACKGROUND/ LITERATURE REVIEW

2.1 Sanitation, Water Scarcity, and Agriculture: the Need for Wastewater Reuse

As the demand for freshwater increases with growing global population and affluence, water scarcity may force some members of society to depend on lower quality water sources to meet their everyday needs. There is also a great threat to agricultural farming in a water scarce world because crop irrigation is currently the greatest user of water (Mara, 2004). More farmers are thus turning to wastewater as a source of water for irrigation due to water shortages, especially in developing countries such as Bolivia. Bolivian farmers currently utilize both treated and untreated wastewater for agricultural production. For example, farmers in Cochabamba, Bolivia use approximately 42,300 m^3 per day of effluent wastewater treated by facultative ponds to irrigate crops (Jimenez, 2008). Unfortunately, the use of untreated and/or improperly treated wastewater poses human health risks from excreta-related pathogens, such as fecal bacteria and viruses that are transmitted to farmers and consumers of the irrigated produce (Drechsel et al., 2010).

Human exposure to water borne pathogens can lead to diarrheal diseases. In Bolivia, there were more than 1.9 million incidences of diarrheal diseases reported from 2001 to 2003, of which 80 % were children under the age

of five (Fretes-Cibils et al., 2006). Bolivia is a lower middle income country that has limited access to improved sanitation and clean water (WHO, 2013). Important demographics of Bolivia concerning water, agriculture, and sanitation are shown in Table 5. The high incidences of diarrheal diseases are attributed to Bolivia's relatively low coverage of their population with improved water and sanitation.

Table 5: Bolivia's demographics of water, agriculture, and sanitation

Demographic	Value	Description
Total Renewable Internal Freshwater Sources	304 billion m^3	Internal river flows and groundwater from rainfall
Improved Water Sources, Rural	71%	Percentage of Bolivia's rural population that has access to improved water sources
Annual Freshwater Withdrawals, Agriculture	57%	Percentage of total freshwater withdrawals for agriculture (irrigation and livestock)
Agricultural Land	34%	Percentage of arable land area being used for permanent crops
Improved Sanitation Facilities	27%	Percentage of Bolivia's population with access to improved sanitation facilities that effectively prevent human, animal, and insect contact with excreta

Source: The World Bank (2013)

Properly treated wastewater that meets water reuse guidelines, in terms of pathogen and nutrient removal, can safely be used for agriculture and decrease the stress on freshwater withdrawals. Reclaimed wastewater is a sustainable, alternative irrigation source that provides water and nutrients (e.g. nitrogen and phosphorus) necessary for plant growth. Removal of pathogens from wastewater prior to reuse is important because it decreases human exposure to excreta-

related pathogens which in turn decreases public health risks related to waterborne diseases.

Pathogen removal can be achieved through various wastewater treatment technologies such as sedimentation, aerobic biodegradation, anaerobic biodegradation, filtration, advanced oxidation, and disinfection through chlorination. In developing countries, such as Bolivia, more affordable yet effective wastewater treatment options are typically used. A few of the most commonly used treatment systems in developing countries include upflow anaerobic sludge blanket (UASB) reactors, trickling filters, and oxidation ditches (Mara, 2004). More natural wastewater treatment systems, such as waste stabilization lagoons and constructed wetlands, are also widely used in developing countries because they offer sustainable wastewater treatment (Mara, 2004; Muga and Mihelcic, 2008).

2.2 Upflow Anaerobic Sludge Blanket (UASB) Reactor

Treatment of domestic wastewater using UASB reactors has proven to be an effective wastewater treatment option in developing countries, especially in South America (Crites and Tchobanoglous, 1998). Today, there are more than 1000 UASB reactors operated worldwide (Tiwari et al., 2006). UASB reactors are high rate anaerobic reactors with short hydraulic retention times (HRT), ranging from 6 to 12 hours (Mara, 2004). There are numerous advantages associated with UASB reactors: (1) small reactor volume results in low land requirements, (2) low construction and operating costs, (3) low energy requirements, (4) low

sludge production, and (5) efficient BOD removal (Augusto de Lemos Chernicharo, 2007).

The BOD removal efficiency of an UASB reactor without post treatment can be as high as from 56 to 86% and the TSS removal efficiency without post treatment can range from 44 to 90% (Oliveira and Von Sperling, 2009). UASB reactors are more efficient at removing BOD and TSS than removing nutrients and pathogens. For that reason, post-treatment of the reactor effluent is required in order to meet wastewater reuse guidelines (Chong et al., 2010). The key mechanisms that contribute to pathogen reduction in UASB reactors are sedimentation and adsorption; therefore removal of the larger (more dense) pathogens is higher than removal of the smaller (less dense) pathogens. Larger size pathogens, such as helminth eggs and fecal coliforms, tend to settle to the bottom of the reactor, while smaller pathogens may adsorb to suspended solids (Gerardi and Zimmerman, 2005). In a recent study by Chernicharo et al. (2001), pathogen removal efficiencies in a UASB reactor reached approximately 70 % for helminth eggs and 1-log unit for fecal coliform. UASB reactors are less efficient at removing nitrogen and phosphorus than they are at removing pathogens from the wastewater. For example, a study by von Sperling et al. (2005) reported organic nitrogen and total phosphorus removal efficiencies in a UASB reactor to be 12% and 1%, respectively. In UASB reactors, anaerobic digestion converts organic nitrogen and phosphorus to ammonia and phosphate. These compounds are released into the liquid phase, thus very little nutrient removal is achieved (Khan et al., 2011).

UASB reactors are comprised of two parts: a circular or rectangular reactor column and a gas- liquid- solid separator (Lettinga, 1991). A standard schematic of a UASB reactor is shown in Figure 3. Wastewater is treated through an anaerobic process that can accept high loading rates (Chong et al., 2010). Figure 3 shows how the influent wastewater enters the bottom of the reactor and flows upward through a sludge bed (Crites and Tchobanoglous, 1998). The sludge bed is the bottom-most layer in the reactor, composed of densely packed sludge granules, or flocs, that have high settling properties. The granules or flocs are small aggregates of inert organic and inorganic material with a diameter of 1 to 5 mm (Chong et al., 2010). Located directly above the sludge bed is a layer of liquid containing suspended aggregates of anaerobic bacteria called the sludge blanket (Von Sperling et al., 2005). In this zone, organic matter is adsorbed onto the aggregates of bacteria in dispersed growth and anaerobically digested, which reduces the BOD concentration (Von Sperling et al., 2005). Anaerobic digestion produces biogas that contains methane and carbon dioxide that also helps to mix the contents of the reactor as the gas rises to the top of the reactor. This also aids in the formation of the biomass aggregates (Crites and Tchobanoglous, 1998). The wastewater continues to flow upward with a few active sludge particles and into the phase separator (Mara, 2004).

The phase separator separates the gas (methane and carbion dioxide), liquid (effluent), and solids (biomass). The phase separator is designed in the shape of an inverted cone, which enables suspended sludge particles to settle out by decreasing the upflow hydraulic velocity and increasing the flow area

(Mara, 2004). Sludge particles settle back down to the sludge blanket and sludge bed zones, which helps to maintain the high biomass concentration within the reactor (Crites and Tchobanoglous, 1998). Gas produced in the reactor is captured and collected in the gas collection dome area of the phase separator. The biogas can be used for energy production or can be flared (Von Sperling et al., 2005). Effluent wastewater exits the reactor and may be sent for post-treatment, such as disinfection, to further remove nutrients and pathogens.

Sludge production in a UASB reactor requires maintenance to ensure that the reactor functions properly. Due to the high retention capacity of sludge, UASB reactors have high solids residence times greater than 30 days (Augusto de Lemos Chernicharo, 2007). Excess sludge (or wasted sludge) is wasted from the reactor every 2 to 3 weeks and diverted to sludge drying beds for dewatering. After drying, the sludge can be landfilled immediately or can be stored for a minimum of 3 months and applied to agricultural land (Mara, 2004).

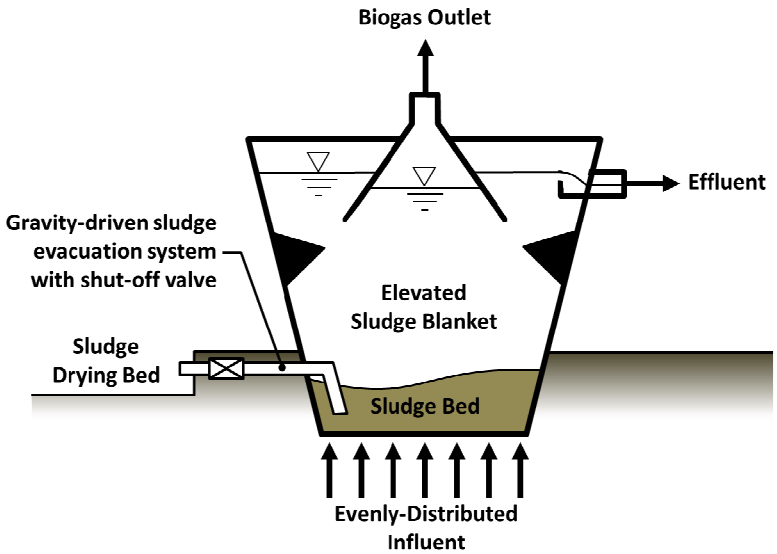

Figure 3: Typical design of an upflow anaerobic sludge blanket reactor

2.3 Waste Stabilization Lagoon Systems

Waste stabilization lagoons are natural wastewater treatment systems that utilize energy inputs from the sun and wind as well as biological processes to treat raw wastewater. Lagoons are shallow basins or reservoirs that are excavated in the earth and lined with polyethylene or vinyl sheeting, clay, or soil-cement (World Health Organization EMRO, 1998). A significant amount of land is required for lagoon construction, however, maintenance and operational requirements are low (Mara, 2004). Waste stabilization lagoons are considered the simplest forms of wastewater treatment. They provide numerous advantages including opportunities for wastewater reuse (Crites, 1998).

Commonly used in rural communities of developing countries, waste stabilization lagoons provide inexpensive and sustainable methods for treating domestic wastewater. Operational costs for lagoon systems are affordable because wastewater treatment depends on natural mechanisms and requires fewer inputs from skilled workers. The natural processes that occur in a waste stabilization lagoons are supplied by the microorganisms and algae present in the lagoon system. Microorganisms oxidize unwanted chemicals within the influent wastewater, while the algae perform photosynthesis to provide the oxygen needed for oxidation. Oxygen is also transferred from air to the water by wind and diffusion (Mihelcic et al., 2009). Temperature and solar radiation are two important energy inputs necessary for effective wastewater treatment in lagoon systems; therefore waste stabilization lagoons are best designed for wastewater treatment in warm climate regions (Mara, 2004). Unlike conventional

wastewater treatment options, lagoons have a slower oxidation process and thus require a longer hydraulic retention time (Mara, 2004). The retention times typically range from 1 to 180 days depending on the type of waste stabilization lagoon being used (Mihelcic, 2009). There are several types of waste stabilization lagoons, which are generally categorized by the presence and source of oxygen (Crites and Tchobanoglous, 1998). Table 6 lists and describes the main types of waste stabilization lagoons.

Table 6: Types of waste stabilization lagoons and their characteristics

Type of Lagoon	Presence of Oxygen	Typical Depth (m)	Hydraulic Retention Time (days)	Purpose
Anaerobic	No oxygen	2 – 5	20 – 50	BOD/TSS removal (for higher-strength wastewaters)
Facultative	Dissolved oxygen on the top layer (produced by algae and diffusion from the air), no oxygen on the bottom layer	1.2 – 2.5	20 – 180	BOD/TSS removal and some pathogen removal
Maturation	Dissolved oxygen throughout the pond profile (produced by algae)	1 – 1.5	10 – 15	Pathogen removal and polishing
Partial Mixed Aerated	Dissolved oxygen throughout the pond profile (produced partially by mechanical means)	2 – 6	3 – 20	BOD/TSS removal

Source: Crites (1998), Mihelcic, et al (2009)

Waste stabilization lagoons are often designed in series. Typically, stabilization ponds in series are ordered in the following sequence: anaerobic pond(s), followed by facultative pond(s), followed by maturation pond(s) (Mihelcic, 2009). The effluent water quality is higher when the wastewater is treated through a series of lagoons rather than using a single lagoon (Mara, 2004). The series of lagoons work similarly to a plug flow reactor in that the wastewater has an extended hydraulic retention time than in a single pond, thus increasing removal efficiency of various wastewater constituents (Mara, 2004).

Waste stabilization lagoons are efficient at removing suspended solids, BOD, and pathogens, depending on the lagoon type, but the removal efficiency of nitrogen and phosphorus is not as high. Some lagoon treatment designs may include pre-treatment may precede lagoon treatment systems in order to remove large floating solids. Pre-treatment options commonly used prior to wastewater lagoon systems include screens to remove large solids and grit chambers to decrease high sediment loading (Mihelcic et al., 2009). Wastewater constituents are removed in waste stabilization lagoons by various biological processes (listed in Table 7).

Table 7: Removal mechanisms of wastewater constituents by waste stabilization lagoons

Constituents	Concentration in Typical Wastewater	Removal Efficiency	Reduction Mechanisms
Total Suspended Solids (TSS)	240 mg/L	55 – 95%	Sedimentation
Biological Oxygen Demand (BOD)	200 mg/L	70 – 95%	bacteria oxidation

Table 7: Continued

Nitrogen	35 mg N/ L	up to 80%	NH_3 volatilization, algal uptake, sludge, decomposition, adsorption, nitrification/denitrification
Phosphorus	10 mg P/L	15 – 25%	minimal removal
Pathogens	3 million coliforms/ 100mL	Viruses: 2 – 4 log Bacteria: 2 – 6 log Helminths: 2 – 4 log	sedimentation, solar radiation, pH, dissolved, oxygen, temperature predation, natural die off

Source: Crites and Tchnobanoglous (1998), Mihelcic and Zimmerman (2010), Oakley (2005), EPA (2002)

The total suspended solids in the lagoon treatment system are comprised of the suspended solids in the influent wastewater, microbial biomass produced in the lagoon, and the algae growing in the lagoon. Suspended solids are removed by the settling or sedimentation, which is dependent upon hydraulic retention time. The longer the hydraulic retention time, the smaller the concentration of suspended solids present in the effluent. Algae in the lagoon also settle to the bottom of the lagoon as a result of die-off. Overall, the TSS removal efficiency in waste stabilization lagoons varies depending on the lagoon type. The effluent TSS may range from 60mg/L in partial mixed lagoons to 150mg/L in facultative lagoons (EPA, 2002).

Influent wastewater also contains both soluble and particulate BOD. Particulate BOD is removed through sedimentation, while soluble BOD is removed by biodegradation (Crites and Tchobanoglous, 1998). Aerobic bacteria use oxygen supplied by algae to degrade soluble BOD in wastewater, through the process of oxidation (Von Sperling, 2007). This biological process occurs in all lagoon types except in anaerobic lagoons. The absence of oxygen in

anaerobic lagoons promotes the growth of two main groups of anaerobic bacteria, methanogens and acetogens. Acetogens convert organic compounds into volatile fatty acids such as acetic acid and methanogens convert volatile fatty acids, hydrogen, carbon dioxide into methane (EPA, 2002). Facultative lagoons contain both aerobic and anaerobic zones; therefore BOD reduction occurs by both aerobic and anaerobic processes. Of all the lagoon types, the highest level of BOD removal occurs in facultative lagoons (Mihelcic et al., 2009). In waste stabilization lagoons, BOD removal depends on hydraulic retention time and water temperature (Crites and Tchobanoglous, 1998). For example, anaerobic lagoons were reported to have a five-day BOD reduction of 60 to 80% at water temperatures of 25 to 30^{o}C and retention time of 1 to 2 days (World Health Organization EMRO, 1998).

Nutrients (nitrogen and phosphorus) are somewhat removed in a lagoon treatment system; however, removal efficiencies are lower in lagoons than conventional wastewater treatment systems. Nitrogen removal in waste stabilization lagoons involve various mechanisms listed previously in Table 7. The total nitrogen concentration in average strength domestic wastewater is approximately 35 mg/L (Mihelcic and Zimmerman, 2010). Conversion of organic nitrogen (urea and amino acids) into other nitrogenous compounds (ammonia, nitrate, and nitrite) decreases the amount of total nitrogen in the wastewater (Middlebrooks et al., 1983). Organic nitrogen is converted to ammonia through the process of ammonification in facultative and maturation (Mara, 2004). The ammonia is removed from wastewater by algae (Crites and Tchobanoglous,

1998. The algae uptake nitrogen for cell growth and settle out of the wastewater when they die, thus removing nitrogen from the wastewater). In facultative lagoons, additional organic nitrogen conversion may occur through two processes: nitrification and denitrification (Mihelcic et al., 2009). Nitrification is an aerobic process that converts ammonium in the presence of oxygen to nitrate. The nitrate formed by nitrification is converted to nitrogen gas by the process of denitrification. Nitrogen gas then escapes into the atmosphere which contributes to nitrogen removal from the system. Although nitrogen removal is typically minimal in many of the lagoon types, facultative lagoons have been reported to achieve nitrogen reduction levels as high as 99 % (Middlebrooks et al., 1983).

The removal of phosphorus in waste stabilization lagoons is much lower than nitrogen removal. Phosphorus may be removed through adsorption, coagulation, precipitation, and organism uptake (Middlebrooks et al., 1983). Microorganisms uptake phosphorus for use in metabolic functions and storage for future use (Middlebrooks et al., 1983). In addition, some phosphorus is also lost through precipitation and sedimentation.

Another objective of waste stabilization ponds is the removal of pathogens, such as fecal bacteria, helminth eggs, and viruses (Crites and Tchobanoglous, 1998). Lagoons are designed to have shallow depths to enable solar UV penetration, a major disinfection mechanism. Properly designed and functioning lagoon treatment systems can have higher pathogen removal efficiency than conventional wastewater treatment systems (Mara, 2004). Waste stabilization lagoons can remove up to 6 log units of fecal bacteria, 100 % of

helminths, and up to 4 log units of viruses (Mara, 2004). Although pathogen removal occurs in all the waste stabilization lagoons types, the highest removal efficiency of pathogens is accomplished in maturation lagoons.

2.4 Maturation Lagoons

Primarily designed to remove pathogens from pre-treated wastewater, maturation lagoons are considered an economical alternative to conventional disinfection methods (Von Sperling, 2007). The effluent from maturation lagoons may contain pathogen levels that meet water quality standards for agricultural or aquaculture wastewater reuse and discharge into surface water bodies (Mara, 2004). The removal of BOD, TSS, and nutrients are minimal in maturation lagoons; thus, maturation lagoons are placed last in series to polish the effluent of the preceding ponds. In wastewater treatment designs, maturation lagoons may be used as post-treatment for effluent water from anaerobic reactors (Von Sperling, 2007).

The major pathogens removed from maturation lagoons are protozoan parasites, helminth eggs, bacteria, and viruses (Mara, 2004). The mechanisms for pathogen removal vary for different types of pathogens, but include sunlight-mediated mechanisms, sedimentation, temperature, pH, predation, and natural die-off. The main contributing mechanism to pathogen inactivation in maturation lagoons is solar irradiation, with UVB, UVA, and photosynthetically active radiation (Bolton et al., 2010). Solar UV sunlight causes direct photoinactivation of microorganisms, damaging RNA, DNA, and other cell components (Bolton et al., 2010). Inactivation of pathogens by solar radiation is dependent upon light

penetration of the water column and thus the depth of the lagoon (Davies-Colley et al., 1999). Maturation ponds are designed to be shallow (typically 1- 3 m in depth), to ensure solar diffusion throughout the lagoon system (Bolton et al., 2010). High algal concentrations can decrease the amount of sunlight that infiltrates the water column, thus decreasing pathogen inactivation (Davies-Colley et al., 1999). In waste stabilization ponds, the inactivation of pathogens vary with the different types of solar radiation (Davies-Colley et al., 1999). *E. coli* and fecal coliforms are predominantly affected by UVB radiation (Davies-Colley et al., 1997). In another study, it was found that total coliforms and fecal coliforms were more suseptible to UV inactivation than coliphages (Bourrouet et al., 2001). Overall, high light intensity (>450 nm wavelength), high pH (>9.4), and high DO create the ideal lagoon environment for optimal pathogen destruction (Drechsel et al., 2010).

Solar radiation also interacts with pH and dissolved oxygen (DO) to inactivate pathogens (Maiga et al., 2009). The combination of sunlight, pH, and DO naturally disinfect wastewater in maturation lagoon systems. Photoinactivation of pathogens varies with the level of dissolved oxygen and becomes more efficient at higher pH values (Hosetti and Frost, 1998). The high level of algal photosynthesis in lagoons contributes to high pH levels (Bolton et al., 2010). The process of photosynthesis consumes carbon dioxide, which decreases the concentration of carbon dioxide in the lagoon. The low concentration of carbon dioxide disturbs the carbonate-bicoarbonate equilibrium, thus increasing the concentration of hydroxide ions, which raises the pH of the

lagoon water (Mara, 2004). High pH values enhance pathogen cell damage needed for inactivativation. Pathogens are not able to survive in water at pH values greater than 9.4 (Drechsel et al., 2010). The high pH of the lagoon must be maintained to ensure the longevity of the pathogen inactivation time. If the pH of the lagoon fluctuates away from the recommended pH levels and the retention time is not long enough, then pathogen reactivation may occur, which will increase the concentration of viable pathogens in the effluent (Gerardi and Zimmerman, 2005). In addition to raising the pH, algal photosynthesis also increases the dissolved oxygen concentration (Dixo et al., 1995). The increased intensity of sunlight enhances photosynthetic activity which in turn increases the concentration of dissolved oxygen in the lagoon system. Rapid pathogen inactivation requires high pH and DO levels (Mara, 2004). In a recent study by Bolton et al. (2010), UVA solar inactivation of pathogens occurred fastest in water environments with a high dissolved oxygen concentration and pH of 9.5.

Pathogens are also removed from maturation lagoons through sedimentation. Larger and more dense pathogens, such as helminth eggs, tend to settle to the bottom of the lagoon while smaller and less dense pathogens adsorb to suspended solid particles, which settle over time. Ohgaki et al. (1986) found that in aerobic waste stabilization lagoons, viruses tend to adsorb to algal particulates, which settle out. The long hydraulic retention times of maturation lagoons allow settling of pathogens and solids to occur for efficient removal of pathogens.

Temperature is another factor that contributes to pathogen inactivation. Waste stabilization lagoons are best suited for wastewater treatment in arid or semi-arid countries because it provides the necessary sunlight needed to raise water temperature for pathogen destruction (Mara, 2004). Previous research indicates that water temperatures ranging from 20 to 40^{o}C are favorable for virus reduction in waste stabilization lagoons (Berg, 1966). In another study, Rao et al. (1981) reported virus reduction from 88 to 99 percent from wastewater treated by waste stabilization ponds with 20 to 35^{o}C water temperatures. Although the lagoons are most efficient in warm climates, they can still function in cold climates but with lower removal efficiency. The lagoons must be designed with a higher hydraulic retention time and greater surface area to reduce the pathogen levels in wastewater (Drechsel, 2010).

2.5 Bacteriophage Overview and Importance

Bacteriophages, also referred to as phages or coliphages, are viruses that infect bacteria (Kutter and Sulakvelidze, 2005). Ubiquitous in the environment, bacteriophages exist naturally in fresh water, salt water, soil, plants, animals, and humans (Kutter and Sulakvelidze, 2005). Although bacteriophages are found in the human intestinal tract, these viruses are not harmful to humans (APHA, AWWA, and WEF. 2012). As the largest known viral group, at least 5,136 bacteriophage groups have been identified (Calendar, 2004). Classification of bacteriophages into groups can be done according to three characteristics: strain of bacteria host, morphology, and genome sequence (Abedon, 2008).

Similar to pathogenic viruses in size and structure, bacteriophages have a head or “capsid” that contains genetic material, a tail or “sheath”, and tail fibers used to attach the phage to the host cell (Bitton, 2005). Infection of the host cell occurs through the transfer of genetic material from the bacteriophage to the host cell. Bacteriophages attach to the cell by adsorption of the tail fibers. After attachment, the phage’s genetic material is injected into the host cell and the lytic cycle begins, as shown in Figure 4 (Kutter and Sulakvelidze, 2005). The host cell becomes a production site, where the virus’ genetic material is replicated to form numerous progeny bacteriophages (Bitton, 2005). The host cell then ruptures, destroying the cell and releasing the new bacteriophages to infect more cells.

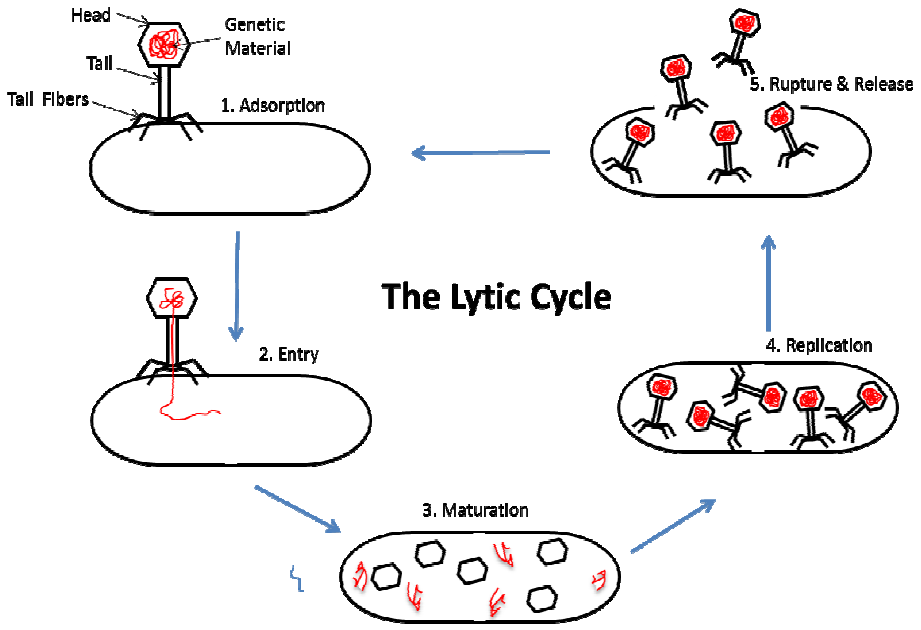

Figure 4: The viral lytic cycle

Viral reproduction occurs at an exponential rate, but this rate can be decreased when viruses become inactivated. Damage to the viral head causes virus inactivation by: (1) damaging the genetic material, which inhibits survival of

the virus after adsorption, (2) preventing viral attachment to the host cell, and (3) preventing entry of the genetic material into the host cell (Abedon, 2008). In wastewater treatment, pathogenic viruses and bacteriophages can be inactivated by various mechanisms. Waste stabilization lagoons, in particular, reduce bacteriophage concentrations by means of natural disinfection. As discussed previously, environmental factors that inactivate bacteriophages include: solar radiation, heat, pH, and the presence of hydrolytic enzymes produced by other microorganisms (Abedon, 2008).

Human feces contain a high concentration of bacteriophages, which contributes to the presence of bacteriophages in domestic wastewater at concentrations ranging from 10^5 to 10^7 phage particles/L (Bitton, 2005). Bacteriophages are often used in evaluating water and wastewater quality for the purpose of monitoring pathogens. Bacteriophages are resistant to wastewater treatment. The direct quantification of pathogens in wastewater is a complex and expensive process. Enteric viruses are very difficult to detect using tissue cultures; therefore requiring more complex, technological methods of detection, such as the Polymerase Chain Reaction (PCR) methods (Bitton, 2005). Measuring bacteriophages involves a fairly simple plaque assay method that requires no special training or expertise and yields overnight results. Since bacteriophages are easy to detect and quantify, they are generally used as pathogenic indicators for water quality assessment. There are discrepancies in literature as to whether bacteriophages serve as better indicator organisms for the detection of enteric viruses compared to using fecal coliforms as an indicator.

Previous research has indicated that bacteriophages a potential to be good indicator organisms of enteroviruses in water treatment quantifications (Stetler, 1984; Eaton et al., 2000), while other studies suggest that bacteriophages are better indicators of fecal coliforms (Wentsel et al., 1982; Gantzer et al., 2001). It is important to understand the characteristics that make a good indicator organism for the purpose of determining the applicability of bacteriophages as a pathogenic indicator.

According to the characteristics of a good indicator organism, bacteriophages seem to qualify as a suitable indicator organism for enteric viruses. Bacteriophages are present in wastewater at concentrations well above enteric virus concentrations (Stetler, 1984). As previously described above, bacteriophages have are very similar in composition to pathogenic viruses and carry out the same cycle of reproduction (lytic cycle). Additionally, bacteriophages are more resistant to environmental and wastewater treatment factors than pathogenic viruses (Ashbolt et al., 2001).

Three groups of bacteriophages (somatic coliphages, male-specific coliphages, and coliphages that infect *Bacteroids fragilis)* are regularly used as indicators of fecal contamination in wastewater quality monitoring (Bitton, 2005). Table 8 shows compares somatic, male-specific, and *B. fragilis* coliphages according to their characteristics. Out of the three coliphage groups presented in Table 8, somatic coliphages have the highest concentration in human feces, the highest resistance to environmental inactivation, and one of the highest concentrations in wastewater.

Table 8: Characteristics of somatic coliphage, male specific coliphage, and bacteriophage

Characteristic	Somatic Coliphage	Male – Specific Coliphage	*B. fragilis Coliphage*
DNA	Single Stranded	Double Stranded	Double Stranded
Group Homogeneity	Homogenous	Heterogeneous	Heterogeneous
Host Bacteria	*E. coli CN 13*	*E. coli* F_{amp}	*Bacteroids fragilis HSP40*
Infection Route	Bacteria cell wall	F pilus of bacteria	Bacteria cell wall
Replication	Intestinal tract of humans and warm-blooded animals	Intestinal tract of humans and warm-blooded animals	Intestinal tract of humans and warm-blooded animals
Concentration in Wastewater	$10^3 – 10^4$ ml^{-1}	$10^3 – 10^4$ ml^{-1}	$<1 – 10^3$ ml^{-1}
Concentration in Human Waste	Intermediate	Low	Low
Probability of Replication in the Environment	Intermediate	Low	Very low
Resistance to Inactivation in Environment	Intermediate	Low	High
Ease of Detection	Easy to detect	Somewhat easy to detect; host bacteria must be in log phase	More labor-intensive, time consuming, and expensive

Sources: Calendar (2004), Gerardi and Zimmerman (2005), Grabow (2001)

Somatic coliphage concentrations are generally higher than male-specific coliphage concentrations (Grabow, 2001). The detection of somatic coliphage is simple, inexpensive, and yields overnight results. For these reasons, somatic coliphage should be further researched as an indicator organism for enteric viruses in wastewater.

2.6 Enteric Virus Overview

Enteric viruses are a group of pathogenic viruses that infect humans. They are composed similarly to bacteriophages in that they have a capsid that

contains the virus' DNA and the capsid is attached to a tail (Gerardi and Zimmerman, 2005). Reproduction of enteric viruses happens through the same replication process in which bacteriophages reproduce, i.e., the lytic cycle. These viruses replicate within the human intestinal tract and are released from the body through human excreta. The presence of enteric viruses in water is a result of contamination of human excreta, which is the source waterborne disease outbreak. There are about 160 identified enteric virus groups in existence (National Research Council of the National Academies, 2004). A few enteric viruses that are important in public health monitoring include: rotavirus (causes diarrhea), poliovirus I (causes paralysis), hepatitis A virus (causes liver disease), and norovirus (causes diarrhea) (Bitton, 2005; Henze et al., 2008).

In wastewater, the concentration of enteric viruses varies depending on the number of infected persons in a population. One study by Oragui and Mara (1996) found that in Brazil the concentration of one type of enteric virus, rotavirus, to range from 1.06×10^4/ L to 2.66×10^5 / L in raw wastewater. The wastewater was treated by a five-series waste stabilization lagoon system (anaerobic lagoon, facultative lagoon and three maturation lagoons) where rotavirus was removed by 4 log units (Oragui and Mara, 1996).

Quantification of enteric viruses is an extensive and long process that does not always provide an accurate estimation of the number of viruses present in wastewater because only a fraction of enteric viruses are able to be cultivated (Payment, et al., 2004). Therefore, bacteriophages are often used as indicator organisms for enteric viruses in wastewater treatment. Comparing enteric virus

removal to bacteriophage removal in waste stabilization lagoons, Locas et al. (2010) reported that bacteriophage removal up to 3 log units and enteric virus removal up to 1 log unit in an aerated lagoon system. Enteric viruses are more resistant to wastewater treatment than bacteriophages. There are several factors that can be attributed to enteric virus removal in waste stabilization ponds: solar radiation, temperature, sedimentation, presence of algae and bacteria, and ammonia (Mara and Horan, 2003). Overall, enteric viruses are pathogenic organisms that are resistant to wastewater treatment, which increases public health concerns with wastewater reuse in agriculture; therefore it is important to identify a good indicator organism for wastewater quality monitoring.

2.7 Fecal Coliform Overview

Fecal coliforms, also referred to as thermotolerant coliforms, are a group of non-pathogenic bacteria that are the only bacteria able to breakdown lactose at 44^{o}C temperatures (Mara, 2004). These bacteria are larger in size than viruses, ranging from 0.1 to 1 µm and reproduce by binary fission (Gerardi and Zimmerman, 2005). Examples of fecal coliforms include *Escherichia coli* and *Klebsiela pneumonae* (Bitton, 2005). Fecal coliforms reside in the intestinal tract of humans and warm blooded animal, in which the presence of fecal coliform in human excreta contributes to its presence in wastewater. Typical wastewater concentrations of fecal coliforms range from 10^{6} – 10^{7} CFU per 100 mL of wastewater (Henze et al., 2008). They are commonly used in water quality monitoring to indicate the presence of fecal contamination in water. The detection of fecal coliforms involves methods such as membrane filtration and most

probable number tests, which require a sterile laboratory environment, expertise of the procedure, and a sufficient amount of time to conduct the procedure (Elmund et al., 1999).

Despite the intensive detection methods, fecal coliforms are widely used as indicator organisms because they provide a good indication of the presence of both bacterial and viral pathogens (Mara and Horan, 2003). Although fecal coliforms are used as traditional indicator organisms, there are limitations that restrict fecal coliforms from being considered a good indicator of viruses in wastewater. First, fecal coliforms have similar characteristics (such as size, composition, reproduction, and persistence) as bacteria, which indicate that they behave more closely to bacterial pathogens in wastewater treatment than viruses (Bitton, 2005). Secondly, fecal coliforms are less resistant to wastewater treatment and disinfection than viruses (Bitton, 2005). For example, in a study by Chivukula et al. (2005) that measured the concentration of indicator organisms in the effluent of six wastewater treatment plants, the percentage of indicator organisms present in the effluent following the disinfection process was 27% fecal coliforms, 31% enteric viruses, and 40% male-specific coliphages. That study showed that fecal coliforms are less resistant to disinfection, whereas enteric viruses and bacteriophages are similar in resistance to disinfection.

In waste stabilization lagoons, fecal coliform reduction is attributed to sedimentation (fecal coliforms are dense and settle out in the sludge), low water turbidity which allows solar inactivation, natural die-off (increases with decreasing turbidity), and predation (Alkan et al., 2005). Maturation lagoons have been

reported to remove fecal coliforms by approximately 90 % (Vorkas, 1999). In another study, Oragui et al. (1987) found that fecal coliforms were removed by 3.5 log units in a five series waste stabilization lagoon system consisting of an anaerobic pond, facultative pond, and three maturation lagoons. Fecal coliform removal has been found to be higher than removal of bacteriophages and enteric viruses in waste stabilization lagoons. In a three-series aerated waste stabilization lagoon system, Locas et al. (2010) reported an average removal of 4.9 log units of fecal bacteria, 2.5 log units of somatic coliphage, and 1.0 log units of enteric viruses. It is evident that fecal coliforms do not share characteristics or similar resistances to viral pathogens; therefore bacteriophages should be considered as a possible alternative indicator organism for viral pathogens in wastewater treatment.

2.8 Relevant Research and Literature Gap

Bacteriophages have been a prevalent subject of study in regards to their removal in wastewater treatment systems and their potential as an indicator of viral pathogens in water and wastewater. Much of the research has been done on wastewater treatment facilities worldwide, with the majority of studies being in Spain and South America. Table 9 highlights research that has been done on bacteriophages that relate to the research in this thesis. The six studies were examined with special attention to the concentration and removal efficiencies of indicators (somatic coliphages, male –specific coliphages, and fecal coliforms) and viral pathogens (enteric viruses).

Table 9: Relevant research on bacteriophage, fecal coliform, and enteric virus removal in wastewater treatment systems

Ref.	Wastewater Treatment System	Bacteriophage Removal	Enteric Viruses Removal	Fecal Coliforms Removal	Bacteriophage Detection Method	Research Location
[1]	Three aerated waste stabilization ponds	**Somatic coliphage:** 2.5 ± 0.4 $\log_{10}$ units reductions **Male – Specific coliphage:** 3.4 ± 0.4 $\log_{10}$ units reduction	1.0 ± 0.7 $\log_{10}$ units reduction	4.9 ± 0.7 $\log_{10}$ units reduction	EPA Method 1602	Quebec, Canada
[2]	6 wastewater treatment plants: 4 activated sludge plants, 1 nitrification plant, & 1 biological nutrient removal plant	Up to 5.5 $\log_{10}$ units reductions	Up to 4.6 $\log_{10}$ units reduction	Up to 7.9 $\log_{10}$ units reduction	Agar overlay method by Adams	United States
[3]	Two anaerobic ponds followed by a facultative & a maturation pond	2.0 $\log_{10}$ reductions	N/A	5 $\log_{10}$ units reduction	Double Agar Layer Method	Leon, Spain
[4]	Two nonaerated facultative ponds and two oxidative ponds	**Somatic coliphage:** 2.9 $\log_{10}$ units reduction **Male-Specific coliphage:** 2.4 $\log_{10}$ units reduction	N/A	3.5 $\log_{10}$ units reduction	Double Agar Layer Method – ISO 10705-2 standard	Santa Gregori, Spain
[5]	Two facultative lagoons in series	**Somatic Coliphage:** 4.6 $\log_{10}$ units reduction **Male-Specific coliphage:** 3.5 $\log_{10}$ units reduction	N/A	3.4 $\log_{10}$ units reduction	Double Agar Layer Method	Choconta, Colombia
[6]	Pilot- scale advanced pond system (APS): anaerobic reactor followed by a high-rate pond, a deep algal setting pond, & two-cell maturation pond	**Somatic coliphage:** 2.2 $\log_{10}$ units reduction	N/A	>4 log10 units reduction	Single Agar Plating Method	Hamilton, New Zealand

[1] Locas et al.(2010), [2] Harwood et al. (2005), [3] Reinoso et al. (2011), [4] Lucena et al. (2004), [5] Campos et al. (2002), [6] Davies – Colley et al. (2005)

Locas et al. (2010) studied the removal of indicator and enteric viruses in wastewater treated by a three-series aerated lagoon treatment system in Quebec, Canada. The system receives an incoming flow of approximately 36,000 m^3/ day of wastewater (Locas, 2010). The influent wastewater contained an approximate concentration of 45 MPNIU/L of enteric viruses, 1 x 10^4 PFU/100 mL of somatic coliphages, 12,000 PFU/ 100 mL for male-specific coliphages, and 4 x 10^6 CFU/ 100 mL of fecal coliforms (Locas, 2010). The three-series aerated lagoon system removed the indicator organisms with the efficiency of 2.5 ± 0.4 log units for somatic coliphages, 3.4 ± 0.4 log units for male-specific coliphages, and 4.9 ± 0.7 log reduction units for fecal coliforms (Locas, 2010). The results from the Locas et al. (2010) study shows that fecal coliforms had the highest log removal indicating that they are less resistant to wastewater treatment in this aerated lagoons system than bacteriophages and enteric viruses. Enteric viruses were barely removed from the system, while bacteriophages had less removal than fecal coliforms but higher removal than enteric viruses.

In another study, Harwood et al. (2005) researched the removal of pathogens (*enterococci*, *Giardia*, *C. perfringens*, and *Cryptosporidium*) and indicator organisms (coliphages, total coliforms, and fecal coliforms) in six wastewater reclamation facilities in the United States that use biological treatment and disinfection. The flow of the treatment facilities ranged from 60,480 m^3/ day to 3,456 m^3/ day (Harwood, 2005). Looking specifically at fecal coliforms, somatic coliphages, and enteric viruses, the $\log_{10}$ average influent concentrations of these organisms in the six facilities were approximately 6.2 CFU/ 100 mL for

fecal coliforms, 4.6 PFU/ 100 mL for 700691 strain of coliphages, 4.7 for 15597 strain of coliphages, 3.8 MPN/ 100 L for enteroviruses, and 2.3 MPN/ 100 L for *Cryptosporidium* (Harwood, 2005). Results from the study showed that coliphages reached up to 5.5 log removal units, enteric viruses reached up to 4.6 log removal units, and fecal coliforms reached up to 7.9 log removal units (Harwood, 2005). Harwood et al. (2005) found that there were no significant correlations between enteric viruses and coliphages. It was also concluded that the concentration of the coliphage indicator organism has a weak correlation to the concentration of the enteric virus pathogen (Harwood, 2005). Furthermore, fecal coliforms were removed at a higher degree than both coliphages and enteric viruses. Enteric viruses were the most resistant to wastewater treatment than all the other organisms quantified.

In a study by Reinoso et al. (2011), the removal of various types of indicators in a wastewater treatment plant in Spain that consists of two anaerobic lagoons followed by a facultative lagoon and a maturation lagoon. The wastewater treatment system receives an average flow of 3,200 m^3/ day (Reinoso, 2011). The summer influent wastewater contained an average concentration of 6.5 ± 0.7 log CFU/ 100 mL of *E.coli* and 5.3 ± 1.3 log PFU/ 100 mL of coliphages (Reinoso, 2011). Reinoso et al. (2011) reported log reductions of 2 for coliphages and log reduction units of 5 for *E.coli*. Coliphages were more resistant to wastewater treatment in the lagoon system than *E.coli.* Reinoso et al., (2011) stated that anaerobic ponds were most effective at removing pathogens than facultative and maturation ponds; although the minimum

retention time necessary for efficient pathogen removal in waste stabilization ponds is unknown.

Lucena et al. (2004) studied the removal of specific indicators (fecal coliforms, enterococci, sulphite-reducing clostridia, somatic coliphages, male specific coliphages, and coliphages infecting *Bacteroides fragilis*) in wastewater treated by two non-aerated lagoons followed by two oxidative lagoons. The wastewater treatment facility is located in Santa Gregori, Spain and has an approximate incoming flow of 240 m^3/ day (Lucena et al., 2004). In the summer, the four-series waste stabilization lagoon system removed fecal coliforms, somatic coliphage, and male specific coliphage with an average removal efficiency of 2.7 log units, 3.7 log units, and 2.9 log units, respectively (Lucena et al., 2004). Results of the study show that fecal coliforms were less resistant to wastewater treatment by waste stabilization lagoons than somatic coliphages and male-specific coliphages. Comparing the bacteriophages, the male-specific coliphage group was more resistant to wastewater treatment than the somatic coliphage group. It was concluded that of all the indicators quantified in the study, somatic coliphage serves as the best indicator to use in wastewater quality monitoring because they are easy to detect and quantify (Lucena et al., 2004).

In another study, Campos et al. (2002) compared the removal of viral indicators (somatic coliphages, male specific coliphages, and coliphages infecting *Bacteroides fragilis)* to the removal of fecal indicators (fecal coliforms, *E.coli*, *Streptococcus faecalis*, and *Clostridium perfringens*) in a wastewater treatment system in Colombia. The treatment facility receives a mean flow of

1,555 m^3/ day of wastewater (Campos et al., 2002). The results of the microbial quantification were reported for two years, 1999 and 2000 (Campos et al., 2002). In the earlier study, the influent wastewater contained a concentration of 5.5 log CFU/ 100 mL of fecal coliforms, 5.6 log PFU/ 100 mL of somatic coliphage, and 4.8 log PFU/ 100 mL of male-specific coliphage (Campos et al., 2002). The influent concentrations of the microorganisms were slightly different in 2000 with 6.5 log CFU/ 100 mL of fecal coliform, 5.6 log PFU/ 100 mL of somatic coliphages, and 4.8 log PFU/ 100 mL of male-specific coliphages. Campos et al. (2002) found higher removals of fecal coliforms, somatic coliphages, and male-specific coliphages in the year 2000, with log reduction units of 4.6 for somatic coliphage, 3.5 for male-specific coliphage, and 3.4 for fecal coliforms. Unlike to previously mentioned studies, somatic coliphages and male specific coliphages were reduced at a higher concentration than fecal coliforms in the two-series facultative lagoon. Campos et al., (2002) concluded that the removal rates of viral and bacterial indicators varied in the wastewater treatment lagoons depending on the sampling time period. In addition, it was concluded that bacteriophages are easy, economical, and quick to quantify which makes them a good alternative fecal indicator (Campos et al., 2002.

Davies-Colley et al. (2005) compares the removal of somatic coliphages to the removal of *E. coli* in a pilot-scale advanced pond system (APS) located in New Zealand. The advanced pond system treated 2 m^3/ day of wastewater which is treated by the APS consisting of an anaerobic digestion followed a high-rate pond, a deep algal settling pond, and two maturation ponds (Davies-Colley et al.,

2005). The average concentration of *E.coli* and somatic coliphage in the summer influent wastewater was 5.9 CFU/ 100 mL and 4.6 PFU/ 100 mL, respectively (Davies-Colley, 2005). Davies-Colley et al., 2005 found that *E.coli* removal (>4 log reduction) was greater in the APS system than somatic coliphage (2.2 log reduction). The study showed that solar UVB radiation was a major factor in the reduction of somatic coliphage in the APS system (Davies- Colley et al., 2005). Thus the removal of somatic coliphage in the APS system during the summer suggests that enteric virus removal in the same system in summer months would be more efficient than removal rates in the winter (Davies-Colley et al., 2005).

All of the studies in Table 9 measured bacteriophage removal in wastewater treatment using waste stabilization lagoons, with the exception of the Harwood (2005) study that focused on removal of indicators and pathogens in various conventional wastewater treatment plants (ex. activated sludge plant, nitrification plant, and biological treatment plant). Only two of the wastewater treatment systems in the studies looked at indicator organism removal in maturation lagoons (Reinoso, 2011; Davies-Colley, 2005). Bacteriophage quantification was done in sterile laboratories using various methods of both double and single agar layer assays. From the research results, somatic coliphage removal ranged from 2.1 to 4.6 log units. Generally, virus removal in waste stabilization ponds can reach up to 4 log units, which is higher than conventional wastewater treatment systems, which can range from 1 to 2 log units (Mara, 2004). The studies in Table 9 achieved virus removal within this

range (Locus, 2010; Reinoso, 2011; Lucena 2004; Campos, 2002; Davies-Colley, 2005).

In comparing somatic coliphage reduction to enteric virus and fecal coliform reduction, waste stabilization lagoons were more efficient at removing somatic coliphages than enteric viruses (Locus, 2010) but had an even higher reduction in fecal coliform concentrations (Locus 2010; Reinoso, 2011). This concentration and removal information is useful in determining which organism is a better indicator of enteric viruses: somatic coliphages or fecal coliforms.

There are numerous studies on pathogen removal from waste stabilization ponds, but there is a lack of information in the following areas: (1) somatic coliphage removal in maturation lagoons, (2) somatic coliphage potential as an indicator organism for pathogenic viruses, (3) bacteriophage removal in wastewater treated by a community managed wastewater treatment system, and (4) quantification of pathogen using a modified method for a developing world field setting. Therefore, this research focuses on bacteriophage removal in a community-managed system, which uses an UASB reactor followed by two maturation lagoons.

The research presented in this thesis will contribute to the understanding of the fate of somatic coliphage in wastewater treatment in the developing world, particularly removal by maturation lagoons. The research also investigates the potential of somatic coliphage as an indicator organism of pathogenic viruses. Furthermore, the research presents a modified double agar layer method that

can be conducted in the field for the quantification of bacteriophage in order to assess wastewater quality.

CHAPTER 3: METHODS

3.1 Description of Research Site

Sapecho is a small, rural community located in the South American country of Bolivia (shown in Figure 5). The community of Sapecho sits at an elevation nearly 405 meters above sea level and has an approximate population of 1,300 (Verbyla, 2012). Domestic wastewater generated by the community is managed by a treatment system composed of an UASB reactor followed by two waste stabilization lagoons in series (Figure 6). The incoming flow rate of the system was measured to be 58.1 m^3/day (Verbyla 2012).

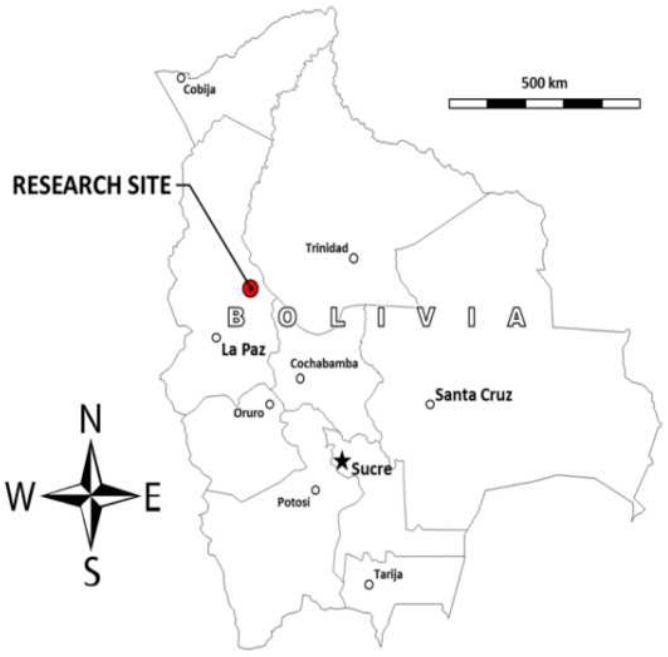

Figure 5: Map of research site location in Sapecho, Bolivia

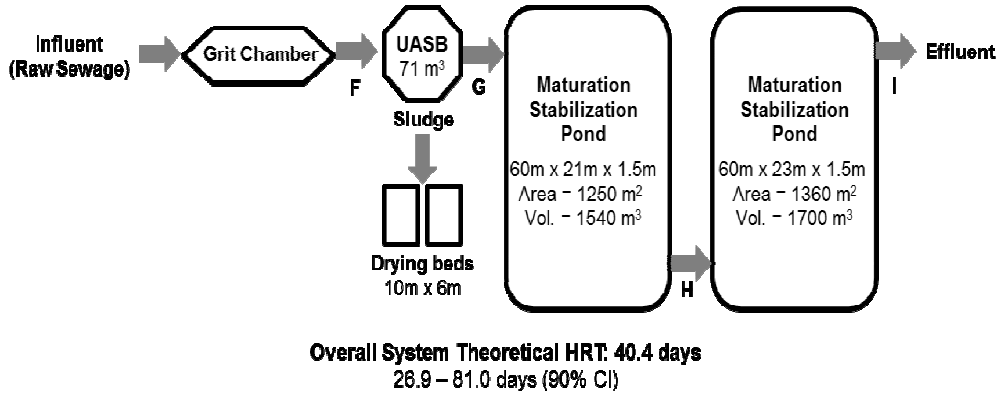

Figure 6: Sapecho, Bolivia wastewater treatment system design

From 2010 until the end of the project, there were complications with the USAB reactor due to maintenance difficulties. The discharge pipe that manages the sludge level within the reactor was clogged, which prevented sludge from being removed from the reactor (Verbyla, 2012). By June 2012, the sludge level nearly reached the top of the reactor; however sludge accumulation within the reactor did not hinder the normal water flow rates (Verbyla, 2012).

3.2 Overview of EPA Method 1602

Coliphage can best be quantified by the double agar layer assay. This method is relatively inexpensive to perform and the procedure is fairly simple to understand. In order to determine the coliphage concentration in water samples collected from the wastewater treatment facility at the research site in Bolivia, this study employed EPA Method 1602: Male-specific (F+) and Somatic Coliphage in Water by Single Agar Layer (SAL) Procedure. The specified Double Agar Layer (DAL) assay instructions indicated throughout this procedure were used.

The EPA Method 1602 is a double agar layer assay that uses two agar layers to assess the concentration of bacteriophage in a water sample. The top

agar layer combines the log phase host, *E.coli,* with the water sample containing the bacteriophage and the bottom agar layer contains agar that supports *E.coli* growth. Bacteriophages infect and inactivate *E.coli* causing the formation of plaques. The numbers of plaque forming units (PFU) are representative of the concentration of coliphage in the water sample once converted to PFU/ mL.

3.3 EPA Method 1602 Modifications

The procedure used for this research was modified to be performed in the field setting. Table 10 provides information about the modifications made to the EPA method 1602.

Table 10: Modifications made to the EPA Method 1602: Male-specific (F+) and Somatic Coliphage in Water by Double Agar Layer (DAL) Procedure

Modification	Comment
Small volume equipment	All the equipment needed to conduct the DAL procedure was transported to Bolivia. The equipment had to be scaled down to meet weight and volume requirements for ease of air and in country ground travel.
Less sterilized environment	There was no facility with a laboratory in the local community of the research site; therefore a small hotel room with screened windows was used as a field laboratory in the nearby town of Palos Blancos.
Incubator without a shaker	For ease of travel, a smaller incubator had to be used. There were no small incubators available to the research group that had a built in shaker.
Power wattage	The power outlets in Bolivia have a 200 voltage source which is higher than the equipment voltage capacity (120 volts); therefore a voltage converter had to be used.

The DAL Assay was conducted for the quantification of both male-specific and somatic coliphages. Due to discrepancies in the results yielded by the male-specific coliphage trials in this research, only the somatic coliphage results are

presented and discussed in this thesis. The male-specific coliphage results are presented in Appendix E – Appendix H.

3.4 Sample Collection and Storage

Grab samples were collected at two points of the Sapecho wastewater treatment plant at 10:30 am on June 17, 2012. Figure 7 shows the location of the sampling points. These two collection points were chosen to compare findings from this research with other studies of the same system related to fecal coliform removal (Verbyla, 2012) and enteric virus removal (Symonds, 2013 unpublished data). The grab samples were obtained by filling two separate 100-mL, plastic sample collection bags with 100 mL of the appropriate wastewater samples. The bags were placed in a dark container and immediately transported to the laboratory location where the samples were stored at 4^{o}C until use.

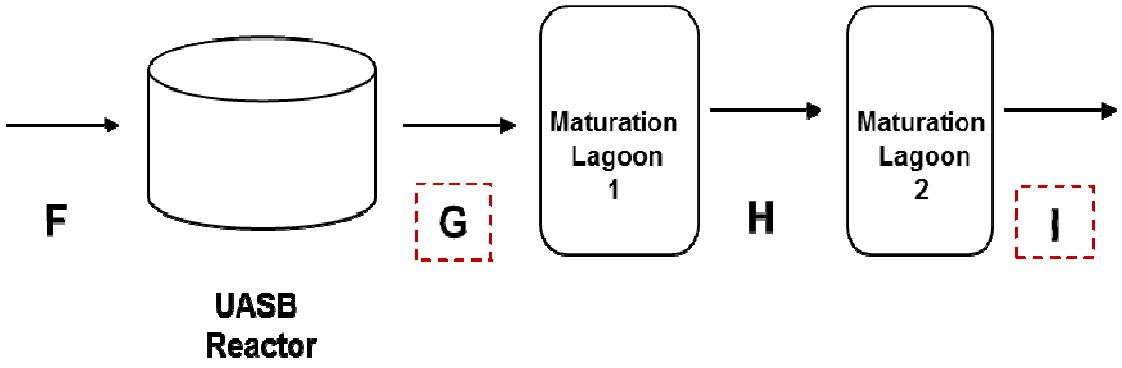

Figure 7: Sample points selected at the Sapecho wastewater treatment facility: sample point G (effluent of UASB) and sample point I (effluent of maturation lagoon 2)

3.5 Field Laboratory Configuration

A field laboratory was assembled in a small hotel room with approximate dimensions of 10 ft.- by-10 ft. All furniture was removed from the room for

thorough cleaning. The floor was swept and spider webs were removed from the windows. Five tables (3 medium sized tables and 2 miniature tables) were wiped down with a 10 % bleach solution and covered with plastic. Laboratory equipment (e.g. packaged petri plates, packaged tryptic soy broth, etc.) was stored in large duffle bags and stored underneath the medium sized tables.

In the room, there was one small glass window that connected to one of the bathroom stalls and a large screened in window with fabric curtains that opened to the outside common area. The door to the room was also screened in and had a fabric curtain. In addition, the room only had one electrical source with a single outlet. Figure 8 shows the interior and exterior view of the field laboratory prior to setup and Figure 9 shows the schematic of the field laboratory setup.

Figure 8: Interior and exterior pictures of the field laboratory space prior to room setup

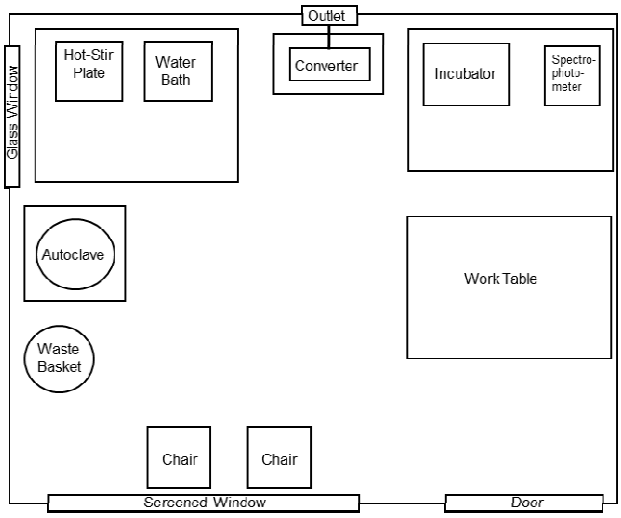

Figure 9: Schematic configuration of the field laboratory set up near the research site in Palos Blancos, Bolivia

One table was designated as the work table, in which sample dilutions and agar plates were prepared. Directly behind the work table, another table was arranged with the incubator and spectrophotometer. A miniature table was place on the center of the back wall (location of the only outlet in the room) on which the converter was placed. The water bath and heat-stir plate were placed on the table to the left of the converter. Figure 10 shows the actual setup of the field laboratory.

Figure 10: Photo of the field laboratory in a small hotel room near research site in Palos Blancos, Bolivia

The laboratory was kept clean by sweeping the floor and wiping down the tables with 10 % bleach solution and ethanol every day after trials were completed. Upon entering the laboratory, researchers put on laboratory coats, gloves, and safety goggles.

3.6 Preparation of Antibiotic Stocks, Tryptic Soy Broth, and 1.5% Tryptic Soy Agar

A nalidixic antibiotic stock solution was prepared and stored for later use in the DAL procedure. The nalidixic acid (Fisher Scientific, Fair Lawn, New Jersey) was pre-measured in the USF Analytical lab using an analytical balance and put into plastic micro-test tubes for safe transportation of the antibiotics to Bolivia.

A 100-mL beaker was filled with deionized water and covered with a piece of aluminum foil. The beaker was then autoclaved in a 12-L portable autoclave steam sterilizer (Health and Medical Sales, Inc., Cedar City, Utah), for 15 minutes at 121^{o}C and 15 psi. After autoclaving was complete, the beaker was removed and allowed to cool to room temperature. Pre-measured 1g of nalidixic acid was added to the beaker and mixed thoroughly. Next, the solution was aseptically drawn into a 60 mL syringe and passed through a 0.22 -µm filter. The filtered solution was then dispensed into 15 -mL plastic freezer vials (5 mL into each vial). Vials were properly labeled and put into the freezer at -20 ^{o}C until later use.

Next, the tryptic soy broth (TSB) solution was made by combining 200 mL of DI water and 6 g of double strength tryptic soy broth mix (Environmental

Express, Charleston, South Carolina) in a 500 -mL beaker with screw cap. The beaker was placed on a ceramic heat-stir plate (IKA Works, Inc., Wilmington, North Carolina) at a temperature of 150 ^{o}C and brought to a boil. A stir bar was inserted into the beaker and the solution was allowed to mix at a moderate speed for 5 minutes. Once the contents were completely dissolved, the beaker was allowed to cool to room temperature, capped, and, stored in the refrigerator at 4^{o}C. A second beaker of tryptic soy broth solution was prepared for the next step of preparing the bottom layer agar plates.

The 1.5% agar (bottom layer agar) plates were made to be used for two parts of the double agar layer (DAL) assay: (1) growth of host *E. coli CN-13* bacteria and (2) bottom layer agar for the assay. The beaker containing 200 mL of TSB was placed on the hot plate set at a temperature of 150 ^{o}C and brought to a slight boil. Next, 3g of agar (Acros Organics, Fair Lawn, New Jersey) was mixed into the TSB until contents were fully dissolved. The top of the beaker was then covered with a piece of foil and autoclaved for 15 minutes at 121^{o}C and 15 psi. After being autoclaved, the beaker was removed from the autoclave and placed in a 2-L water bath (PolyScience, Inc., Niles, Illinois) at 45^{o}C to keep the TSA from solidifying. A thermometer was used to check the temperature of the TSA. A single vial of nalidixic stock solution was removed from the freezer and thawed. Once the solution cooled to 45 ^{o}C, 2 mL of nalidixic stock solution was added to the tryptic soy agar (TSA) using a 100 µL micropipette (Eppendorf Research Plus, Hamburg, Germany).

The bottom of 14 plastic 100 -mm petri plates was appropriately labeled: 12 labeled with the dilution value and sample point location (G or I), 2 blanks, and the remaining petri plates were left blank. TSA was removed from the water bath and aseptically poured into the petri plates. After filling the petri plate halfway with TSA, the lid was placed back onto the plate and the agar was allowed to cool for solidification. All petri plates were then inverted, placed into a plastic sleeve, and stored at 4 ^{o}C.

3.7 Sample Dilutions

In order to determine the appropriate dilution to use that yields the best results for quantifying somatic coliphage, the wastewater sample was diluted. Four dilutions were prepared: 0.1, 0.01, 0.001, and 0.0001. Triplicate testing was done on each dilution to determine precision of the results; therefore each dilution was prepared in triplicates.

Four test tubes with screw caps were placed in a test tube rack and labeled with dilution values (0.1, 0.01, 0.001, and 0.0001). Using a 1000 µL micropipette, 9 mL of tryptic soy broth (without antibiotics) was dispensed into each test tube. Next, the wastewater sample bag was mechanically mixed (by shaking) for 5 seconds to ensure even distribution. A 1 mL aliquot was then taken from the undiluted wastewater sample and added to the test tube labeled “0.1”. The test tube was then capped and mechanically mixed (by shaking). Another 1 mL aliquot was then taken from the test tube labeled “0.1” and added to the test tube labeled “0.01”. The same procedure was followed to obtain the remaining dilutions of “0.001” and “0.0001” as shown in Figure 11.

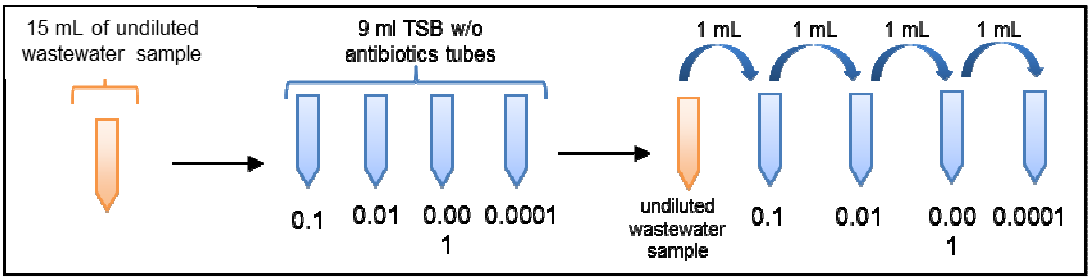

Figure 11: Preparation of wastewater sample dilutions for the double agar layer assay

3.8 Preparation of Host Bacteria Culture

ATCC No. 700609 *E.coli CN-13* freeze-dried bacteria (ATCC, Manassas, Virginia) were revived according to the ATCC instructions (see Appendix M). Once revived, the *E. coli* bacteria solution was transferred into a glass test tube and capped.

Four of the pre-made, unlabeled agar plates were taken from the refrigerator for the culturing of the bacteria using the streak plate method. An inoculating loop was sterilized before use by passing it over a flame and allowing it to cool for 5 seconds before use. The inoculating loop was then swirled around twice in the test tube containing the *E.coli* bacteria solution. Using the streak pattern in Figure 12, the bacteria were streaked onto one of the plates. After streaking the plate, the inoculating loop was passed through the flame again for re-sterilization. The streak method was repeated for the remaining three plates. Once all the plates were streaked with the bacteria, the plates were inverted and incubated overnight at 35^{o}C in an incubator (Boekel Scientific, Feasterville, Pennsylvania). After incubation, the plates were stored (inverted) in plastic sleeves at 4^{o}C.

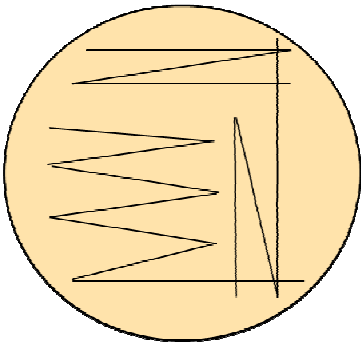

Figure 12: Streak plate method used to culture *E.coli CN-13* host bacteria

3.9 Preparation of Log Phase Host Bacteria

In a 125-mL Erlenmeyer flask, 40 mL of tryptic soy broth was combined with 0.4 mL of nalidixic acid stock solution and swirled to mix. Next, an individual colony from one of the bacteria streak plates was selected and aseptically inoculated into the flask using a sterilized inoculating loop. The flask was mixed by swirling. The flasks were then capped with a piece of foil and incubated for 18 hours at 35^{o}C ± 5^{o}C without shaking. After incubation, a 2 mL aliquot from the incubated flask was added to a second 125 mL Erlenmeyer flask containing 40 mL of tryptic soy broth and 0.4 mL of nalidixic acid stock solution. The flask was mixed by swirling and then incubated for 1 hour at 35^{o}C ± 5^{o}C without shaking.

After 1 hour, the flask was removed from the incubator to check the absorbance reading in the spectrophotometer (HACH, Loveland, Colorado). The spectrophotometer was set to a 520 nm wavelength for the absorbance reading. Next, the spectrophotometer was zeroed using a blank made up of 2 mL of tryptic soy broth and 0.02 mL of nalidixic acid stock solution. A micropipette was used to dispense a 2 mL aliquot from the incubated flask into a plastic curvette. The absorbance reading of the curvette was taken and recorded. Log phase

growth is indicated by an absorbance reading of 0.1 to 0.5 optical density units. If log phase was not reached, the flask was placed back into the incubator and the absorbance was checked every 30 minutes until desired reading was obtained.

3.10 Preparation of 0.7% Tryptic Soy Agar

Next the 0.7% TSA (top layer agar) was prepared. A 200-mL beaker of tryptic soy broth was prepared as described above (Section 3.6). After the TSB was prepared, 1.4 g of agar was added to the beaker while on the heat-stir plate. The contents of the beaker was heated and stirred on the stir heat plate until agar was thoroughly dissolved. Next, the beaker was cooled to room temperature. Once cooled, 0.1 mL of nalidixic acid stock solution was added to the beaker and mixed (by swirling the beaker). Before proceeding, 14 pre-made bottom layer agar petri plates were taken from the refrigerator, allowed to cool to room temperature, and appropriately labeled with the date, dilution value, and sample point (two of the plates were labeled as Blank 1 and Blank 2 with date and indication of sample point location).

Thirteen appropriately labeled test tubes were placed in a test tube rack and placed into a 45^{o}C water bath to keep agar from solidifying before use (see Figure 13). Next, a micropipette was used to aseptically dispense 5 mL of the agar into each test tube. For the twelve tubes labeled with dilution values, 0.5 mL of the respective wastewater dilution was added to the test tube in addition to 0.1 mL of log phase *E.coli CN-13*.

The two remaining test tubes in the water bath served as negative blanks. The purpose of the negative blank was to confirm the validity of the DAL assay

results by indicating contamination (if plaques formed on the petri plate) or non-contamination (if plaques did not form). The blanks did not contain the wastewater sample; instead tryptic soy broth (without antibiotics) was added to the test tube in place of the wastewater sample. Using a micropipette, 0.5 mL of tryptic soy broth (without antibiotics) and 0.1 log-phase *E.coli CN-13* was added to the test tubes.

All the test tubes were then mixed by mechanically inverting the test tube three times. After mixing, the contents of each test tube were poured into their respective bottom layer agar plate and given time to solidify. The plates were immediately inverted and incubated overnight at 35^{o}C ± 5^{o}C.

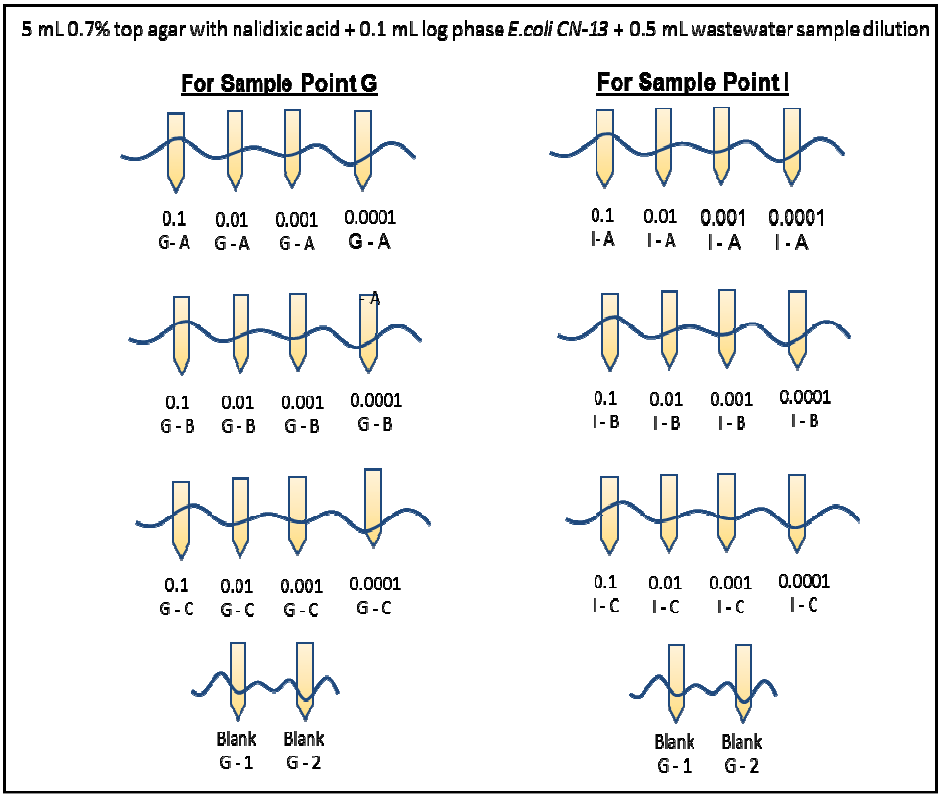

Figure 13: 0.7% Top agar layer test tubes of dilution triplicates 0.1, 0.01, 0.001, 0.0001 for sample points G and I

3.11 Quantification of Plaques

The plates were removed from the incubator and plaques that formed were counted. Plates were placed on a home-made light box (Appendix K) so that the plaques could be seen clearly. The plaques varied in size from small to large. Small plaques received a count of one. When multiple plaques form in the same area, a large plaque is formed. The large plaques that formed on plates with small, countable plaques were also assigned a valued count of 1. The final concentration of somatic coliphage was calculated using Equation 1 (EPA, 2001).

$$C_a = \left(\frac{P \times D}{V} \right) \times 100 \qquad (1)$$

where C_a = somatic coliphage concentration in PFU/100 mL, P = total number of plaques on a single plate, D = reciprocal of dilution made on the inoculum before plating, and V = the volume of sample added to the plate. Results were quantified according to the EPA Method 1602 Section 13.1 (EPA, 2001). Plates that have an un-countable number of plaques are reported as too numerous to count (TNTC) and plates that have no plaque formations are reported as zeros. In calculating the average concentration of somatic coliphage for the influent and effluent, all countable plates (for all dilutions) was averaged. Countable plates are considered as plates that have between 0 – 100 plaque formations.

Liquid waste generated throughout the DAL assay was safely disposed of at the conclusion of the research in the local sewer system after the addition of chlorine. Solid wastes were disposed of through the community waste management system.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Plaque Formation and Quantification Results

Multiple dilutions (0.1, 0.01, 0.001, and 0.0001) were used in the plaque assay to determine which dilution would yield the best results for bacteriophage quantification for the modified double agar layer field method. In plaque quantification, it is important that the appropriate dilution is used so that plaque counts provide a precise count of the number of bacteriophage present in the sample. Plaques should form in small individual circles that are clear and easy to count. In this study, the smallest dilution, 0.1 dilution plates, contained plaque formations that were too numerous to count. The culmination of small plaques forming one large plaque makes it difficult to accurately quantify the bacteriophage concentration. The 0.01 dilution generated more individual, small plaques but still generated the formation of large sized plaques. A dilution of 0.001 had small plaque formations that were clear and easy to count. The highest dilution, 0.0001, had minimal plaque formation. On some of the 0.0001 dilution plates, no plaques formed. Table 11 shows the concentration of somatic coliphage calculated from the plaques formed on each sample dilution plate. According to the plaque formation results for each dilution, the 0.01 dilution yielded the best plaque formation for quantification of somatic coliphage in the influent wastewater collected at point G of the Sapecho wastewater treatment

system while the 0.001 dilution yielded the best results at the effluent of the treatment system for point I.

Table 11: Concentration (PFU/100 mL) of somatic coliphage at sample point G (influent) and sample point I (effluent) at 0.1, 0.01, 0.001, and 0.0001 dilutions

Sample	Somatic Coliphage Concentration (PFU/ 100ml)			
	0.1	0.01	0.001	0.0001
G-A	TNTC	2.20E+06	6.00E+06	1.00E+07
G-B	TNTC	3.70E+06	7.00E+06	0.00E+00
G-C	TNTC	3.50E+06	7.00E+06	0.00E+00
I-A	TNTC	3.00E+05	0.00E+00	0.00E+00
I-B	2.90E+05	1.00E+05	0.00E+00	0.00E+00
I-C	2.30E+05	1.20E+06	1.00E+06	0.00E+00

*TNTC – too numerous to count
*Zero values reported in the table had no plaque formations

The results of the modified double agar layer method presented in this thesis were validated using two negative controls. Negative controls are used to test for outside contamination of samples to ensure that the procedure is being conducted efficiently and producing true results. Two negative blank plates were prepared the same way as the other DAL sample plates with the exception of adding the wastewater sample to the 0.7% top layer agar. In place of adding 0.1 mL of the wastewater sample, 0.1 mL of TSB was added to the top layer agar. After overnight incubation, the negative blanks had no plaque formations, indicating that the double agar layer procedure had no contamination and that the results were valid.

4.2 Somatic Coliphage Removal in Maturation Lagoons

The two maturation lagoons in the Sapecho wastewater treatment system reduced the somatic coliphage concentration within the wastewater. Concentrations at the influent (sample point G) were higher than the concentrations at the effluent (sample point I) for all dilutions except the 0.1 dilution. Table 12 shows the average somatic coliphage concentration of each dilution at both sample points. Dilutions 0.01 and 0.001 are highlighted in Table 12 because as previously stated these dilutions yielded the best results for plaque quantification at the Sapecho wastewater treatment system during the time that the samples were taken.

Table 12: Average concentration at sample point G and I at 0.1, 0.01, 0.001, and 0.0001 dilutions

Dilutions (mL)	Concentration (PFU/100 mL)	
	Avg. Conc. Point G	Avg. Conc. Point I
0.1	TNTC	2.60E+05 (± 4.2E+04)
0.01	3.13E+06 (± 8.1E+05)	5.33E+05 (± 5.8E+05)
0.001	6.67E+06 (± 5.7E+05)	3.33E+05 (± 5.7E+05)
0.0001	3.33E+06 (± 5.7E+06)	0.00E+00

*A zero value indicates no plaque formations; TNTC – plaques too numerous to count.

The log removal of somatic coliphage by the two-series maturation lagoons was calculated by averaging the PFU/100 mL for all countable plates at sample points, point I and point G. There was a 1.05 log (91%) reduction in somatic coliphage concentrations achieved solely through maturation lagoon wastewater treatment. The reduction levels found in the Sapecho wastewater treatment system is less than those reported in previous studies. Somatic

coliphage concentrations in wastewater have been reported to decrease by 2.1 to 4.6 log units in wastewater treated by waste stabilization lagoons (Locus, 2010; Lucena 2004; Campos, 2002; Davies-Colley, 2005).

Using the EPA Method 1602 Section 13.1 instruction for quantification of results (EPA, 2011), the average of all countable plates for the influent samples (point G) and effluent samples (point I) were obtained. The average concentration of somatic coliphages in the influent was determined to be 4.38 x 10^6 PFU/ 100 mL (standard deviation = ± 3.7E+06, n = 9) and the average effluent concentration in the effluent was determined to be 3.90 x 10^5 PFU/ 100 mL (standard deviation = ± 4.5E+05, n = 8).

The reduction of somatic coliphage across the two series maturation lagoon system in Sapecho can be attributed to various mechanisms, primarily solar radiation and sedimentation. There is a lack of research on virus removal mechanisms in waste stabilization lagoons, but research shows that waste stabilization lagoons have good removal rates of pathogens and indicators (Maynard et. al., 1999). The dynamics of the maturation lagoons (shallow depths and long hydraulic retention times) enhance the removal mechanisms by increasing the exposure period to solar radiation and giving time for particles to settle (Berg, 1973; Maynard et al. 1999). Short circuiting within the reactor can result in insufficient hydraulic retention times. As a consequence, the lagoon will not function properly, resulting in insufficient treatment and removal of wastewater constituents and pathogens (Berg, 1973; Lizima, 2012). In a tracer study conducted on a three pond facultative lagoon system in San Antonio

(Bolivia), Lizima (2012) reported that the lagoon treatment system was short-circuiting due to a design flaw of one of the ponds that prevents mixing of incoming wastewater with the standing lagoon water.

Solar radiation (specifically ultraviolet light) penetrates the water column causing somatic coliphage inactivation by damaging phage DNA. Solar inactivation is highest near the surface of the water column because ultraviolet light exposure is highest at the surface and decreases down the water column. Sunlight penetration of the water column depends on the turbidity of the water and the depth of the lagoon. The maturation lagoons in Sapecho are designed with a shallow depth of 1.5 m to allow for solar inactivation of pathogens. The two maturation lagoons of the Sapecho system are shown in Figure 14.

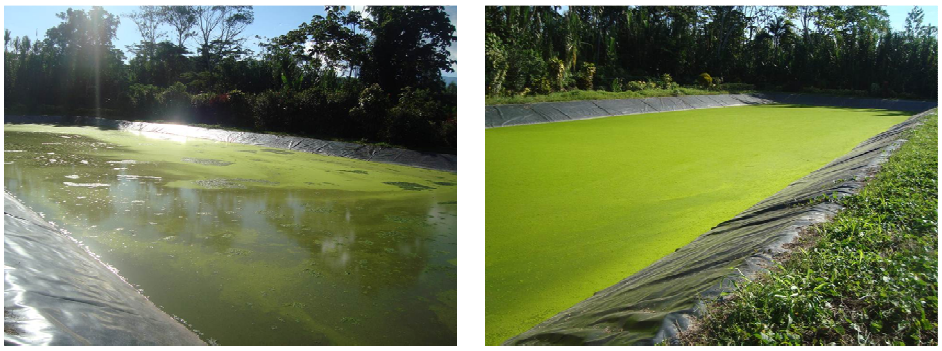

Figure 14: Two - series maturation lagoons in the Sapecho wastewater treatment system

Samples were collected on a partly cloudy, sunny day in mid-June. Solar radiation contributed to the reduction of somatic coliphages found in the results from the modified double agar layer assay. Somatic coliphage reduction levels were lower than the expected reduction levels due the presence of algae and

aquatic plants in the two maturation lagoons which decreased solar penetration of the water.

Ultraviolet inactivation is hindered in water that has a high turbidity because the light is absorbed preventing the light from penetrating the water column. As seen in Figure 14, both maturation lagoons contain a dense layer of algae and an aquatic plant called Lemna cover the top of the water column decreasing solar radiation from reaching lower depths of the water column. In research, Davies-Colley et al. (1997) found that ultraviolet wavelengths caused bacteriophage inactivation in waste stabilization ponds. Therefore, the presence of Lemna that grows on the surface in both Sapecho maturation lagoons decreases the solar inactivation of pathogens within the lagoon system. The low reduction of somatic coliphage (1.05 log removal) found in this research may be attributed to the decrease in solar radiation of the water column.

Sedimentation is another mechanism that attributed to the reduction in somatic coliphage concentration in the two series maturation lagoons. Coliphages absorb to suspended solid particles in the wastewater which successively settle out. Approximately 60 to 100 percent of bacteriophages in waste stabilization lagoons adsorb to suspended solids (Wellings et al., 1976; Vasl and Kott, 1982). In research, coliphages were found to adsorb to suspended solids in an oxidation pond under aerobic conditions (Ohgaki, 1986). Under these conditions and a 20 day retention time, Ohgaki et al., (1986) reported a 1 log unit removal of coliphage in the oxidation pond.

4.3 Somatic Coliphage as an Indicator Organism

Bacteriophages have been identified by many researchers as a good indicator of pathogens in wastewater treatment. Inconstancies in the research and literature raise the question as to which indicator is a better indicator of enteric viruses: bacteriophages or fecal coliforms? Using the findings in this research, somatic coliphage was evaluated using the specifications of a good indicator organism to determine if somatic coliphage can be applied to enteric viruses as a better indicator. The data presented on fecal coliforms and enteric viruses in Table 13 was supplied from studies conducted by two other USF graduate students (Verbyla, 2012; Symonds, unpublished). Table 13, shows the concentrations and log removal of somatic coliphage, enteric viruses, and fecal coliforms in wastewater treated by two maturation lagoons.

Table 13: Concentrations and log removal of somatic coliphages, fecal coliforms, and enteric viruses in influent and effluent treated wastewater at the study site

	Somatic Coliphage Concentration (PFU/ 100mL)	**Fecal Coliforms Concentration** (CFU/ 100 mL)	**Enteric Viruses Concentration** (IU/ 100 mL)
Point G (Influent)	4.38E+06 (±3.7E+06)	1.00E+07(±0)	2.00E+03(±0)
Point I (Effluent)	3.90E+05(±4.5E+05)	7.40E+04(±0)	9.10E+02(±0)
Log removal	**1.05**	**2**	**0.3**

Sources: Concentrations for fecal coliform (Verbyla, 2012) and enteric virus concentrations (Symonds, unpublished)
*Somatic coliphages measured in plaque forming units (PFU); enteric viruses measured in international units (IU); fecal coliforms measured in coliform forming units (CFU).

The reduction of enteric viruses and the two indicators, somatic coliphages and fecal coliforms, can be ranked from highest to lowest: fecal coliforms >somatic coliphages >enteric viruses. Similar results were obtained by

Locas et al. (2010) indicating that wastewater treated by aerated lagoons reduced fecal coliform concentrations up to 3 log units, somatic coliphage concentrations by 2 to 3.4 log units, and enteric viruses were reduced by a lesser extent with removal under 1 log unit.

The data from Table 13 was analyzed according to the specifications of good indicator organisms to determine if somatic coliphage is a better indicator of enteric viruses or fecal coliform in treated wastewater. These characteristics are evaluated below:

1. The indicator should be present when the pathogen is present. Somatic coliphage, enteric viruses, and fecal coliforms were detected in both the influent and effluent wastewater in the Sapecho system. Influent wastewater contained 4.38 E+06 (±3.7E+06) PFU/ 100 mL, 2.00 E+03 IU/ 100 mL, 1.00 E+07 CFU/ 100 mL concentrations of somatic coliphage, enteric viruses, and fecal coliforms, respectively. The treated effluent contained lower concentrations of somatic coliphage, enteric viruses, and fecal coliforms present at 3.90 E+05 (±4.5E+05) PFU/100 mL, 9.10 E+02 IU/ 100 mL, and 7.4 E+04 CFU/ 10 mL, respectively.
2. The indicator should be absent when the pathogen is absent. This characteristic was not examined in this research.
3. Indicator concentration must be higher than the pathogen concentration. In this research, it was found that the concentration of somatic coliphage was higher than the concentration of enteric viruses

in both influent and effluent wastewater samples. The concentration of fecal coliforms was higher than the concentration of enteric viruses in both influent and effluent wastewater samples. Somatic coliphages are more resistant to wastewater treatment than fecal coliforms (Kott, 1966). Overall, both somatic coliphage and fecal coliforms indicators were present at higher concentrations than the pathogen, enteric viruses, in the maturation lagoon system influent and effluent wastewater samples.

4. Indicator and pathogen occur in a constant ratio. The ratio of somatic coliphages to enteric viruses in the influent was approximately 10^3:1 and 10^2:1 in the effluent. The ratio at which somatic coliphage and enteric viruses occurs in wastewater at the Sapecho research site is somewhat constant. In a study by Kott et al (1973), the concentration ratio of bacteriophages to enteric viruses in wastewater treated by oxidation ponds averaged to 10^3:1. The ratio found in the influent of the Sapecho two-series maturation lagoon system was consistent with the ratio found by Kott et al (1973). The ratio of fecal coliforms to enteric viruses was approximately 5×10^3:1 in the influent and 8×10^1:1 in the effluent. The ratio of fecal coliforms to enteric viruses is not very consistent, meaning that the removal of fecal coliforms from the wastewater treatment system is considerably higher than the removal of enteric viruses.

5. Similar characteristics to pathogen. As discussed in Chapter 2, somatic coliphage has very similar characteristics to enteric viruses including size, basic structure, composition, morphology, and reproductive cycle (Grabow, 2001). Fecal coliforms do not have the same characteristics as enteric viruses. Fecal coliforms are bacteria whereas enteric viruses are a virus.
6. Easy Quantification of Indicator. Somatic coliphages are the easiest bacteriophage group to detect and quantify, even in a field setting (Muniesa et al., 1999; Guzman et al., 2007). In this thesis, somatic coliphage was detected using a modified double agar layer assay. Samples were analyzed using this method and yielded results within 24 hours. In comparison, fecal coliforms are more difficult to quantify than somatic coliphage because they require a more sterile laboratory environment to conduct the quantification procedure.
7. Same resistances to environmental conditions and disinfection of pathogen. The presence of somatic coliphage, enteric viruses, and fecal coliforms in the treated effluent shows that both viruses and coliforms are somewhat resistant to wastewater treatment, in this case, wastewater treatment by two maturation lagoons. In Sapecho's two series maturation lagoons, pathogens and indicator organisms are removed by sedimentation, solar radiation, pH, temperature, predation, and natural die-off. The primary mechanisms attributed to the reduction of enteric viruses and somatic coliphages are solar radiation and

sedimentation (Rao et al., 1980; IAWPRC Study Group, 1990). Fecal coliforms, on the other hand, are removed primarily through sedimentation because they are dense and tend to settle out (Verbyla, 2012; Feachem, 1983; Shuval, 1986). Somatic coliphages had a higher reduction level (1.05 log removal) in the maturation lagoon system than enteric viruses (0.3 log removal). The removal of fecal coliforms compared to both viruses was higher at a value of 2 log removals. The level of removal for somatic coliphages is closer to the removal level of enteric viruses by 0.75 log units.

8. The indicator test is applicable to all water types. This indicator characteristic was not examined in this research.
9. The indicator test only detects the specified pathogen and does not yield false positives. This parameter was not examined in this research.

Somatic coliphages meet many of the specifications listed and therefore qualify as a better indicator for enteric viruses than fecal coliforms in wastewater, according to evaluation done in this research. Somatic coliphage concentrations in the treated wastewater were consistently higher than enteric virus concentration which yielded appropriate ratios. Previous research shows that bacteriophage removal in wastewater treatment is significantly higher than enteric virus removal which was removed at nearly 1 log unit (Locas, 2010). Enteric viruses and somatic coliphages are both similar in size, composition, and have the same reproductive cycle; on the other hand, fecal coliforms bacteria

and are much less similar to enteric viruses. Additionally, the log reduction of enteric viruses (0.3 log removal) was closer in value to the log reduction of somatic coliphages (1.05 log removal) within the Sapecho two-series maturation lagoon system; therefore indicating that enteric viruses and somatic coliphages have similar resistances to wastewater treatment. The modified double agar layer method used to detect somatic coliphage was simple and yielded results comparable to previously conducted research related to this thesis. Somatic coliphage met many of the indicator specifications related to enteric viruses. This supports previous research and literature that identifies bacteriophages as a good indicator of enteric viruses (Stetler, 1984; Wentsel, 1982; Eaton et al., 2000; Duran et al., 2002).

4.4 Evaluation of Modified Double Agar Layer Assay for Field Usage

The modified double agar layer assay method was fairly simple to conduct and yielded somatic coliphage quantification results similar to those found in previous studies. This modified method was conducted in a developing world setting for measuring bacteriophage concentrations in treated wastewater. Modifications made to the EPA Method 1602 enabled the performance of the double agar layer assay using materials and field laboratory conditions available in the developing world. Table 14 compares and contrasts the EPA Method 1602 with the modified field method used in this thesis.

The field conditions presented challenges for conducting the double agar layer method. Sterile conditions are essential in conducting microbial laboratory procedures.

Table 14: Comparison of double agar layer assay methods: EPA Method 1602 vs. modified field method

EPA Method 1602	Modified Field Method
Sterile lab	Screened in room
Multiple power sources for use of electrical equipment	One outlet for use of electrical equipment
DI water	Distilled water / DI water
Bunsen burner	Lighter
1 liter volume solutions	500 mL volume (or less) solutions
Incubation at 35^{o}C	Incubation at 35-40^{o}C
Log Phase Bacteria Growth: 16-hour bacteria culture with continuous shaking	Log Phase Bacteria Growth: 18-hour bacteria culture without shaking

The field laboratory that was set-up near the research site presented the following challenges:

- Single outlet available for use of multiple electricity powered devices
- Dusty lab conditions presented a challenge for keeping the space sterile
- Screened in windows opened lab space to unfavorable conditions (e.g. humidity)

Utilizing a single outlet for the electricity powered devices (heat-stir plate, water bath, incubator, autoclave, and spectrophotometer) was the most difficult challenge to handle in the field setting. The laboratory space had a single outlet that had a 220 voltage current; therefore a voltage converter was necessary for the use of the electrical devices which had a 120 voltage capacity. In using a single outlet, the use of the electrical devices had to be alternated in a systematic manner. For example, the autoclave would be used at the beginning of the work

day to sterilize the glassware and make tryptic soy broth or tryptic soy agar needed for the day. The recommended incubation temperature for the double agar layer assay is generally 35^{o}C. During the times that the bacteria were incubated for log phase growth, the incubator had to be unplugged so that the water bath and heat-stir plate could be used. To ensure that the incubator temperature did not fall below 35^{o}C, the temperature was raised to 38 to 40^{o}C prior to being unplugged. After using the other devices, the incubator was checked and the temperature decreased by two or three degrees. The incubator was maintained a temperature range of 35 to 40^{o}C to achieve log phase bacterial growth because studies show that optimum *E. coli* growth occurs at this temperature range (Rattanabumrung et al., 2012).

In order to keep the lab as clean as possible while conducting research, the floors were swept every day before and after work, all glassware and metal equipment were sterilized in the autoclave before and after use, and tables and equipment were wiped down with a 10% bleach solution and ethanol after work. Cloth curtains were placed over the screened in windows to keep out dust particles, minimize humidity, and prevent insects from entering the laboratory space. Furthermore, sterile equipment was stored in large, zipped canvas bags underneath the table to prevent exposure from outside contamination. These preventive measures attribute to the success of the modified field method double agar layer and the validity of the method's results obtained in Bolivia.

Humidity is a negative factor that can cause discrepancies in the results of the double agar layer method. As mentioned previously in this research, male-

specific coliphage was quantified using the same modified double agar layer method presented in Chapter 3. Samples were collected at the same two sample points (point G and point I) of the Sapecho wastewater treatment system. The male-specific coliphage was quantified one day after the somatic coliphage, where it rained continuously throughout the day. The precipitation increased the humidity in the air, increasing the humidity in the field laboratory. The increase in humidity is attributed to the inconsistent results and contaminated negative blanks in the quantification of the male-specific coliphage. The humidity prevented the solidification of a few of the top layer agar plates which yielded faulty results. Humidity is a field laboratory challenge that can interfere with the modified doulble agar layer method and cause errors in the quantification results.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

The integrity of the global water supply is threatened by the increasing demand of water due to the growing global population and demand. Water scarcity is a global concern that impacts many developing countries forcing people to depend on unclean water sources for domestic, agricultural, and industrial needs. The agricultural sector consumes the highest amount of water. Wastewater is an alternative water source that also contains nutrients needed for crop growth.

The objective of this research was to develop a modified double agar layer assay method that can be conducted in the field to quantify bacteriophage to assess the quality of wastewater for agricultural reuse. Furthermore, results from the modified double agar layer assay were used to investigate the potential of somatic coliphage as a better indicator organism for enteric viruses. It was hypothesized that a modified EPA double agar layer method can be developed and deployed in a developing world rural community to effectively quantify the concentration of somatic coliphage in a community managed wastewater treatment system and thus serve as a good indicator of enteric viruses in the water.

5.1 Conclusions

A modified double agar layer assay was developed from the EPA Method 1602 in order to measure the concentration of bacteriophage in wastewater. The modified double agar layer assay can be used in the developing world field setting to detect and quantify bacteriophage for the purpose of water and wastewater quality monitoring. In conducting the modified DAL method at the Sapecho wastewater treatment system in June, the 0.01 dilution yielded the best results for somatic coliphage quantification in the influent wastewater sample and the 0.001 dilution yielded the best results for somatic coliphage quantification in the effluent wastewater sample. These two dilutions yielded the best results for this particular research that was being conducted at that time. The characteristics of wastewater vary with location, seasons, and population dynamics; therefore multiple dilutions are needed in order to determine the best dilution for a particular study.

The average concentration of somatic coliphages in the influent was 4.38 x 10^6 PFU/ 100 mL (standard deviation = ±3.7E+06, n = 9) and the average effluent concentration in the effluent was 3.90 x 10^5 PFU/ 100 mL (standard deviation = ± 4.5E+05, n = 8). Overall, the two series maturation lagoons at the Sapecho research site removed bacteriophage from the wastewater with 1.05 log removal efficiency. It was expected that somatic coliphage would be removed by up to 2 log units by the two series maturation lagoons. The bacteriophage removal efficiency found in this study is lower than the removal efficiencies reported in previous research which ranged from 2.1 to 5.5 log removal units

(Locas et al., 2010; Harwood, et al., 2005; Reinoso et al., 2011; Lucena et al., 2004; Campos et al., 2002; Davies-Colley et al., 2005). Pathogen removal in the two series maturation lagoon system is likely low due to the short hydraulic retention time of the wastewater. In a tracer study, Lizima (2012) found that the two series maturation lagoons had a theoretical hydraulic retention time of 24.1 days.

There are no specific guidelines addressing virus removal in wastewater treatment for wastewater reuse. The WHO standards indicate that wastewater pathogens, in general, must be reduced by 6 to 7 log units for unrestricted irrigation of food crops and 3 to 4 log units for restricted irrigation (WHO, 2004). Somatic coliphages have been detected in raw wastewater at concentrations of 10^4/mL or 10^6/100 mL (Jofre, 2008). Using the higher concentration range, it can be estimated that there was an overall somatic coliphage reduction of 1.3 log units which does not meet the WHO required pathogen reduction standards for wastewater reuse in agricultural irrigation.

Similar to the WHO guidelines, EPA guidelines do not have specific removal standards for viruses relating to wastewater reuse. EPA guidelines measure the quality of wastewater for reuse by the concentration of fecal coliforms per 100 mL; therefore virus concentrations in treated effluent of this study site cannot be used to determine wastewater compliance with EPA standards for wastewater reuse in agriculture. The fecal coliform concentration in the treated effluent was measured to be 7.4E+04 CFU/ 100 mL (Verbyla, 2012). The EPA guidelines require less than or equal to 200 fecal coliforms/ 100mL for

wastewater reuse in agricultural irrigation of food and non-food cops. According to these EPA guidelines, the Sapecho wastewater treatment system's effluent water quality does not meet EPA's agricultural reuse standards.

Somatic coliphages have the potential to be good indicator organisms for enteric viruses according to the specifications of an indicator organism. A few of the characteristics that make somatic coliphages a good indicator organism for enteric viruses are that both viruses are: (1) similar in characteristics, (2) resistant to the same treatment, and (3) present in wastewater. The indicator organism specifications are listed in Table 15 along with the supporting observations from this research.

Table 15: Evaluation of somatic coliphage as an indicator organism for enteric viruses and supporting observations

Met (✓) , Not met (X) in this study, or (NE) Not examined	Indicator Characteristic	Observations
✓	Indicator easy to detect	Somatic coliphages were easily detected and quantified in the field setting using a modified double agar layer method
✓	Indicator should be present when the pathogen is present	Both enteric viruses and somatic coliphages were detected in both the influent and effluent of the maturation lagoon system
NE	Indicator should be absent when the pathogen is absent	This characteristic was not examined in this research
✓	Indicator and pathogen should have similar characteristics	Somatic coliphages are viruses that infect E.coli only. They are similar in size, composition, and have the same reproductive cycle as enteric viruses

Table 15: Continued

NE	Indicator should only detect specified pathogen and not yield false positives	This characteristic was not examined in this research
✓	The test for the indicator should be applicable all water types	This characteristic was not examined in this research but the double agar layer method is applicable for the detection of somatic coliphage in both water and wastewater samples (EPA, 2001).
✓	Indicator concentration in water source should far proceed the pathogen concentration	The somatic coliphage concentration in the influent wastewater was 3.3 log units higher than that of enteric viruses and 2.6 log units higher in the treated effluent
✓	Indicator and pathogen should have similar resistances to treatment and disinfection	Both enteric viruses and somatic coliphage were present in the treated effluent. Both are also inactivated by the same mechanisms, primarily solar radiation and sedimentation. The two series maturation lagoon system removed somatic coliphage by 1.05 log units and enteric viruses by 0.3 log units
X	Indicator and pathogen should exist in water source at same ratio	The ratio of enteric viruses to somatic coliphages in the influent was $1{:}10^3$ and $1{:}10^2$ in the treated effluent; ratio was somewhat consistent but not exactly

Although somatic coliphages meet many of the indicator qualifications for enteric viruses there are limitations, as shown in Table 15. Unlike enteric viruses, somatic coliphage exists naturally in natural water sources such as rivers and streams where they are able to replicate (Sinkova and Cervenka, 1981; Jofre, 2008). Therefore in natural water (freshwater and salt water), somatic coliphages

can be present when enteric viruses are not present giving a false indication of the presence of enteric viruses. Furthermore, the last characteristic item in Table 15 describes the ratio at which enteric viruses and somatic coliphage exist in treated wastewater. This ratio was somewhat consistent by a difference of 1 log. This may give an inaccurate count of the number of enteric viruses that are present in the wastewater in relation to the actual concentration of enteric viruses. Overall, somatic coliphages qualify as a good indicator organism for the presence of enteric viruses in treated wastewater in this research but indication of these organisms does not accurately predict the actual concentration or removal efficiency of the enteric viruses in wastewater treatment by maturation lagoons.

5.2 Recommendations

The wastewater treatment system in Sapecho does not produce quality effluent that meets wastewater reuse guidelines. Pathogen concentrations in the research site effluent exceed the required pathogen concentration standards set by the World Health Organization and Environmental Protection Agency. In order to meet these standards the wastewater treatment system can be improved to increase the efficiency of pathogen reduction. This can be done by increasing the hydraulic retention time of the maturation lagoons. By increasing the hydraulic retention time, pathogens will have longer exposure to natural disinfection mechanisms (e.g. solar radiation, pH, temperature, predation, and natural die-off). In addition, a longer hydraulic retention time will allow more pathogens and particles containing adsorbed pathogens to settle out of the wastewater. Ohgaki

et al. (1987) found that increasing the hydraulic retention time in waste stabilization lagoons can lead to increased reduction of bacterial concentrations. The hydraulic retention time can be increased by adding a third maturation lagoon to the Sapecho wastewater treatment system or by decreasing the incoming flow of wastewater into the treatment system.

Further research is needed to better understand the potential of bacteriophage as an indicator organism for enteric viruses. The three groups of bacteriophages commonly used as pathogenic indicators in wastewater should be quantified and evaluated according to the indicator specifications list. By comparing the three bacteriophages, researchers can determine which bacteriophage group yields the quantification results that are most similar to the actual enteric virus concentrations that exist in the wastewater. Indicators are supposed to be indications of the presence of pathogens and provide a fairly accurate estimation of the actual concentration of pathogens in the wastewater.

In addition, more research should be done on identifying the specific removal mechanisms of bacteriophages and viruses from waste stabilization lagoons, particularly maturation lagoons. Research has reported that viruses in general are removed from waste stabilization ponds through sedimentation, pH, solar radiation, temperature, predation, natural die-off, and the presence of ammonia (Rao, 1980; IAWPRC Group, 1990; Maynard, 1998; Mara, 2004). Each mechanism should be analyzed to better understand to what degree each mechanism contributes to the overall reduction of viral and bacteriophage concentrations in wastewater treated by waste stabilization lagoons.

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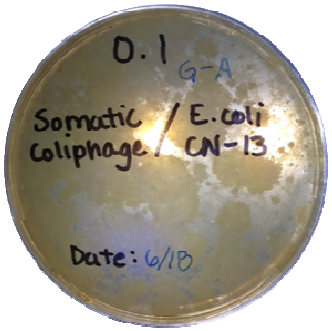
APPENDICES

Appendix A – Somatic Coliphage Quantification Results for Sample Point G

Sample ID	Dilution	Number of Plaques	Volume WW (ml)	Concentration PFU/100 ml	Observations
G-A	0.1	COUNTLESS	0.1	0.00E+00	
G-A	0.01	22	0.01	2.20E+06	21 clear plaques plus large blotch
G-A	0.001	6	0.001	6.00E+06	5 clear plaques plus large blotch
G-A	0.0001	1	0.0001	1.00E+07	
G-B	0.1	COUNTLESS	0.1	0.00E+00	
G-B	0.01	37	0.01	3.70E+06	36 clear plaques plus large blotch
G-B	0.001	7	0.001	7.00E+06	
G-B	0.0001	0	0.0001	0.00E+00	
G-C	0.1	COUNTLESS	0.1	0.00E+00	
G-C	0.01	35	0.01	3.50E+06	34 clear plaques plus large blotch
G-C	0.001	7	0.001	7.00E+06	6 clear plaques plus one large blotch
G-C	0.0001	0	0.0001	0.00E+00	
Blank I-1	N/A	0	N/A	ND	
Blank I-2	N/A	0	N/A	ND	

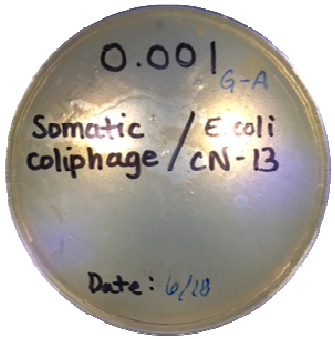
Samples taken at 10:30 am on 6/17/12, analyzed on 6/18/12, and results were read from plates on 6/19/12
*ND = no data
*N/A = not applicable
*TNTC = too numerous to count

Appendix B – Pictures of Somatic Coliphage DAL Plate Results for Sample Point G

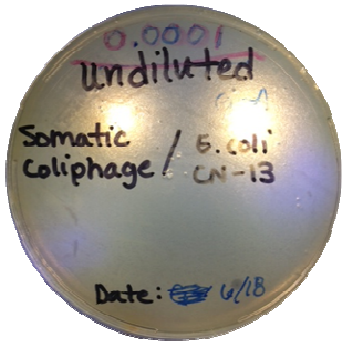


G-A
Dilution 0.1

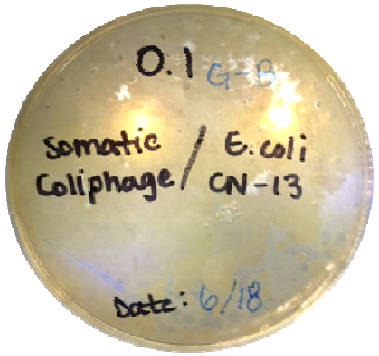
G-A
Dilution 0.01



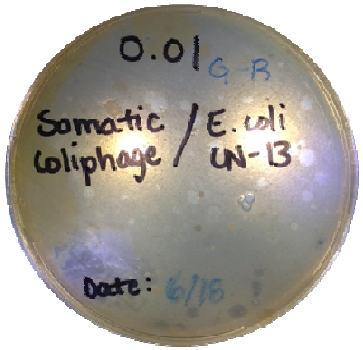
G-A
Dilution 0.001



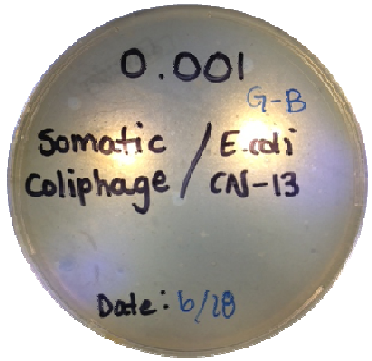
G-A
Dilution 0.0001



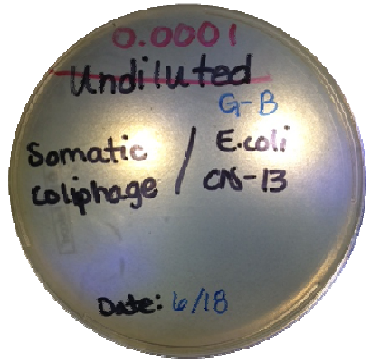
G-B
Dilution 0.1



G-B
Dilution 0.1

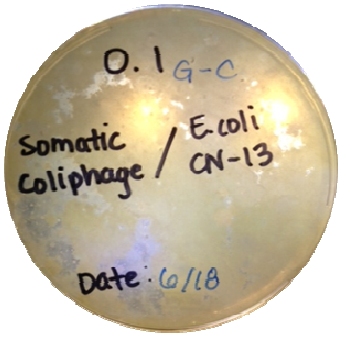


G-B
Dilution 0.1

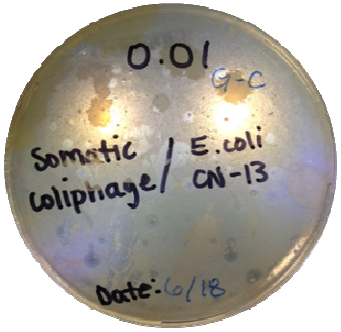


G-B
Dilution 0.1

Appendix B – Continued

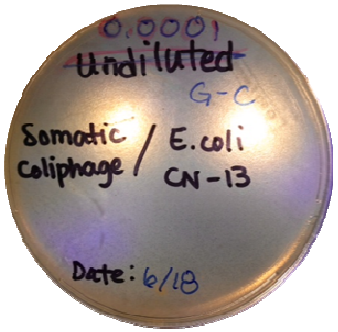


G-C
Dilution 0.1

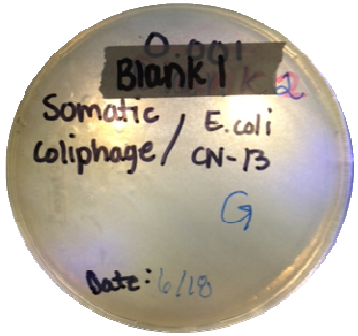


G-C
Dilution 0.01

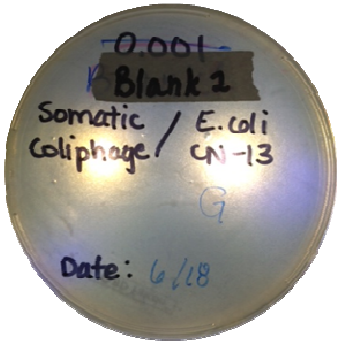
G-C
Dilution 0.001



G-C
Dilution 0.0001



G Blank 1



G Blank 2

Appendix C – Somatic Coliphage Quantification Results for Sample Point I

Sample ID	Dilution	Number of Plaques	Volume WW (ml)	Concentration PFU/100 ml	Observations
I-A	0.1	COUNTLESS	0.1	TNTC	
I-A	0.01	3	0.01	3.00E+05	
I-A	0.001	0	0.001	0.00E+00	
I-A	0.0001	0	0.0001	0.00E+00	
I-B	0.1	29	0.1	2.90E+05	28 clear plaques plus large blotch
I-B	0.01	1	0.01	1.00E+05	
I-B	0.001	0	0.001	0.00E+00	
I-B	0.0001	0	0.0001	0.00E+00	
I-C	0.1	23	0.1	2.30E+05	22 clear plaques plus large blotch
I-C	0.01	12	0.01	1.20E+06	
I-C	0.001	1	0.001	1.00E+06	
I-C	0.0001	0	0.0001	0.00E+00	
Blank I-1	N/A	0	N/A	ND	
Blank I-2	N/A	0	N/A	ND	

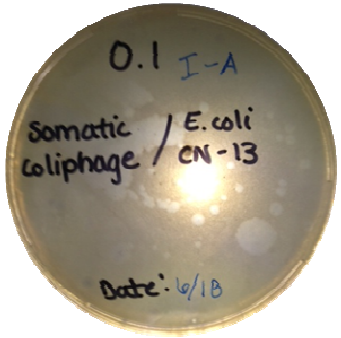
Samples taken at 10:30 am on 6/17/12, analyzed on 6/18/12, and results were read from plates on 6/19/12

*ND = no data

*N/A = not applicable

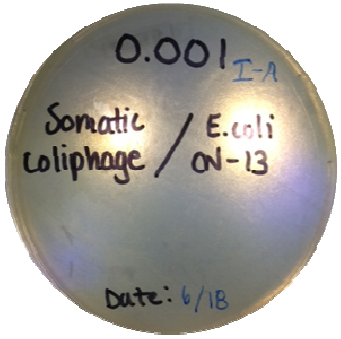
*TNTC = too numerous to count

Appendix D – Pictures of Somatic Coliphage DAL Plate Results for Sample Point I

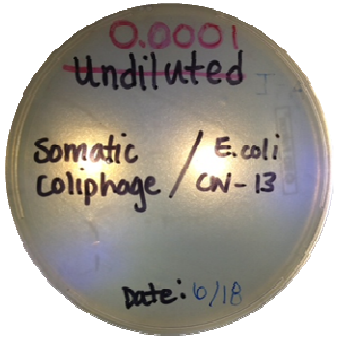


I-A
Dilution 0.1

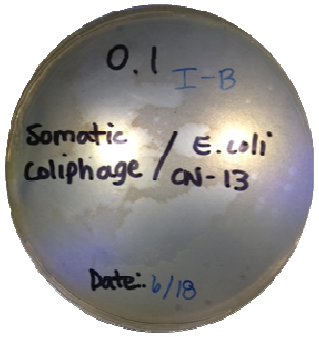
I-A
Dilution 0.01



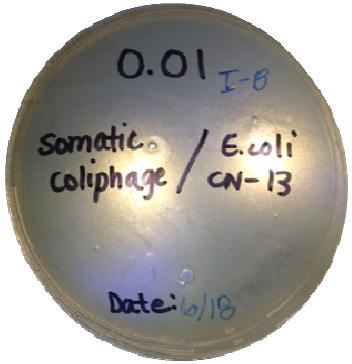
I-A
Dilution 0.001



I-A
Dilution 0.0001

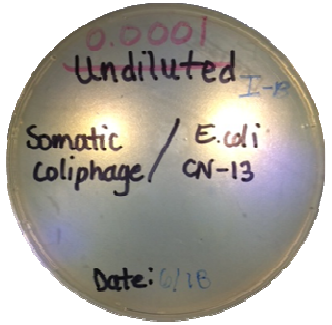


I-B
Dilution 0.1



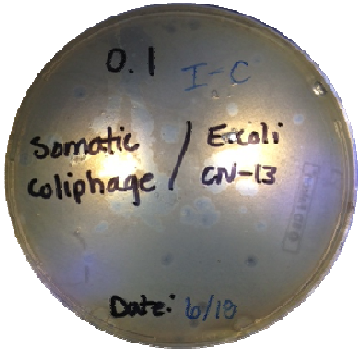
I-B
Dilution 0.01

I-B
Dilution 0.001

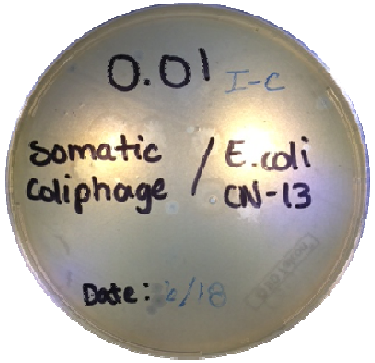


I-B
Dilution 0.0001

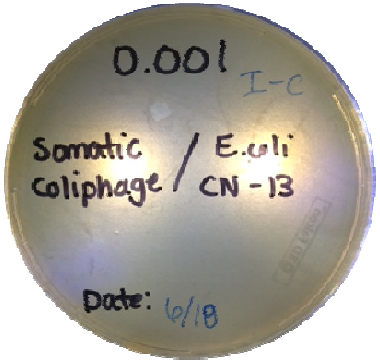
Appendix D – Continued



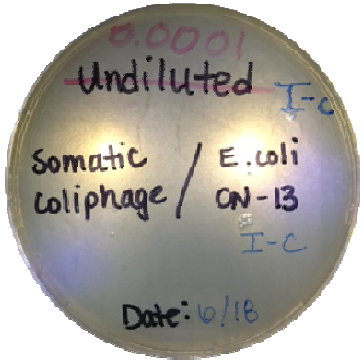
I-C
Dilution 0.1



I-C
Dilution 0.01



I-C
Dilution 0.001



I-C
Dilution 0.0001

I Blank 1

I Blank 2

Appendix E – Male-Specific Coliphage Quantification Results for Sample Point G

Sample ID	Dilution	Number of Plaques	Volume WW (ml)	Concentration PFU/100 ml	Observations
G-A	0.1	DAMAGED	0.1	N/A	Agar did not solidify
G-A	0.01	DAMAGED	0.01	N/A	Agar did not solidify, but one large plaque was visible
G-A	0.001	DAMAGED	0.001	N/A	Agar did not solidify
G-A	0.0001	8	0.0001	8.00E+06	
G-B	0.1	COUNTLESS	0.1	TNTC	
G-B	0.01	0	0.01	0.00E+00	
G-B	0.001	0	0.001	0.00E+00	
G-B	0.0001	2	0.0001	2.00E+06	One large plaque one small plaque
G-C	0.1	COUNTLESS	0.1	TNTC	
G-C	0.01	0	0.01	0.00E+00	
G-C	0.001	0	0.001	0.00E+00	
G-C	0.0001	2	0.0001	2.00E+06	
Blank I-1	N/A	2	0	ND	
Blank I-2	N/A	0	0	ND	

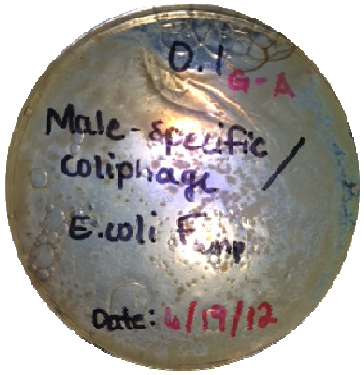
Samples taken at 10:30 am on 6/17/12, analyzed on 6/19/12, and results were read from plates on 6/20/12

*ND = no data

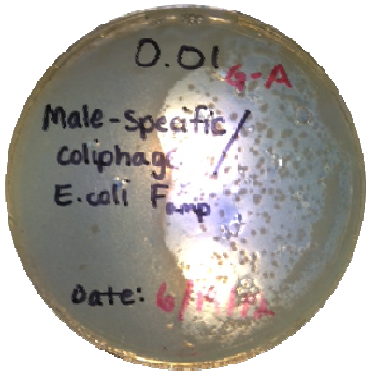
*N/A = not applicable

*TNTC = too numerous to count

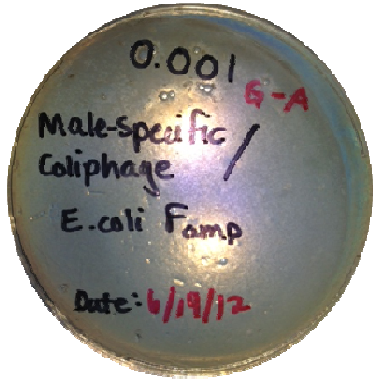
Appendix F – Pictures of Male-Specific Coliphage DAL Plate Results for Sample Point G



G-A
Dilution 0.1

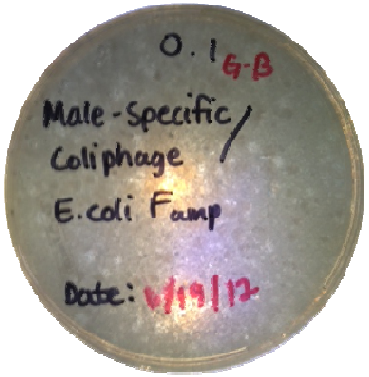


G-A
Dilution 0.01

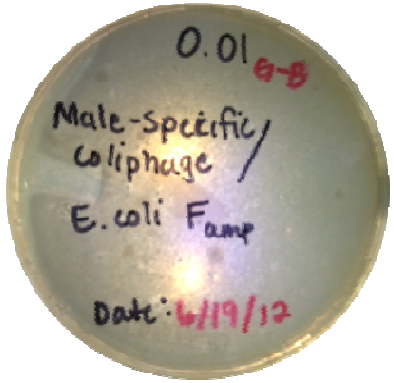


G-A
Dilution 0.001

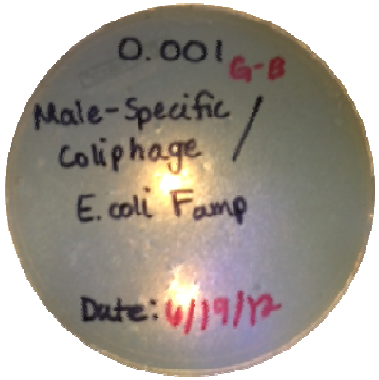
G-A
Dilution 0.0001



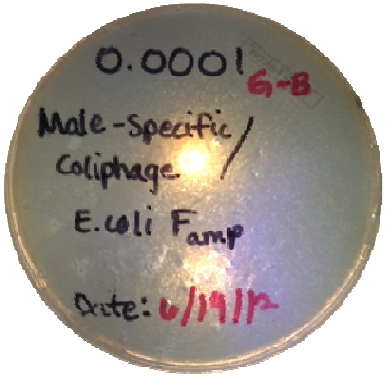
G-B
Dilution 0.1



G-B
Dilution 0.1



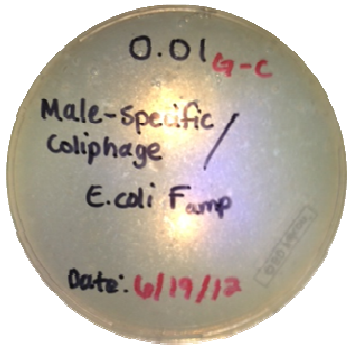
G-B
Dilution 0.1



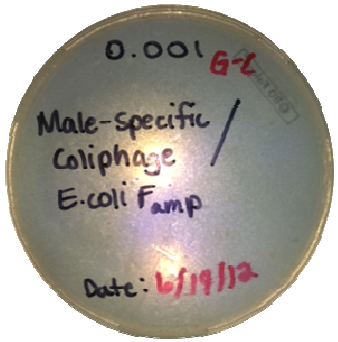
G-B
Dilution 0.1

Appendix F – Continued

G-C
Dilution 0.1

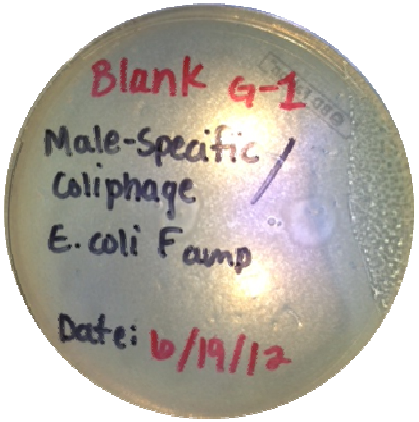


G-C
Dilution 0.01



G-C
Dilution 0.001

G-C
Dilution 0.0001



G Blank 1

G Blank 2

Appendix G – Male-Specific Coliphage Quantification Results for Sample Point I

Sample ID	Dilution	Number of Plaques	Volume WW (ml)	Concentration PFU/100 ml	Observations
I-A	0.1	COUNTLESS	0.1	TNTC	
I-A	0.01	12	0.01	1.20E+05	
I-A	0.001	3	0.001	3.00E+05	
I-A	0.0001	11	0.0001	1.10E+07	
I-B	0.1	33	0.1	3.30E+04	
I-B	0.01	28	0.01	2.80E+05	
I-B	0.001	21	0.001	2.10E+06	
I-B	0.0001	4	0.0001	4.00E+06	
I-C	0.1	0	0.1	0.00E+00	
I-C	0.01	COUNTLESS	0.01	TNTC	
I-C	0.001	13	0.001	1.30E+06	
I-C	0.0001	5	0.0001	5.00E+06	
Blank I-1	N/A	7	N/A	ND	
Blank I-2	N/A	11	N/A	ND	

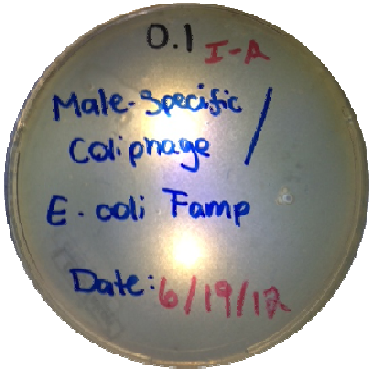
Samples taken at 10:30 am on 6/17/12, analyzed on 6/19/12, and results were read from plates on 6/20/12

*ND = no data

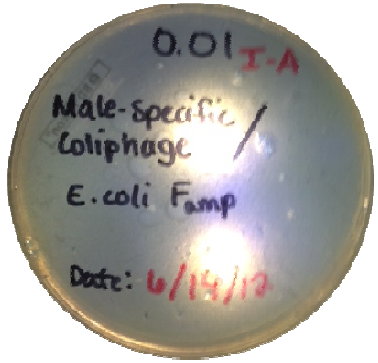
*N/A = not applicable

*TNTC = too numerous to count

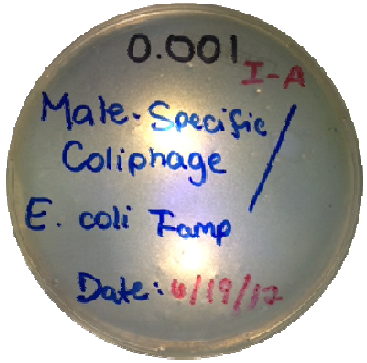
Appendix H – Pictures of Male Specific Coliphage DAL Plate Results for Sample Point I



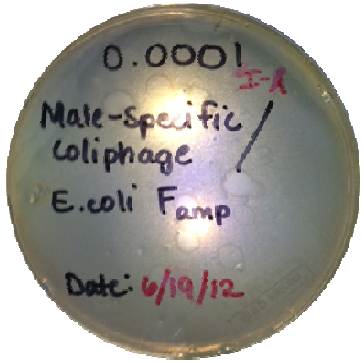
I-A
Dilution 0.1



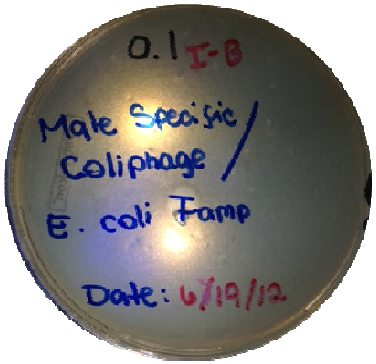
I-A
Dilution 0.01



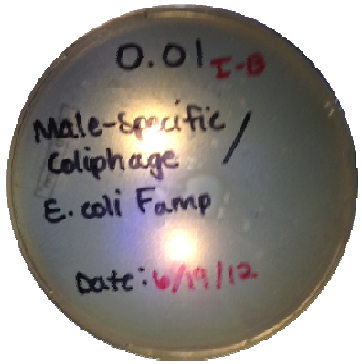
I-A
Dilution 0.001



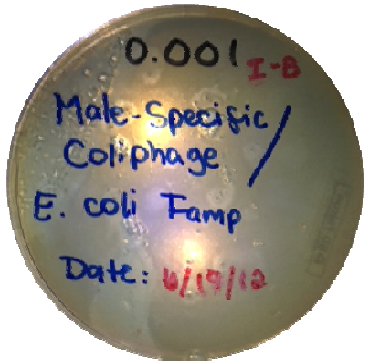
I-A
Dilution 0.0001



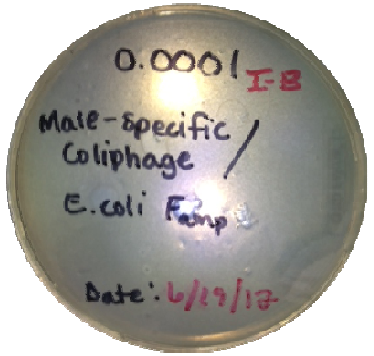
I-B
Dilution 0.1



I-B
Dilution 0.1



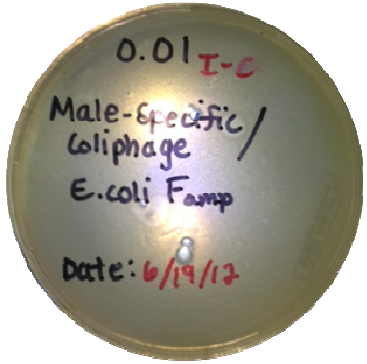
I-B
Dilution 0.1



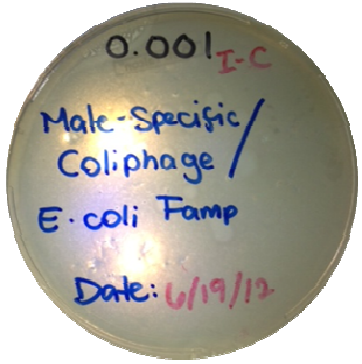
I-B
Dilution 0.1

Appendix H – Continued

I-C
Dilution 0.1



I-C
Dilution 0.01



I-C
Dilution 0.001

I-C
Dilution 0.0001

I Blank 1

I Blank 2

Appendix I – Discussion of Male-Specific Coliphage Double Agar Layer Assay Quantification Results

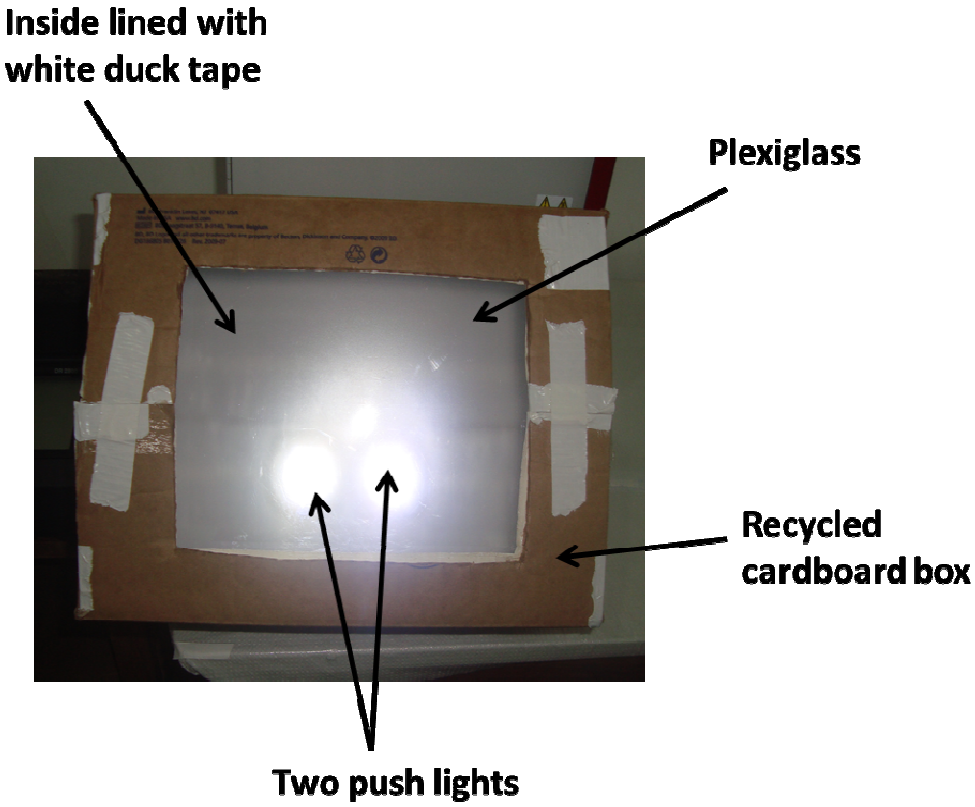
Male-specific coliphages were also detected in the Sapecho wastewater treatment system using the modified double agar layer assay method. In order to detect male-specific coliphages, *E.coli* F_{amp} was cultured and used as the host bacteria. In addition, a streptomycin/ampicillin antibiotic stock was used instead of the nalidixic acid stock. The day that the sample was analyzed, it rained all day. The rain created a humid condition in the field laboratory which made it more difficult for the agar plates to solidifying. Agar plates that did not solidify in the 5 minute time period were still placed into the incubator without being inverted. Results from the male-specific double agar layer assay were inconsistent and the negative blanks had plaque formations which mean that the samples were contaminated by an outside source. Therefore, the results of the male-specific quantification were not considered for this research. The challenge of humid conditions resulted in the contamination of the agar plates.

Appendix J – List of Materials

Autoclave tape
Beaker, 200-mL
Beakers, 100-mL
Deionized water
Escherichia coli CN-13
Erlenmeyer flask
Glycerol
Graduated cylinder
Heat-stir plate
Incubator
Inoculating loop
Labeling tape
Microfilter, 0.22-µm
Micropipette, 100-µL
Micro-test tubes (2-mL, plastic)
Micropipette, 1000-µL
Nalidixic acid
Parafilm
Petri dishes (plastic, 100-mm)
Spoon
Stir bar
Syringes (plastic, 60-mL)
Streptomycin
Test tubes (glass with screw caps)
Test tubes (plastic with screw caps)
Test tube rack
Timer
Tryptic soy broth mix
Tweezers
Water bath
Weighing paper
Weighing scale (with 0.01 accuracy)

Appendix K – Picture of Light Box Used for Counting Plaques

The collapsible light box was constructed from a recycled cardboard box, white duct tape, and a 1ft-by-1ft piece of plexiglass sheet. The inside of the box was lined with white duct tape to help reflect the light for improved visual of the plaques. Two small push lights (battery operated) were placed inside the box as a light source. Petri plates were placed on the light box in order to easily identify and count plaques.



Appendix L – Step-by-Step Modified Double Agar Layer Assay Used in Bolivia

The step-by-step modified double agar layer method used for day 1 is provided below. Day 1 consists of preparing the antibiotic stock solutions, preparing the bottom agar layer plates, and culturing the host bacteria.

1. General Stock Solutions
    - 50% *Glycerol Stock* – Add 5 mL of glycerol to 5 mL of DI water and mix thoroughly to dissolve. Autoclave for 15 minutes at 121^{o}C and 15 psi. Add 2 mL of glycerol stock to 800 mL of log phase bacteria in TSB.
2. Prepare Antibiotic Stocks
    - Fill two 200 mL beakers with 100 mL of the DI water (label one beaker" Nalidixic Acid Stock" and the other beaker "Streptomycin/Ampicillin Stock").
    - Autoclave DI water for 15 minutes at 121^{o}C and 15 psi. Allow to cool.
    - Add appropriate antibiotics to the autoclaved DI water:

      Somatic: Add 1 g of nalidixic acid to one beaker and dissolve.

      Male-Specific: Add 0.15 g of ampicillin sodium and 0.15 g of streptomycin sulfate to the other beaker and dissolve
    - Use a syringe and a 0.22 µm filter syringe cap to dispense 5 mL of the stock into a 15 mL freezer vial. Repeat until both solutions are

completely transferred to the vials (separate set of vials for each antibiotic stock: somatic and male-specific).

- Store unused vials at -2 0^{o}C for up to one year. For reuse: thaw at room temperature or at 36^{o}C ± 1^{o}C in a water bath and mix well.

3. Prepare Tryptic Soy Broth (TSB) Media
   - Pour 200 mL of DI water into a 500 mL beaker with screw cap.
   - Add 6 g of tryptic soy broth mix to the beaker.
   - Place the beaker on a heat-stir plate and insert a stir bar into the media. Heat the media to a boil while stirring to completely dissolve the tryptic soy broth mix and allow to cool.
   - Repeat for a second beaker.

   ** Prepared broth is a clear, yellowish-brown color.*

4. Prepare 1.5% Tryptic Soy Agar (TSA)

   (Used for streak plates and bottom layer of agar)

   - Add 3 g of agar to each Erlenmeyer flask containing TSB media.
   - Put the beaker on a heat-stir plate and insert a stir bar into the media. Heat the media to a boil while stirring to completely dissolve the agar.
   - Autoclave the TSB media for 15 minutes at 121^{o}C and 15 psi.
   - Using a pipette, add the appropriate antibiotic stock to the 200 mL of autoclaved 1.5% TSA:

     Somatic: Aseptically add 2 mL of stock nalidixic acid.

Appendix L – Continued

Male-Specific: Aseptically add 2 mL of stock ampicillin/streptomycin.

**Add antibiotic stock to the beaker and keep solution in the water bath to prevent agar from solidifying.*

- Swirl flasks to mix well.
- Label the bottom of 18 petri plates as shown below.
- Aseptically pour 1.5% TSA into each 100-mm petri plate, filling the plate about half way.
- Aseptically replace the lids and allow the agar to solidify for before use. Once agar is solid, invert the dish.

  ** If not used immediately, store the plates inverted at $4^{o}C \pm 1^{o}C$ for up to 2 weeks.*

5. Revive Freeze-dried Bacteria – refer to Appendix M
6. Attain Isolated Colonies
   - Isolate bacteria colonies by streaking the host bacteria onto 1.5% TSA plates with the appropriate antibiotic stock.

     E.coli CN-13 = stock nalidixic acid

     E.coli F_{amp} = stock ampicillin/streptomycin
   - Incubate the streak plates overnight at $35^{o}C \pm 5^{o}C$.
7. Grow Log-Phase Bacteria

Appendix L – Continued

- Add 40 mL of TSB with stock nalidixic acid to a 125 mL Erlenmeyer flask and add 40 mL of TSB with stock ampicillin/streptomycin to a separate 125 mL Erlenmeyer flask.
- Pick an individual colony from each of the incubated bacteria plates and inoculate into the respective flask.

  E.coli CN-13 = stock nalidixic acid

  E.coli F_{amp} = stock ampicillin/streptomycin
- Cap flasks with foil and incubate the flasks for 18 hours at 37^{o}C ± 1^{o}C without shaking.
- If log phase is not reached, transfer 2 mL from 18-hour culture flask and inoculate into another 40 mL flask of TSB with antibiotics. Incubate for an hour and check absorbance reading. If log phase is not reached, check absorbance every 30 minutes until desired reading is obtained.
- Once desired absorbance is reached, aseptically remove 2 mL of log-phase culture from the flask, dispense into a plastic curvette, and use a spectrometer to read the absorbance at 520 nm (log phase growth is indicated by an absorbance reading of 0.1 – 0.5 optical density units). If the targeted absorbance range isn't reached, place the cultures back into the incubator for an additional 30 minutes. Take a reading every 30 minutes until target absorbance is reached.

Appendix L – Continued

- Store log-phase cultures at $4^{o}C \pm 1^{o}C$ to slow replication until ready for use for up to 48 hours.
- Store remaining bacterial host culture at $4^{o}C \pm 1^{o}C$ overnight to inoculate flasks for preparation of new-log-phase bacterial hosts.

The second day of the modified double agar layer method is described below. Day 2 is the preparation of the sample dilutions, preparation of the top agar layer, and the quantification of results.

1. Prepare Coliphage dilutions
    - Add 10 mL of undiluted wastewater sample to one test tube and 10 mL of undiluted wastewater to another test tube.
    - Add 9 mL of TSB without antibiotics to 6 dilution tubes. Label two tubes for each dilution: 0.1, 0.001, and 0.0001.
    - Mechanically mix the two undiluted tubes for 5 seconds then transfer 1 mL from each undiluted tube into two separate 0.1 dilution tubes.
    - Mechanically mix the two 0.1 tubes for 5 seconds then transfer 1 mL from each 0.1 tube into two separate 0.01 dilution tubes.
    - Mechanically mix the two 0.01 dilution tubes for 5 seconds then transfer 1 mL from each 0.01 tube into two separate 0.001 dilution tubes.
2. Prepare Tryptic Soy Broth (TSB) Media

Appendix L – Continued

- Add 3 g pouch of tryptic soy broth mix to the beaker.
- Put the beaker on a heat-stir plate and insert a stir bar into the media. Heat the media to a boil while stirring to completely dissolve the contents and allow to cool.

  ** Prepared broth is a clear, yellowish-brown color.*

3. Prepare 0.7% TSA – Used for top layer of agar
   - Add 1.4 g of agar each Erlenmeyer flask containing TSB media.
   - Put the beaker on a heat-stir plate and insert a stir bar into the media. Heat the media to a boil while stirring to completely dissolve the agar.
   - Autoclave TSB media for 15 minutes at 121^{o}C and allow cooling.
   - Use a pipette to add the appropriate antibiotic stock to 500 mL of autoclaved 1.5% TSA:

     Somatic: Aseptically add 1 mL of stock nalidixic acid.

     Male-Specific: Aseptically add 1 mL of stock ampicillin/ streptomycin.
   - Add antibiotic stock to the beaker and keep solution in the water bath until use to prevent agar from solidifying.
   - Tubes must be used the day they are prepared.
4. Double Agar Layer Procedure Final Steps
   - Use a pipette to place 5 mL of 0.7% TSA top agar with antibiotics in 18 tubes in a 48^{o}C ± 1^{o}C water bath.

Appendix L – Continued

- Aseptically inoculate the TSA top agar tubes containing stock nalidixic acid with 0.1 mL of log phase *E.coli CN-13*.
- Immediately add 0.5 mL of undiluted somatic coliphage stock.
- Mix the inoculum by briefly rolling the tube in palm of hand.
- Pour contents into the bottom agar plate labeled "undiluted, *E.coli CN-13*, somatic"
- Repeat steps 2 through 5 for each dilution.
- Repeat steps 2 - 6 using *E.coli* F_{amp} and male-specific coliphage.
- Add 0.5 mL TSB to each blank. (Blanks should include: 5 mL 0.7% TSA top agar with appropriate stock antibiotic + 0.1 mL of appropriate *E.coli* + 0.5 mL of TSB)
- Mix the inoculum by rolling briefly in palm of hand.
- Pour contents of the tubes into the appropriately labeled bottom agar dishes
- Allow the top agar layer to harden, replace the top lid, and invert the plates and incubate for overnight at $35^{o}C \pm 5^{o}C$. Remove the plates after the allotted time has passed and count the number of plaques.

Appendix M – ATCC *E.coli* Revival Directions

Revive E. coli strains using the following ATCC directions to ensure optimal recovery of the bacteria (ATTC, 2013). If the bacteria are not revived immediately, store vials at 4℃ until use.

1. Prepare tryptic soy broth solution before opening the bacteria package.
2. Carefully open the packaged *E.coli* stain
3. The top of the glass vial containing the freeze-dried bacteria was broken using tweezers. Next, the cotton plug was removed from inside the vial.
4. Aseptically add 1.0 mL of tryptic soy broth to the vial using a micropipette and mix well.
5. Next, 0.1 mL of this mixture was added to 0.9 mL of tryptic soy broth.
6. A sterilized inoculating loop was dipped into the vial solution and streaked onto an agar plate.
7. The agar plate was incubated overnight to allow culture growth.

Appendix N – Aseptic Techniques

The general aseptic techniques used for the modified double agar layer method is listed below:

1. Disinfect workspace with bleach solution prior to working.
2. Always work under near a Bunsen burner flame to prevent contamination of solutions, samples, and petri plates.
3. Sterilize inoculation loop over Bunsen burner and allow to cool before use.
4. Sterilize the end of the uncapped tubes over Bunsen burner before and after use.
5. Always label the bottom of the petri plates (not the lid).
6. When pouring agar into the petri plate, open the petri plate only half-way to prevent contamination.
7. Slide lid back onto the petri plate by pushing hand across back of the plate.
8. Don’t lean over the petri plate when closing the lid.

Appendix O –Storage of Stock Solutions and Antibiotics

Solution	Storage Time	Storage Instructions
50% Glycerol Stock	1 year	Store at 4^{o}C
Antibiotic Stocks	1 year	Store frozen at -20^{o}C in plastic vials
1.5% Tryptic Soy Agar	2 weeks	Store in sterile, capped container at 4^{o}C
0.7%Tryptic Soy Agar	3 months	Store in sterile, capped container at 4^{o}C
Tryptic Soy Broth	3 months	Store in sterile, capped container at 4^{o}C
Agar Petri Plates	2 weeks	Store dishes in sterile sleeve bags at 4^{o}C (warm to room temperature before use)

ABOUT THE AUTHOR

Sakira graduated from the University of Florida with a Bachelor of Science in Environmental Science in 2010. Sakira gained an interest in engineering while working as a receptionist at the Tampa office of Golder Associates, Inc. in 2004. Sakira worked closely with engineers and planners on various projects pertaining to transmission line routing. The main project that Sakira worked on was the Progress Energy Levy County Nuclear Power Plant.

In 2009, Sakira transferred to the Gainesville office to continue her internship. In this office, she researched information for various projects and assisted with writing proposals. In her last semester of undergrad, Sakira was assigned to the Lake Okeechobee Water Quality Project. This project sparked Sakira's interest in water reuse and wastewater treatment.

In 2011, Sakira was accepted to the University of South Florida as a graduate student in the Civil & Environmental Department to pursue a Master of Science in Engineering Science. The summer of 2011, Sakira was awarded a research fellowship by the National Science Foundation which allowed her to travel to Bolivia to conduct research. Sakira researched the detection of coliphage in wastewater effluent using a modified EPA Method 1602 Double Agar Layer (DAL) Assay. After graduation, Sakira aspires to obtain a career working with an environmental engineering consulting firm.