Assessment of the fate of Cryptosporidium parvum, Giardia lamblia and Ascaris lumbricoides in class A composting and class B lime stabilization biosolids treatment processes

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Abstract
The management of biosolids in the United States has become an issue of public health concern in regard to disposal. Congress created the Clean Water Act in 1972 to protect waters in the United States and as a result, no longer can human waste be dumped into oceans or rivers. Means of disposal of biosolids are focused on land application today. The practice of land application takes into consideration the classification of the biosolids material and management practices to control access to and growth of crops on the land. Biosolids are classified as either Class A or Class B depending on which process was used to produce the material. Class A biosolids are “virtually” pathogen free and Class B have pathogens present in reduced numbers.

The only parasite indicator organism that is required by the Environmental Protection Agency’s Part 503 Rule to screen for in biosolids is Ascaris lumbricoides. Protozoans including Cryptosporidium parvum and Giardia lamblia were not considered human pathogens when the Part 503 rule was written, but are present in biosolids materials using the recovery methods developed in this project. Through the use of these methods, the protozoa were found to be at least as prevalent as Ascaris lumbricoides. A. lumbricoides cannot be used as an indicator of the presence of all parasites since it has a variable geographic distribution in the United States.

Two biosolids treatment methods were assessed to determine the persistence of parasites in Class B lime stabilization and Class A composting. Results of the lime stabilization procedure show evidence that this method is effective in reducing bacteria concentration, but that the parasites persisted throughout the treatment process. The Class A treatment process, on the other hand, was effective in reducing C. parvum, G. lamblia and A. lumbricoides.

The Class A composting process involves heat inactivation and is a method that has shown to reduce pathogens more effectively than Class B treatments where no heat is required. The ability to demonstrate that pathogens are reduced after a particular treatment is dependent on the availability of efficient recovery methods and viability/infectivity assays.

Keywords
Biology, Microbiology, Engineering, Sanitary and Municipal

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ASSESSMENT OF THE FATE OF CRYPTOSPORIDIUM PARVUM,
GIARDIA LAMBLIA AND ASCARIS LUMBRICOIDES IN CLASS A
COMPOSTING AND CLASS B LIME STABILIZATION BIOSOLIDS
TREATMENT PROCESSES

BY

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DISSERTATION
SUBMITTED TO THE UNIVERSITY OF NEW HAMPSHIRE
IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy
In
Microbiology

December, 2003
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Ph.D. DISSERTATION

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12/4/2003
Date
DEDICATION

This dissertation is dedicated to my husband Scott for wholeheartedly believing in me. I could not have achieved this goal without your love and support. Thanks also to my family, friends and animals for offering encouragement and unconditional love.
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by

Christine L. Bean

University of New Hampshire, December, 2003

The management of biosolids in the United States has become an issue of public health concern in regard to disposal. Congress created the Clean Water Act in 1972 to protect waters in the United States and as a result, no longer can human waste be dumped into oceans or rivers. Means of disposal of biosolids are focused on land application today. The practice of land application takes into consideration the classification of the biosolids material and management practices to control access to and growth of crops on the land. Biosolids are classified as either Class A or Class B depending on which process was used to produce the material. Class A biosolids are “virtually” pathogen free and Class B have pathogens present in reduced numbers.

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CHAPTER ONE

AN OVERVIEW OF LEGISLATION AND PATHOGEN STANDARDS
FOR THE TREATMENT AND LAND APPLICATION OF BIOSOLIDS

INTRODUCTION

Clean Water Act

In 1972, Congress enacted the first comprehensive national clean water legislation in response to growing public concern for serious and widespread water pollution by amending the Federal Water Pollution Control Act of 1948 and creating the Clean Water Act. The Clean Water Act is the primary federal law that protects waters in the United States including lakes, rivers and coastal areas from pollution and is implemented by a joint effort of the Environmental Protection Agency (EPA) and individual states. Today, the quality of our waters has improved as a result of the implementation of pollution control programs established by this Act. One of the national goals of this legislation is to eliminate the discharge of pollutants like sewage into waters. In addition to improving the nation’s water quality standards, the legislation provided billions of dollars for construction of sewage treatment plants. In 1972, sewage treatment plants served only 85 million people whereas today modern wastewater facilities serve 173 million people (NRC, 2002).
Biosolids

As a result of the Clean Water Act, the generation of biosolids has increased to over thirteen million tons per year in the United States (EPA, 1999a). The term biosolids is used to refer to domestic wastewater sludge that meets EPA standards for use as a fertilizer or soil conditioner. The term "sludge" was replaced with the term biosolids after a wide spread campaign started in 1991 to clarify terminology.

There are several alternatives for disposal of these biosolids, which include incineration, surface disposal in landfills, and land application. Incineration and landfilling of biosolids pose environmental and economic costs. Incineration of biosolids is the burning at high temperatures in an enclosed device. Concern over the production of dioxins from these biosolids materials has resulted in a movement away from incineration (NIOSH, 2000). Surface disposal occurs when biosolids are placed on an area of land for final disposal. Examples of sites for surface disposal include landfills, lagoons and waste piles. Land application is the application of biosolids to land to either condition the soil or to fertilize crops or other vegetation grown in the soil. Techniques to perform land application include spraying or spreading on the soil surface or tilling into the soil after being surface applied. Land application has increased from 33% of biosolids generated to 60% from 1988 to 1998 and has become the focus for disposal of biosolids (EPA, 1999A).
Land Application

The practice of land application to dispose of and recycle biosolids has advantages and disadvantages. Benefits of land application include the effective recycling of nutrients like nitrogen and phosphorus and organic matter, which improves the water holding capacity of the land on which it has been applied. Reductions have also been seen in the cost to farms for fertilizer as well the use of inorganic fertilizers. Land application is considered a cost-effective means of disposal.

The risks associated with land application include the potential for pathogen and heavy metal exposure. Municipal wastewater may contain four major types of human pathogenic organisms including viruses, protozoa, bacteria and helminthes (Table 1).
Table 1
Principal Pathogens of Concern in Domestic Sludge and Sewage Sludge Considered in Establishing the Part 503 Rule

<table>
<thead>
<tr>
<th>BACTERIA</th>
<th>PROTOZOA</th>
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<tbody>
<tr>
<td><em>Salmonella</em> sp.</td>
<td><em>Cryptosporidium</em></td>
</tr>
<tr>
<td><em>Shigella</em> sp.</td>
<td><em>Entamoeba histolytica</em></td>
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<tr>
<td><em>Yersinia</em> sp. <em>Vibrio cholerae</em></td>
<td><em>Giardia lamblia</em></td>
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<tr>
<td><em>Campylobacter jejuni</em></td>
<td><em>Balantidium coli</em></td>
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<tr>
<td><em>Escherichiae coli</em></td>
<td><em>Toxoplasma gondii</em></td>
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<table>
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<tr>
<th>ENTERIC VIRUSES</th>
<th>HELMINTH WORMS</th>
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<tr>
<td>Hepatitis A virus</td>
<td><em>Ascaris lumbricoides</em></td>
</tr>
<tr>
<td>Adenovirus</td>
<td><em>Ascaris suum</em></td>
</tr>
<tr>
<td>Norwalk virus</td>
<td><em>Trichuris trichuria</em></td>
</tr>
<tr>
<td>Caliciviruses</td>
<td><em>Toxocara canis</em></td>
</tr>
<tr>
<td>Rotaviruses</td>
<td><em>Taenia saginata</em></td>
</tr>
<tr>
<td>Enteroviruses</td>
<td><em>Taenia solium</em></td>
</tr>
<tr>
<td>Polioviruses</td>
<td><em>Necator americanus</em></td>
</tr>
<tr>
<td>Coxsackieviruses</td>
<td><em>Hymenolepsis nana</em></td>
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<tr>
<td>Echoviruses</td>
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<tr>
<td>Reoviruses</td>
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<td>Astroviruses</td>
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There is also a risk of exposure to heavy metals such as lead, cadmium and mercury (NRC, 2002). The long-term effects of land application are not understood and the cumulative loading issues for heavy metals are still being addressed. Another disadvantage is the negative public perception of land application. There
continues to be a need for public education to overcome this issue. Potential effects on
ground water quality need to be considered when biosolids are stockpiled in large
amounts for periods of time. Runoff from these stockpiles also has the potential for
contaminating surface waters.

**Environmental Protection Agency's Part 503 Biosolids Rule**

When the Clean Water Act was again amended in 1987, the EPA Part 503
Biosolids Rule was written which established standards for the use or disposal of sewage
sludge (EPA, 1996). The intention of the Part 503 Rule was to protect public health and
the environment from any adverse effects of pollutants that may be present in sludge.
The potential risk posed by pollutants in sewage sludge to affect public health and the
environment through a number of different routes of exposure was assessed.
Transmission routes evaluated included inhalation, direct ingestion of soil fertilized with
sewage sludge, direct dermal contact with sewage sludge, consumption of crops grown
on this soil, and contamination of drinking water or surface water with biosolids. The
Part 503 Rule identified the potential pathogens present using the best scientific
practices at that time and because it has not been revised, newly emerging pathogens
since the mid-1980's have not been addressed or considered in the Part 503 Rule.

**Class A and Class Biosolids**

The Part 503 pathogen reduction alternatives ensure that pathogen levels in
biosolids are reduced to levels considered safe for the biosolids to be land applied.
Criteria were developed to classify biosolids as Class A or Class B with respect to the
level of pathogens present. A designation of Class A is used when the pathogen *Salmonella* sp., enteric viruses, viable helminth ova and fecal coliforms are below detectable levels. These levels are less than 3 Most Probable Number (MPN) per 4 grams (g) of total solids for *Salmonella* sp., less than 1 plaque-forming unit per 4 g total solids for enteric viruses, less than 1 viable helminth ovum per 4 g total solids and less than 1,000 MPN per gram for fecal coliforms (EPA, 1999a)(EPA, 1999b). Class A biosolids are therefore considered “virtually” pathogen free because none of the above listed pathogens are detectable.

The Class B designation is given to those biosolids when pathogens are detectable, but have been reduced to levels that do not pose a threat to public health and the environment as long as actions are taken to prevent exposure to the biosolids after their use or disposal. The land application restrictions allow natural processes like ultraviolet light and air-drying to further reduce pathogens in the biosolids before the public has access to the site and through these practices it is thought to protect public health to the same extent as the Class A requirements.

Because Class A biosolids are virtually pathogen-free, uses include public sites such as golf courses and the use on foods such as root crops. Class B biosolids have more site restrictions which include such stipulations as animals shall not graze on the land for 30 days after application and public access to land with a high potential for public exposure shall be restricted for 1 year after application. Additionally, food crops with harvested parts that touch the biosolids/soil mixture and are totally above the land surface shall not be harvested for 14 months after application (NRC, 2002).

The designation Class A or Class B can be achieved by the use of a treatment
process to meet the pathogen reduction requirements. Examples of accepted treatment processes for meeting the Class A pathogen requirement are thermally treating biosolids, treating with high pH temperature process or composting. Class B biosolids are a result of the application of a Process to Significantly Reduce Pathogens (PSRP) (NRC, 2002), which includes aerobic digestion, anaerobic digestion and lime stabilization. The Part 503 regulations established a requirement for the frequency of monitoring for pathogens based on the amount of sludge used or disposed. For example, if equal to or greater than 15,000 metric tons of sewage is used or disposed of per year, monthly monitoring is required whereas if less than 290 metric tons per year are used or disposed of, monitoring must be performed only one time during that year (EPA, 1999a) (EPA 1999b).

Pathogens

Pathogens that propagate in the enteric system of humans are excreted in feces and therefore pose a risk to public health. The level of the four major types of human pathogens in sewage; bacteria, viruses, protozoa and helminthes, varies depending on both the overall health status of the local community and the reduction achieved by the treatment process utilized. Pathogen species and density in wastewater will also vary with the time of year as many of these organisms show seasonal distribution.

Exposure to an infectious agent and the resulting development of disease depends on the infectious dose. *Ascaris lumbricoides* has an infectious dose of one viable helminth ova (VHO). *Cryptosporidium parvum* has been shown to have an infectious dose as low as thirty oocysts (Garcia, 2002). Possible routes of direct
exposure to pathogens include contact when handling soil from home gardens where biosolids have been applied or by inhaling airborne microbes. Examples of indirect exposure routes include consumption of water or food contaminated with pathogens. The study of these exposure routes led to the use of site restrictions imposed by the Part 503 Rule.

The Part 503 Rule also requires direct monitoring of viable helminth ova, specifically *Ascaris* ova, as an indicator for several other helminth species such as *Trichuris* and *Hymenolepsis*. Routine monitoring for all parasites is unrealistic due to cost and lack of methods for all potential pathogens. Therefore, monitoring for indicator organisms has become a routine part of monitoring programs (NRC, 2002). At the time the Part 503 Rule was written, work by Yanko et al. in Los Angeles (Yanko, 1988) showed that *Ascaris* ova were the hardiest of pathogens. On soil surfaces, helminth ova were found to die off after four months, but up to fifty percent of the ova might still persist after four months if the ova were within the soil matrix (Yanko et al., 1988). For this reason, *Ascaris* was chosen as the indicator organism for treatment effectiveness for all helminthes.

*Ascaris lumbricoides*

**Life Cycle.**

The adult nematode, *Ascaris lumbricoides*, measures 20-35 cm long (female) and 15-31 cm long (male). Adults mature and mate in the intestine of humans and produce eggs, which are passed in stool. The adult female may generate nearly 300,000 eggs during her life span (Garcia, 2002). Fertilized and unfertilized eggs are passed
in human feces. Fertilized eggs become infective within two weeks in warm, moist soil and may remain viable for months to years (Garcia, 2002). The eggs are 75 um long and 50 um wide and have a thick, albuminous coat. Unfertilized eggs are larger, 90 um long, and have a minimal outer coat.

Human infection is acquired by ingesting fertilized eggs from soil. Once ingested, the eggs hatch in the stomach and duodenum and larvae penetrate the intestine wall. Larvae are carried by the circulatory system to the heart and then migrate into the pulmonary system where they are filtered out by capillaries. Larvae break into the alveoli of the lung and migrate to the trachea and pharynx where they are coughed up and swallowed to continue the autoinfective cycle. It takes eight to twelve weeks to complete the cycle from egg ingestion to egg passage from the adult female.

**Clinical Disease and Treatment.**

Symptoms of infection vary depending on what stage of the life cycle the host is experiencing. Migration of larvae through lung tissue may cause early symptoms of pneumonitis. Tissue reactions and increased eosinophil counts may also occur with reinfection and larval migration. *Ascaris* pneumonitis results in allergic reactions and symptoms of dry cough and fever suggestive of a viral infection. Sputum at this stage may contain larvae.

Infections with low numbers of adult worms may show no symptoms and symptoms occur only when the number of adults in the intestine becomes large. Adult worms may migrate and cause intestinal blockage, enter the bile duct; liver and even migrate out of the body through the anus, mouth and/or nose (Garcia, 2002). The
adults may carry other pathogens with them like fecal coliforms and cause severe systemic infections especially in immunocompromised patients. In young children, malnutrition may also occur.

Antihelmintic drugs like mebendazole and albendazole are available to eliminate the adult worms although these drugs are not effective against tissue larval stages. Surgery has been indicated when extraintestinal body sites are involved.

**Epidemiology and Prevention.**

*Ascaris lumbricoides* is second only to the parasite pinworm, *Enterobius vermicularis*, in prevalence worldwide (WHO, 1986). It is more prevalent in warm, moist climates like the southeastern part of the United States, but it can survive in temperate zones like the northeast. It is unclear exactly how prevalent *Ascaris* infection is in the United States since the disease is not reportable to state public health departments or the Centers for Disease Control. Primary prevention to control *Ascaris* infection is proper sanitation to prevent the contamination of soil with sewage. Once ova are deposited in soil, there is no way to kill them and they may exist as a public health risk for months to years (Garcia, 2002). The use of human feces as fertilizer has been recognized as a transmission route and is evidence that the land application of biosolids could also pose a risk.
Protozoa

No monitoring for protozoans is currently required in the EPA regulations, even though organisms such as *Cryptosporidium parvum* and *Giardia lamblia* have been identified as pathogens of concern in municipal wastewater and sewage sludge. There may be a significant risk associated with *Giardia lamblia* and land application of biosolids as more than 900 cysts per gram wet weight have been found in anaerobically digested biosolids (NRC, 2002). Even after composting and storage of biosolids for more than one year, the cyst levels remained high and cysts survived over twelve weeks in the soil environment. Raw sewage samples examined in the County Sanitation Districts of Los Angeles County contained an average of $2.5 \times 10^6$ *Giardia* cysts/100 L (Yanko, 1988).

At the time the Part 503 Rule was written, *Cryptosporidium parvum* had not been identified as a human pathogen and was still considered a veterinary pathogen. In one study by Rose et al (1991), *Cryptosporidium* oocysts were detected in 17% of 36 finished drinking water samples collected from various areas of the United States. These protozoa and other newly emerging organisms such as Microsporidia sp., which cause disease in humans, must be considered as potential public health risks especially since their small size may allow them to move more easily through soils into water supplies. *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts are highly resistant to numerous disinfectants and environmental pressures and survive for several months outside the host (Garcia, 2002) (NRC, 2002).

The current Part 503 regulations require that only VHO of *Ascaris* be screened for as an indicator of parasite removal and treatment effectiveness. There are
geographic differences in the prevalence of *Ascaris* in the United States. When biosolids samples are tested for VHO and *Ascaris* ova are absent, it cannot be concluded that no protozoans are present in the sample since it is not clear whether *Ascaris* is an appropriate indicator of other protozoans. If no *Ascaris* is present in sludge prior to processing into biosolids, *Ascaris* cannot act as a measure of treatment effectiveness. One goal of this research is to assess the prevalence of *Cryptosporidium parvum*, *Giardia lamblia* and *Ascaris lumbricoides* in biosolids samples from sites around the United States. *Cryptosporidium parvum* and *Giardia lamblia* are commonly found in surface water (NRC, 2002). The ubiquitous occurrence of these two protozoans in water, and as a consequence in the general population (Garcia, 2002), makes these parasites potential candidates for assessing the fate of protozoans in biosolids treatment processes. Published data document high levels of each protozoan in treated effluents and in raw sewage (Yanko, 1988).

*Cryptosporidium parvum*

**Life Cycle**

*Cryptosporidium parvum* is an intestinal coccidian, protozoan parasite that has emerged as an important cause of diarrhea in humans and animals. The developmental stages of the parasite occur intracellularly within a vacuole of the host cell and multiplication does not occur outside the host cell. The vacuole is located at the microvillous surface of the host cell. Oocysts undergo sporogony while in the host cell and are immediately infective when passed in feces. Some oocysts, instead of forming the thick-walled, environmentally resistant form, develop into thin-walled autoinfective forms. The autoinfective oocyst contains four sporozoites surrounded by a single
membrane, which ruptures when the oocyst is released from the host cell. The released sporozoites invade the microvillous region of other intestinal cells (Garcia, 2002). Sporozoites are motile and adhere to and invade epithelial cells lining the gastrointestinal tract. Invasion involves molecules discharged from the parasite organelles found in the apical end of the sporozoite. After attachment and discharge of the contents of organelles, microvilli are disrupted and Cryptosporidium enters the host cell. The host cell membrane is enveloped around the organism as it enters the cell. Intracellularly, the parasite is in its vacuole and is separated from the host cell cytoplasm where it replicates into eight merozoites. The merozoites rupture out of the host cell and infect other cells and complete the asexual stage of the life cycle. Merozoites eventually differentiate into gamonts, which undergo sexual reproduction and regenerate oocysts that are excreted in feces (Garcia, 2002).

**Clinical Disease and Treatment**

Cryptosporidiosis is usually self-limiting in immunocompetent adults. Symptoms may include nausea, fever, abdominal cramps, anorexia and diarrhea. In immunocompromised hosts, however, this disease may be chronic and life threatening. This is due to the impaired immune system especially in AIDS patients who have CD4 cell counts less than 180-cells/mm³ (Garcia, 2002). Four clinical categories of AIDS-related cryptosporidiosis have been identified: a cholera-like illness requiring rehydration therapy, chronic diarrheal illness, intermittent diarrheal illness and transient diarrheal illness (Garcia, 2002). Other organs have been involved in
Cryptosporidiosis and include the lungs, ears, pancreas, stomach and the biliary tract, which is the most common site of extraintestinal infection.

*Cryptosporidium* is resistant to antimicrobial drugs and there is no cure to date. The establishment of the host cell vacuole may protect *Cryptosporidium* from antimicrobial agents. Treatment options are not indicated in immunocompetent hosts. In immunocompromised hosts, treatment involves partial restoration of immune function. Cases have shown that the use of highly active anti-retroviral therapy (HAART) may restore CD4 cell counts and resolve diarrhea (Clark, 1999). Bovine hyperimmune colostrum has also shown some promise in these patients (Garcia, 2002). Antibiotics have been used such as paromomycin and azithromycin with some partial efficacy of treatment. Nitazoxanide has recently been tested in Mexico and cured nearly 100% of patients with *Cryptosporidium* (Garcia, 2002). Controlled clinical trials with this drug are currently under way in the United States.

**Epidemiology and Prevention.**

*Cryptosporidium* has been isolated in chickens, cattle, turkeys, mice, rats, guinea pigs, horses, dogs, cats, sheep, pigs, rhesus monkeys and humans (Bowman, 1999). Zoonotic transmission is one means of exposure to humans. Two major genotypes within *Cryptosporidium parvum* have been identified. Genotype 1 is exclusive for humans and genotype 2 has a broad host range. Different species show marked host specificity and evidence exists to consider *C parvum* genotypes 1 and 2 as different species. Hundreds of fecal samples from humans were analyzed in a study performed by McLauchlin et al (2000). Genotype 1 was recovered in 38% of the samples and genotype 2 in 62%. Fecal
samples from livestock animals were also analyzed and yielded genotype 2 only. Animals serve as potential reservoirs for humans.

Transmission occurs through the fecal-oral route. Nosocomial infections, daycare center outbreaks and waterborne outbreaks have been documented. Cases of *Cryptosporidium parvum* are reportable to the State Department of Public Health regardless of the age of the patient. Cumulative cases in the United States in 2002 totaled 3,512 (MMWR, 2003). Because oocysts are fully infective when passed in feces, person-to-person transmission occurs when stool material is contacted. Direct contact may occur through sexual practices and indirect contact may occur by exposure to contaminated water supplies. Since the oocyst is only 4-5 um in diameter, filtration of public water supplies may not be sufficient for removal. The oocysts have a demonstrated resistance to chlorination (Guerrant, 2001).

The dose required for infection, the infectious dose (ID) has been proven to be as low as 30 oocysts (Garcia, 2002). Surveys indicate prevalence rates of cryptosporidiosis in Europe and North America to be between 25% to 35% (MMWR, 2003). For the disease to be sustained in a community, susceptible and infected hosts must exist along with oocysts in the environment for the life cycle to propagate.
**Giardia Lamblia**

**Life Cycle**

The life cycle of *Giardia lamblia* begins with the ingestion of the cyst form, which reaches the small bowel, and, in response to the low pH, excysts into the motile, feeding trophozoite form. Using flagella and the ventral disk, the trophozoite moves from the small intestine to the duodenum and jejunum where it attaches to the intestinal epithelium. Trophozoites do not appear to penetrate the epithelium and may remain attached to the mucosal surface until it sloughs off the tip of the villus approximately every 72 hours (Garcia, 2002). The trophozoites are pear-shaped and have a concave ventral disk, which they use for attachment. They have four pairs of flagella, two nuclei, two axonemes, and two median bodies and are 10 to 20 um long and 5 to 15 um wide.

Trophozoites encyst as they move down through the colon. During encystation, flagella are retracted, the cytoplasm becomes condensed and the cyst wall is secreted. This process of encystation is a key step in the life cycle of *Giardia* that allows the organism to survive between hosts outside the human body. The cysts can survive for three months in water at 4°C (Olson et al., 1999). They are usually oval-shaped; contain four nuclei, axonemes and median bodies. They measure 11 to 14 um in length and 7 to 10 um wide.

**Clinical Disease and Treatment.**

The cyst form is infectious and an inoculum of 10 to 100 cysts is required to infect humans (Garcia, 2002). The incubation time to infection ranges from 12 to 20 days before the acute stage begins, which lasts only a few days. The organisms are
present in the duodenal mucosa, but do not cause pathology, as they do not penetrate the mucosa. Symptoms of the acute phase can include nausea, anorexia, fever, chills and a sudden onset of explosive, foul-smelling diarrhea. Stools may have increased amounts of fecal fat and mucus, but no blood is present. Fat absorption may be disrupted due to the presence of trophozoites coating the mucosal lining. Rapid multiplication of trophozoites by binary fission creates a barrier between the intestinal epithelial cells and the lumen causing interference with nutrient absorption (Garcia, 2002).

A chronic phase may follow the acute phase and induces symptoms of brief, recurrent diarrhea followed by normal stools or even constipation. Antigenic variation is a factor in the development of this chronic stage as certain surface antigens may allow the organism to survive better in the intestinal tract. Some other patients are asymptomatic and continue to pass cysts in stools.

Giardiasis occurs often in patients with hypogammaglobulinemia, but has not become the problem it was expected to in AIDS patients. AIDS patients do not appear to be more susceptible to Giardia than do healthy people and when cases occur; treatment is available and effective for this protozoan (Garcia, 2002).

In summary, giardiasis causes infection ranging from an asymptomatic carrier state to severe malabsorption syndrome. Factors contributing to the variation in the effects of exposure include the strain of Giardia, dose, age of the host and immune system function of the host.

Once an infection is diagnosed, treatment is recommended for all cases of Giardia for several reasons. First, the infection may cause subclinical malabsorption. The symptoms may be periodic and if a carrier state develops, asymptomatic carriers are
potential sources of infection for others with whom they may come in contact. The treatment of choice is metronidazole for all cases except during pregnancy. Other drugs are available and have been used successfully including paromomycin and tinidazole.

**Epidemiology and Prevention.**

Transmission of viable cyst forms can occur through many modes including the ingestion of contaminated food or water, direct contact with an infected individual such as children in day care centers, any group of people living in close quarters or through oral/anal sexual practices, especially in male homosexuals. Areas with poor sanitation, such as those that travelers and/or campers may encounter, have resulted in giardiasis being referred to as "traveler's diarrhea". There are also occupations that increase the likelihood of exposure, including sewage and irrigation workers and clinical laboratory workers handling fecal samples. Waterborne outbreaks have occurred in the United States and have been associated with hikers and campers drinking stream water that was contaminated with fecal material from animal reservoirs, such as beavers.

Increased susceptibility has been demonstrated in patients with decreased gastric acidity and also with malnutrition in young children in developing countries. The disease is more prevalent in children than adults and is now reportable to the State Public Health Department and the Centers for Disease Control only in those patients five years old and younger in the United States. The total number of cases reported in 2002 in the United States was 16,124 (MMWR, 2003). Epidemics among adults in this country are seen in association with fecally contaminated food or water. Dogs, cats and other animals can also carry *Giardia* infection.
To prevent transmission of this disease, personal hygiene must be addressed in all patient populations. Improved sanitation measures can be effective in controlling the person-to-person spread of infection. Travelers are advised to avoid drinking from local water supplies when traveling to foreign countries and to limit intake to bottle water. Iodine disinfection of drinking water as well as the use of filtration systems may serve to allow the decontamination of water sources for campers and travelers.

Recovery Methods

Current Part 503 Methods for Ascaris Recovery

Monitoring for Ascaris VHO under the Part 503 Rule (EPA, 1996) requires the use of magnesium sulfate flotation to recover the ova in biosolids samples (Appendix). One liter of liquid sample or 450 grams of solid sample is blended with a surfactant and allowed to settle overnight. The supernate is removed and a second blending and settling step is performed to remove debris and purify the sample. The Ascaris ova are finally concentrated from the sample by the use of magnesium sulfate to create a density gradient, which will float the ova out of the sample matrix for removal. Samples are analyzed for the presence of Ascaris ova using a Sedgwick-Rafter counting chamber and light microscopy. If ova are recovered, the sample must be incubated for three to four weeks at room temperature to allow for viability testing of the recovered ova. Ova are analyzed microscopically after this incubation period for the presence of active, motile larvae internally which are interpreted as viable if present.
Recovery of Protozoa

Little research has been done on the occurrence and recovery of Cryptosporidium and Giardia in sewage when compared to similar research concerned with water. There are significant differences between water and sewage including numbers of parasites, which suggests that techniques currently used for water may not be suitable for sewage samples. Method 1623 is currently being used to test water for Cryptosporidium and Giardia (Appendix G). This approved EPA method uses filtration, immunomagnetic separation (IMS) and fluorescent antibody (FA) testing to recover and identify these protozoans in water samples. Robertson et al (1992) clearly demonstrated that large volume filtration is less efficient than grab sampling for detecting these two protozoans in sewage effluent.

Bean and Brabants (2001) have adapted the Part 503 protocol for Ascaris recovery to successfully recover both Cryptosporidium parvum oocysts and Giardia lamblia cysts (Bean, 2001). The protocol uses a combination of flotation and centrifugation techniques to concentrate the protozoa. Once the concentration procedure is complete, organisms are recovered using immunomagnetic separation (IMS) and identified by using fluorescent antibody staining (DFA). Bean et al (1999) applied the Dynal® immunomagnetic separation technique (IMS) to concentrate oocysts of Cryptosporidium and cysts of Giardia from biosolids successfully (Appendix). Antibody-coated beads specific for Giardia and Cryptosporidium bind to antigens on the cyst and oocyst walls and because these beads are magnetized, they can be isolated easily from the sample matrix resulting in a clean suspension of oocysts once they are dissociated from the bead particles. Merifluor® direct fluorescent antibody
staining (Appendix) is then performed on the IMS concentration product to identify the protozoa and confirm their identity using fluorescently labeled antibodies which are specific for the oocyst and/or cyst cell wall antigen target. The IMS sample is incubated with the fluorescein isothiocyanate-conjugated monoclonal antibodies directed against the antigenic components of Cryptosporidium oocysts and Giardia cysts. The stained preparation is examined under a fluorescent microscope for the simultaneous detection and enumeration of Cryptosporidium oocysts and Giardia cysts.

**Viability Assays**

In addition to optimizing recovery rates, another goal of this project is to assess methods of viability testing for both Cryptosporidium parvum and Giardia lamblia. It is mandatory to test for organism viability when helminth ova or protozoan cysts are recovered to measure risk of exposure. If all organisms recovered are non-viable, then treatment has been effective and the organisms do not pose a public health risk. The viability of Ascaris ova is determined using an incubation and light microscopy to visually identify viable larval forms (EPA, 1996). For the two protozoa in this study, viability was measured using the benchmark of animal infectivity, which is thought to be the most sensitive method for determining infectivity of oocysts. Hence, the animal infectivity assay was used to evaluate the performance of other assays. Because animal infectivity is costly, labor intensive and slow to provide results, other methods to determine protozoan viability were evaluated.

The first comparison evaluated infectivity in neonatal mice and detection of the β-tubulin messenger RNA (mRNA) for Cryptosporidium parvum. The mRNA technique is based on the principle that mRNA in oocysts, as in all cells, is
subject to rapid post-mortem decay (Widmer, 1999). The presence of a selected mRNA transcript, as detected by reverse-transcription PCR (RT PCR) can therefore be used as a marker of viability. Viability of *Cryptosporidium parvum* was also assessed using excystation of oocysts and vital staining with DAPI/PI (Widmer, 1999).

For *Giardia lamblia*, infectivity in mongolian gerbils and vital dye staining was compared. The cysts were stained with fluorescein diacetate and propidium iodide (FDA/PI) and then examined with a fluorescent microscope. FDA-positive cysts fluoresce intensely green and represent viable cysts. PI-positive cysts fluoresce bright orange and represent nonviable cysts.

**Bench Scale Treatment Processes**

The fates of these parasites in bench scale Class A and Class B treatment processes were assessed in the final phase of the project. A composting model using a biosolids extract was used to assess heat inactivation of these parasites in Class A treatment process. A bench scale model of Class B lime stabilization processing was also set up to test for inactivation of the pathogens. There are currently no data available for the fates of either *Cryptosporidium* or *Giardia* in Class B sludge treatment processes such as lime stabilization. Better data on the fate of these organisms during treatment processes will assist in determining the relative risks of exposure to parasites associated with land application of biosolids. Class A biosolids processes have been shown to inactivate representative model pathogens and therefore require no parasite testing. It is assumed that the combination of using a documented process in conjunction with a bacterial limit to document process performance will result in the inactivation of all parasites. Documentation of how biosolids treatment methods affect these parasites
will be beneficial in assessing the public health risk associated with land application of biosolids. If a process can inactivate protozoa and helminthes, the resulting biosolids may be beneficially used in a timely manner.
REFERENCES


NIOSH (National Institute for Occupational Safety and Health). 2000. Workers exposed to class B biosolids during and after field application.


Yanko, W. 1988. Occurrence of pathogens in distribution and marketing municipal sludges. EPA/600/1-87/014. USEPA, Research Triangle Park, NC.
CHAPTER TWO
RECOVERY OF PARASITES

INTRODUCTION

EPA Requirements for Parasite Evaluation in Biosolids

The U.S. Environmental Protection Agency (EPA) revised its guidelines in October 1999 for the detection of *Ascaris* ova as an indicator of parasites in water, wastewater, sludge and composted biosolids (Environmental Regulations and Technology: Control of Pathogens in Vector Attraction in Sewage Sludge). The intention of the revised guidelines is to increase sample volume, providing for a more representative sample for analysis and increasing the likelihood that parasitic ovum will be detected if they are present. *Ascaris* was chosen as the indicator organism based on the knowledge that the ova are capable of surviving in the environment for long periods of time and are resistant to environmental effects (Yanko, 1988). There are currently no requirements for analyzing biosolids for the presence of protozoa. When *Ascaris* was chosen as the indicator organism, protozoa such as *Cryptosporidium parvum* were not yet recognized as human pathogens.

Recovery of *Ascaris* Ova

The most significant change to the original protocol is the substantial increase in the volume of sample tested— from 50 g to 300 g of dry or thick sample, and from 450 mL to 1 L or more of liquid sample (estimated to contain at least 50 g of dry solids). Other
protocol changes include the additional steps of soaking thick samples overnight, blending and settling to ensure liquefaction. The revised protocol utilizes 7X limbro detergent in place of Tween 80 as a surfactant, and includes an additional process of coating glassware with organosilane. Both protocols utilize density gradient centrifugation to create a layer of ova; however, the revised protocol makes use of magnesium sulfate to accomplish this, whereas the previous protocol utilized zinc sulfate solution. The revised protocol employs a 400-mesh sieve to capture the *Ascaris* ova, which are subsequently quantified using a Sedgwick-Rafter counting chamber.

Recovery efficiency, processing time, final volume of sample generated for microscopic analysis, and the amount of materials and reagents required to complete the analysis vary depending on the type of sample and the percentage of total solids. For example, a highly liquid sample with a low percentage of total solids (<5%) produces a smaller packed pellet for processing and subsequent examination, thereby requiring fewer materials and reagents and less processing time. In addition, such a sample will generate a relatively "clean" product for microscopic analysis, minimizing solids and debris that could interfere with counting *Ascaris* ova. However, a sample with a high percentage of total solids (>50%), as is found with composted biosolids, requires additional processing time because the sample may be difficult to pass the through the initial screening. Also, high total solids samples must be separated into smaller aliquots for centrifugation.

Processing times for samples containing 5% to 50% total solids vary depending on overall sample consistency. If the volume of packed sediment exceeds five milliliters, which will occur in a solid sample, the sediment must be redistributed evenly among additional tubes and centrifugation must be repeated, which tends to result in a larger
number of tubes and increases processing time. Using the revised protocol, processing
time for hands-on sample manipulation is between 85-100 minutes. The entire turn­
around time from start of sample processing to the final sample read is three days. These
times are slightly longer than what was required for the previous protocol: 60-70 minutes
of direct sample processing and a turn-around time of two days. Using the revised
protocol, the final report can be ready in 3 days if no *Ascaris* ova are present in the initial
reading; however, the sample must be incubated for 3 to 4 weeks to assess viability if ova
are present. This additional incubation increases reporting time to a minimum of 3 weeks.

Seeding and recovery experiments were conducted using three samples varying in
percentage total solids and type of treatment. The samples included untreated wastewater,
lime-stabilized biosolids and composted biosolids. Using the revised protocol guidelines,
which now require 300 grams or 1 liter of sample, recovery results were compared with
those obtained using the previously required 50 gram or 450 ml sample sizes.

In the seeding study, ova recovery data differed significantly depending on
sample type. Solid samples were processed prior to seeding and evaluated for the
presence of viable helminth ova and larvae. Recovery rates were obtained by seeding
1450 ml of untreated wastewater (0.44%TS), 350 g of lime-stabilized biosolids
(66.6%TS), and 350 g of composted biosolids (50.3%TS) with 3500 *Ascaris* ova.
Original ova counts were performed in triplicate using a haemocytometer. Following
stirring, two aliquots were removed from each material sample, 300 g or 1000 ml and 50
g or 450 ml, depending on the material. All aliquots were processed using the revised
protocol and final results reported as the total number of *Ascaris* ova, unembryonated
*Ascaris* ova, and first-, second-, or third-stage larvae per gram dry weight (GDW).

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Recovery data for the three sample types and volumes is presented in Table 1. The recovery rate of *Ascaris* ova and larvae from untreated wastewater seeded with ova was 12.02% using a 1-L sample with the revised protocol. When analyzing the 450-ml sample aliquot, the recovery rate was only 2.75%. For lime-stabilized biosolids, the recovery rate was 4.38% using a 300-gram aliquot and 7.99% when processing the 50-gram aliquot. The recovery rate for the composted biosolids was 4.5% for the 300-gram aliquot and 9.61% for the 50-gram aliquot.

Table 1

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Amount processed</th>
<th>% Total Solids</th>
<th>Final Volume</th>
<th># Ova per *GDW</th>
<th># Larva per *GDW</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>1 Liter</td>
<td>0.04%</td>
<td>10 ml</td>
<td>7.25</td>
<td>2.75</td>
<td>12.02%</td>
</tr>
<tr>
<td></td>
<td>450 ml</td>
<td></td>
<td>10 ml</td>
<td>1.66</td>
<td>2.22</td>
<td>2.75%</td>
</tr>
<tr>
<td>Limed</td>
<td>300 grams</td>
<td>66.60%</td>
<td>9.4 ml</td>
<td>0.658</td>
<td>0.14</td>
<td>4.38%</td>
</tr>
<tr>
<td></td>
<td>50 grams</td>
<td></td>
<td>10 ml</td>
<td>1.2</td>
<td>0</td>
<td>7.99%</td>
</tr>
<tr>
<td>Compost</td>
<td>300 grams</td>
<td>50.30%</td>
<td>1.5 ml</td>
<td>0.895</td>
<td>0.318</td>
<td>4.50%</td>
</tr>
<tr>
<td></td>
<td>50 grams</td>
<td></td>
<td>8 ml</td>
<td>1.91</td>
<td>17.17</td>
<td>9.61%</td>
</tr>
</tbody>
</table>

*GDW: Gram Dry Weight

*Larva per GDW is not included in the calculation for % Recovery*

Table 1: Ascaris recovery efficiency in raw, lime stabilized and composted biosolids
Ascaris ova were spiked into three types of biosolids; raw sludge, limed and composted biosolids, with varying %total solids ranging from 0.04% (raw) to 66.60% (limed) to evaluate recovery of ova using different volumes of sample. The sample volumes included those previously required in the VHO protocol (EPA) and the new requirements of sample volume based on the revised protocol.
Composted biosolids samples at 50 and 300 g and lime-stabilized biosolids at 300 g required dilution to minimize interferences when analyzing the sample for the presence of *Ascaris* ova. Total solids in these samples were greater than 50%. A 1:2 dilution was performed for the 50-g composted biosolids sample and a 1:4 dilution was performed for the 300 g composted and lime-stabilized biosolids samples.

Recovery rates were low and may be explained by several factors, including variation in total solids percentage, sample type, and sample volume. Due to the high percent total solids in the composted and the lime-stabilized biosolids, these samples were manipulated a great deal. The large amount of solid material may have acted to bind to the ova and the larval forms. These data show that the percent recovery for the 1 L sample was higher using the revised protocol than that for the 450 mL sample only in the untreated wastewater category, which may be attributed to the sample having lower percent solids and, therefore, requiring less manipulation and having fewer particles to adsorb ova and larvae. The high percent recovery rate for the 1 L untreated wastewater sample may have been due to the fact that the sample contained only 0.44% TS.

Recoveries were lower in the composted and lime-stabilized biosolids samples, which contained more than 50% total solids. The use of lime in the treatment process may degrade parasites, which could influence the loss of organisms. Low recoveries in all samples may also be attributed to inadequate spatial distribution of *Ascaris* ova within the samples.
Percent recovery also varied among the volumes of sample spiked. For example, recovery was 12.02% for the 1-L untreated wastewater sample, but only 2.75% for the 450-mL sample. Ova were spiked into 1450-ml untreated wastewater (2.41 ova per milliliter) before the sample was split into 1-L and 450-mL aliquots, which contained 2410 and 1084.5 ova, respectively. Percent recovery for the 50-g sample was roughly twice that of the 300-g sample for both lime-stabilized and composted biosolids.

**Analysis of Loss of Ova**

The next steps in analysis of *Ascaris* recovery were to determine where loss of ova was occurring in the revised EPA protocol. Initial recovery experiments were performed with *Ascaris lumbricoides* ova seeded at 5 ova/mL into 1 liter of RO water using the revised EPA protocol. The goals of these initial experiments were to evaluate ova recovery in an RO water matrix to determine loss due to sample processing and eliminate the biosolids matrix as a variable in recovery efficiency.

VHO recovery includes an initial step where samples are processed by blending with buffered water containing a surfactant, 7X Limbro. The blended sample is poured through a screen to remove large particles and allowed to settle overnight. The supernatant fluid is decanted and the sediment subjected to density gradient centrifugation using magnesium sulfate (specific gravity 1.20). The flotation procedure concentrates parasite ova in a surface layer and removes them from sample debris. A smaller mesh screen is used to remove particulates and the concentrate is incubated at room temperature (28°C) for three to four weeks until *Ascaris* control ova are fully.

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embryonated. One mL samples are evaluated microscopically using a light microscope with phase contrast and 10x and 40x objectives. Ova are enumerated on a Sedgwick-Rafter counting chamber and classified as either embryonated or unembryonated based on microscopic examination.

Five thousand *Ascaris lumbricoides* ova were seeded into 1 liter of RO water. Seeded ova were enumerated in triplicate 1 mL aliquots using a Sedgwick-Rafter counting chamber. 10 mL of the 1 Liter spiked sample volume was screened in 1 mL aliquots to ensure the presence of 5 ova per mL and allow for statistical accuracy. The sample was processed using the revised EPA protocol. This protocol was reviewed and steps representing significant sample manipulation were chosen for taking samples (Chart 1). These samples represent select steps in the process including blending, aspirating and settling of the sample prior to flotation. At each step in the process, 10 mL of sample was removed and evaluated for the presence of *Ascaris* ova by evaluating all 10 mL in 1 mL aliquots using a Sedgwick-Rafter counting chamber. In addition to taking aliquots at each step, materials that would have normally been discarded were retained and evaluated for the presence of ova in an effort to determine the loss at each major step. This included retaining sediments and aspirates and screening for the presence of ova using a Sedgwick-rafter counting chamber to enumerate the detected ova.

A 25% loss was calculated based on the first five samples collected in the protocol. Sources for loss include washing the sample through a 50-mesh sieve and two aspiration steps in the protocol as indicated in Chart 1. Prior to flotation 75.5% of the ova were recovered. Following flotation the final recovery was 9.1% indicating that the most significant loss occurred during the MgSO₄ flotation step. To further elucidate the
source of the loss the sediment was examined and was found to contain 29.5% of the ova seeded. The 400-mesh sieve was also directly seeded with 100 ova and subsequently washed. It was determined that the use of the 400-mesh did not result in significant loss of the ova by pouring ova through the mesh sieve and recovering 100% of them. A hydrometer was used to routinely measure the specific gravity when preparing the MgSO4. The hydrometer was calibrated to ensure accuracy prior to performing seeding studies. Although vessels were coated with sigmacoat, a silicone solution in heptane that forms a thin film on the glass surface and repels water, it is speculated that the loss not accounted for may be a result of vessel transfer.
Chart 1

Seeded 4905 ova into 1500 ml
Sample 1: 39 ova in 10 mL = 5850 ova in 1500 mL*  
Blend Liquid  
↓  
Settle Overnight with 7X Limbro  
↓  
Aspirate (#1: 0 ova seen) & Blend again with RO water  
Sample 2: 26 ova in 10 mL = 5200 ova in 2-Liters 88.9% recovery 11.1% loss  
Settle 2 Hours  
↓  
Aspirate (#2: 0 ova seen) & Mix on stirrer with 7X Limbro  
Sample 3: 30 ova in 10 mL = 5400 ova in 1800 mL 92.3% (88.9%) recovery  
Strain through 50-mesh sieve (Sieve wash: 22 ova = .38% loss)  
↓  
Settle 2 hours with 7X Limbro  
↓  
Aspirate (Aspirate #3: 9 ova seen = .15%) and mix sediment  
Sample 4: 119 ova in 10 mL = 4760 ova in 400 mL 81.4% recovery 6.97% loss  
Put in 50-ml tubes. Centrifuge & Aspirate supernate  
(Aspirate #4: 3 ova seen = .05%)  
↓  
Sample 5: 138 ova per mL = 4416 ova in 32 mL 75.5% recovery 5.85% loss  
Add MgSO₄ & Vortex. Centrifuge  

*MgSO₄ Sediment: 1728 = 29.5% loss  
↓  
Pour supernate through 400-mesh sieve (MgSO₄ rinse: 2 ova = .03%)  
Rinse 400-mesh sieve to collect Ascaris  
↓  
Centrifuge and Aspirate supernate (Aspirate #5: 0 ova seen)  
↓  
Resuspend pellet in 7 mL acid alcohol  
↓  
Centrifuge, Aspirate Supernate (Aspirate #6: 6 ova seen = .1% loss)  
resuspend pellet in 4 mL 0.1 NH₂SO₄  
↓  
FINAL READ – 531 ova in 5 mL 9.1% recovery 36.77% loss

Chart 1: Flow Chart to diagram recovery of Ascaris ova (Formalin Fixed) from RO Water to assess loss in procedure These subsamples represent blending, aspirating and settling of the sample throughout the protocol prior to flotation. Each 10-mL subsample was removed and evaluated for the presence of Ascaris ova by examining all 10-mL in 1-mL aliquots using a Sedgwick –Rafter counting chamber.
In the second recovery experiment, 450 mL of a raw liquid sample representing <5% total solids was spiked with 2250 Ascaris ova. 10 mL of the spiked sample volume was screened in 1-mL aliquots to ensure the presence of 5 ova per mL and allow for statistical analysis. Processing of the sample using the current EPA accepted protocol for the recovery of viable helminth ova resulted in a 59.29% recovery. Further processing using a modified ethyl acetate sedimentation protocol resulted in recovery of 44.8% of the ova recovered by flotation. The modified ethyl acetate sedimentation procedure is currently used in clinical diagnostic laboratories to process human fecal samples for the recovery of ova and parasites (Garcia, 2001). Sedimentation methods of concentration may lead to recovery of all protozoa, oocysts, ova and larvae in samples, but the concentrate contains more debris than the product of a flotation procedure. Based on this experiment sedimentation of the flotation product from a liquid matrix does not appear to improve recovery.
Table 2

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>% Recovery by Flotation</th>
<th>% Recovery by Sedimentation of Flotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid</td>
<td>59.29%</td>
<td>44.80%</td>
</tr>
<tr>
<td>Semi-Solid</td>
<td>2.67%</td>
<td>87.09%</td>
</tr>
<tr>
<td>Compost</td>
<td>0.78%</td>
<td>9.52%</td>
</tr>
</tbody>
</table>

Table 2: Flotation and Sedimentation of Ova
Recovery of Ascaris lumbricoides ova seeded at 5 ova/mL into 50g or 450-mL sample using current EPA accepted protocol for recovery of viable helminth ova.
During the next phase of recovery experiments, 50 grams of a semi-solid and compost sample, representing 10% and 50% total solids respectively, were spiked with 5 ova/ml following an initial soaking and blending step. 10 mL of the spiked sample volume was screened in 1-mL aliquots to ensure the presence of 5 ova per mL and allow for statistical analysis. Processing of the semi-solid and compost samples, using the current EPA accepted protocol for the recovery of viable helminth ova, resulted in a 2.67% and 0.78% recovery respectively. Further processing of the flotation product using a modified ethyl acetate sedimentation protocol resulted in an 87.09% recovery for the semi-solid sample and a 9.52% recovery for the compost sample (Table 2). Based on this experiment (n=3) sedimentation of the flotation product from a solid matrix does appear to improve recovery.
Table 3

<table>
<thead>
<tr>
<th>Pull Number</th>
<th>% Recovery</th>
<th>% Loss</th>
<th>Sources of Loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>88.9</td>
<td>11.1</td>
<td>0 Aspirate 1</td>
</tr>
<tr>
<td>3</td>
<td>88.9</td>
<td>0</td>
<td>0 Aspirate 2</td>
</tr>
<tr>
<td>4</td>
<td>81.4</td>
<td>7.5</td>
<td>.38 Sieve Wash</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>.15 Aspirate 3</td>
</tr>
<tr>
<td>5</td>
<td>75.5</td>
<td>5.9</td>
<td>.05 Aspirate 4</td>
</tr>
<tr>
<td>FINAL READ</td>
<td>9.1</td>
<td>66.4</td>
<td>29.5 MgSO₄ Sediment</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>.03 MgSO₄ Rinse</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 Aspirate 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>.1 Aspirate 6</td>
</tr>
</tbody>
</table>

Table 3: Recovery of Ascaris ova in RO water
The magnesium sulfate flotation protocol was assessed for step where loss of ova occurs. After the settling, aspirating and sieving steps, 24.5% of the ova were lost. Of the 75.5% recovered after these initial steps, only 9.1% were recovered in the final sample after magnesium sulfate flotation.
Recovery of Protozoa: Cryptosporidium parvum and Giardia lamblia Recovery Experiments

There are no current recommended or validated methods for recovering protozoa from biosolids. Adapting the current EPA White House protocol for Ascaris ova for the detection of protozoa, specifically Cryptosporidium parvum and Giardia lamblia, would allow the evaluation of biosolids samples for protozoa along with helminthes. The strategy put forth in these experiments is based on size differential between Ascaris ova and protozoan oocysts/cysts. At each washing and settling step in the ova procedure, where the ova are settled out or retained on the 400 mesh sieve, the oocysts would remain in the discarded wash solution (aspirate) or in the rinse water that passed through the 400 mesh sieve. The following experiments were conducted to determine if oocysts could be recovered from the aspirate or sieve rinse water using the immunomagnetic separation (IMS) procedure and immunofluorescent detection (DFA).
Table 4

<table>
<thead>
<tr>
<th></th>
<th>Cryptosporidium</th>
<th>Giardia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dynal Recovery</td>
<td>38.40%</td>
<td>27.80%</td>
</tr>
<tr>
<td>Efficiency</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Merifluor Direct</td>
<td>99%</td>
<td>99%</td>
</tr>
<tr>
<td>IFA Efficiency</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seed 1</td>
<td>3.20%</td>
<td>4.80%</td>
</tr>
<tr>
<td>Seed 2</td>
<td>3.90%</td>
<td>12%</td>
</tr>
<tr>
<td>Seed 3</td>
<td>8.00%</td>
<td>3.30%</td>
</tr>
<tr>
<td>Seed 4</td>
<td>3.70%</td>
<td>2.40%</td>
</tr>
<tr>
<td>Seed 5</td>
<td>3.70%</td>
<td>3.90%</td>
</tr>
<tr>
<td>Seed 6</td>
<td>6.70%</td>
<td>12%</td>
</tr>
<tr>
<td>Seed 7 Centrifugation</td>
<td></td>
<td>41.70%</td>
</tr>
</tbody>
</table>

Table 4: Recovery of Protozoa in RO Water
Recovery of Cryptosporidium parvum and Giardia lamblia cysts and oocysts seeded at 3 oocysts and 3 cysts per mL into 1-Liter of RO water using current Whitehouse document protocol for recovery of Viable Helminth Ova.
Three thousand Cryptosporidium parvum oocysts and Giardia lamblia cysts were seeded into 1-Liter of RO water. Cysts and oocysts seeded were enumerated by flow cytometry prior to seeding. Cysts and oocysts were seeded at each of 7 steps within the current Whitehouse document protocol (October 1999), representing significant sample manipulation. These steps represent blending, aspirating and settling of the sample throughout the protocol prior to flotation (Chart 2). Each step was evaluated independently with a separate one-liter sample seeded with 3000 cysts and oocysts and the sample was processed to completion. For example, at seed number seven, 3000 cysts and oocysts were added to 1-Liter of RO water and the sample was centrifuged at 3000 rpm for 10 minutes. Following centrifugation, the supernate was decanted and the pellet was resuspended in 10-mL RO water. The sample was then processed using Dynal® Immunomagnetic Separation (IMS) (Appendix C) with subsequent visualization using Merifluor® Direct Immunofluorescence (DFA) (Appendix D).
Chart 2

(SEED 1) Seeded 3000 cysts & oocysts into 1000-mL Blend Liquid
\[ \downarrow \]
Settle Overnight with 7X Limbro
\[ \downarrow \]
Aspirate & (SEED 2) Blend again with RO
(SEED 3) Settle 2 Hours
\[ \downarrow \]
Aspirate & Mix on stirrer with 7X Limbro
\[ \downarrow \]
(SEED 4) Strain through 50-mesh sieve
\[ \downarrow \]
Settle 2 hours with 7X Limbro
\[ \downarrow \]
Aspirate and mix sediment (SEED 5)
Put in 50-ml tubes. Centrifuge & Aspirate supernate (SEED 6)
\[ \downarrow \]
Add MgSO\(_4\) & Vortex. Centrifuge (SEED 7)
\[ \downarrow \]
Pour supernate through 400-mesh sieve & collect wash through sieve
\[ \downarrow \]
Centrifuge sieve wash, resuspend in 10-mL RO water
\[ \downarrow \]
(SEED 8) Perform Dynal® IMS and Merifluor® DFA

Chart 2: RECOVERY OF PROTOZOA (FORMalin FIXED) FROM RO WATER
The chart outlines the seeding experiments (seeds 1-8) for protozoa. At each seeding point, 1-8, cysts/oocysts were seeded and the process was completed from that point on for recovery.
Recovery calculations for seeds 1-7 are based on the efficiency obtained for Dynal® Immunomagnetic Separation (IMS) and Merifluor® Direct immunofluorescence Assay (DFA), as all samples were concentrated and visualized using these techniques. IMS recovery efficiency is reported by the manufacturer to be 60%-95% in water, and there have been no reports on the use of IMS to recover Cryptosporidium or Giardia in biosolids. Recoveries using IMS in these experiments were 38.4% for Cryptosporidium oocysts and 27.8% for Giardia cysts. There doesn’t appear to be a significant variation in recoveries for steps 1-6. The discrepancy in these recovery rates may be a result of using a different enumeration technique for the seeded oocysts and cysts versus the recovered oocysts and cysts.

**Discussion**

Flotation protocols take advantage of the relative low density of protozoan cysts and helminth ova to achieve a partial purification of those organisms using a single sedimentation or centrifugation step. A flotation method could be used as an initial step to enrich for C. parvum oocysts in complex suspensions, such as sludge matrices. This method would not be used as a stand-alone procedure; it would be combined with other methods, probably immunomagnetic separation, to improve the detection of oocysts and cysts in sludge.
CHAPTER THREE

A SURVEY OF WASTEWATER SOLIDS TO ASSESS THE PREVALENCE OF Cryptosporidium, Giardia and Ascaris lumbricoides

INTRODUCTION

The ubiquitous occurrence of Ascaris, Cryptosporidium and Giardia species in biosolids samples from different regions of the United States was assessed over a 15-month period. Thirty-eight biosolids samples were analyzed from 11 states including the Northeast, Northwest, Southwest and Central regions of the United States (Chart 1). Types of treatment that produce sludge were raw (untreated) sludge, Class B lime stabilized and composted biosolids with % total solids ranging from 3.26 % (raw) to 80.84% (compost). The mean percent total solids of all samples included in this study were 36.04%. The objective of the prevalence assessment was to determine whether the three parasites were present in biosolids using current methods for recovery. Viability of recovered parasites was not assessed and presence/absence only was determined.
Table 1: Regional Sampling and Prevalence of Parasites in Biosolids Samples
States included in the 38-sample study cover the Northeast, Northwest, Southwest and Central United States

<table>
<thead>
<tr>
<th>State</th>
<th>Number of samples</th>
<th>Ascaris</th>
<th>Giardia</th>
<th>Cryptosporidium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Washington</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Wyoming</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Indiana</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pennsylvania</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>New York</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Vermont</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Maine</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Massachusetts</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Maryland</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Texas</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Arizona</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Methodology

Organisms were concentrated by magnesium sulfate flotation following the EPA Part 503 approved methodology for recovery of *Ascaris lumbricoides* ova (EPA Part 503...
Biosolids Rule 1994) and by the ethyl acetate sedimentation technique (Garcia, 2001). The volume of sample used in these experiments was 50-g solid or 450 ml liquid sample.

The sample was blended and allowed to settle overnight before the supernatant was removed. Magnesium sulfate was added to bring the sample volume to 50 ml and the sample was centrifuged. The supernatant fluid was examined for the presence of ova, cysts and/or oocysts.

Ethyl acetate sedimentation was also performed on these samples to compare sedimentation with flotation for parasite concentration. The sample was washed two times by mixing with phosphate buffered saline and centrifuging before the ethyl acetate was added. The sample was then mixed and centrifuged and the resulting sediment was examined for ova, cysts and/or oocysts. Protozoa were purified from 5-ml samples of flotation or sedimentation product using Dynal® immunomagnetic separation (IMS). Purified protozoa (20 ul of the IMS product) were identified using Merifluor direct immunofluorescence (DFA).

Results

Overall prevalence of each parasite is the sum of positive samples obtained by recovery method, flotation, or sedimentation. The protozoans are at least as prevalent as *Ascaris* in these study samples as shown in Table 2.
Table 2

<table>
<thead>
<tr>
<th>Organism</th>
<th>Percent Samples Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ascaris</em></td>
<td>15.8%</td>
</tr>
<tr>
<td><em>Cryptosporidium</em></td>
<td>23.7%</td>
</tr>
<tr>
<td><em>Giardia</em></td>
<td>15.8%</td>
</tr>
</tbody>
</table>

Table 2: Prevalence of Parasites in U.S. Samples
Thirty-eight samples including untreated, lime stabilized and composted biosolids from the United States including the Northeast, Northwest, Southwest and Central regions

*Cryptosporidium* oocysts were recovered from 9 of the samples tested (23.7%) and were the most prevalent of the three parasites in this study (Table 2). Only one sample evaluated contained both *Cryptosporidium* and *Ascaris* ova (2.63%). *Giardia* cysts were recovered from 6 samples (15.8%). None of the samples evaluated contained both *Giardia* cysts and *Ascaris* ova.

Comparison of percent recovery by the two different methods is summarized in Table 3. Ethyl acetate sedimentation is more effective at concentrating protozoa based on the findings of this study. The flotation procedure is more effective for recovery of large helminth ova like *Ascaris*.
Table 3

<table>
<thead>
<tr>
<th>Protozoa Recovery:</th>
<th>Ethyl Acetate Sedimentation</th>
<th>Zinc Sulfate Flotation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Giardia</em> cysts</td>
<td>7.7%</td>
<td>0%</td>
</tr>
<tr>
<td><em>Cryptosporidium</em> oocysts</td>
<td>12.8%</td>
<td>2.6%</td>
</tr>
<tr>
<td>Both</td>
<td>7.7%</td>
<td>2.6%</td>
</tr>
<tr>
<td>Ascaris Recovery</td>
<td>7.7%</td>
<td>10.5%</td>
</tr>
</tbody>
</table>

Table 3: Comparison of percent recoveries
Two concentration methods; ethyl acetate sedimentation and zinc sulfate flotation, were used to evaluate recovery of protozoa

**Data analysis**

Crosstabulations were performed using SPPS statistics software to evaluate the strength of association between the presence of *Ascaris* ova and *Cryptosporidium* oocysts (Table 4), and *Ascaris* ova and *Giardia* cysts (Table 5). One sample contained both *Cryptosporidium* and *Ascaris*. The Pearson chi square was 0.194 and the p value=0.660. There appears to be no association between the presence of *Ascaris* and *Cryptosporidium* in this set of data. No samples contained both *Giardia* and *Ascaris*. The Pearson chi square was 1.336 and the p value=0.248. There appears to be no association between the presence of *Ascaris* and *Giardia* in this set of data.
Table 4

Crosstab

<table>
<thead>
<tr>
<th>Count</th>
<th>Presence/Absence of Crypto</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence/Absence of Ascaris</td>
<td>Positive</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>8</td>
<td>24</td>
<td>32</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>9</td>
<td>29</td>
<td>38</td>
</tr>
</tbody>
</table>

Table 4: Crosstabulation of Presence/Absence of *Ascaris* and *Cryptosporidium* in Biosolids Samples  One sample contained both organisms and a total of 6 were positive for Ascaris ova and a total of 9 were positive for Cryptosporidium oocysts
Table 5

Crosstab

<table>
<thead>
<tr>
<th>Presence/ Absence of</th>
<th>Presence</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of Ascaris</td>
<td>Positive</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>6</td>
<td>32</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>6</td>
<td>38</td>
</tr>
</tbody>
</table>

Table 5: Crosstabulation of Presence/Absence of Ascaris and Giardia in Biosolids Samples

No samples contained both organisms. A total of 6 were positive for *Ascaris* ova and a total of 6 were positive for *Giardia* cysts.
Discussion

Monitoring biosolids samples for the presence of protozoa is not currently required, even though organisms such as Cryptosporidium parvum and Giardia lamblia have been identified as pathogens of concern in wastewater solids. The survival of Giardia poses a significant risk associated with the land application of biosolids. More than 900 cysts per gram-wet weight were discovered in anaerobically digested biosolids (NRC, 2002). When the EPA Part 503 Rule was written, C. parvum was considered a veterinary pathogen and had not been associated with human disease. Giardia and Cryptosporidium must be considered potential public health risks, especially since their small size may allow the cysts/oocysts to move more easily through soils into groundwater as compared to the larger helminth ova.

The prevalence data presented here demonstrate that Ascaris is not a reliable indicator of the presence of either Cryptosporidium or Giardia species. The absence of Ascaris ova from biosolids samples does not indicate that protozoa are also absent from the sample. The prevalence assessment was performed using recovery methods that have been shown in this research to be inefficient. Although these parasites cannot be reliably recovered, the prevalence assessment demonstrated that these parasites do exist in biosolids.
CHAPTER FOUR

EVALUATION OF FECAL COLIFORMS, SALMONELLA, CRYPTOSPORIDIIUM PARVUM, GIARDIA LAMBLIA AND ASCARIS LUMBRICOIDES PERSISTENCE UNDER LIME STABILIZATION CONDITIONS

INTRODUCTION

Biosolids are categorized as Class A or Class B. Class A biosolids contain pathogens below detectable levels whereas Class B sludge contains pathogens which are reduced in number. The requirements for use and disposal of biosolids will depend on the classification. The use of lime to reduce or eliminate pathogen content in sewage sludge represents a simple and inexpensive method of treatment by which Class B sludge can be generated (Farrell 1974). Lime stabilization is a process where calcium hydroxide \((\text{Ca(OH)}_2)\) or calcium oxide \((\text{CaO})\) is added and the pH elevated to 12 for 2 hours and then reduced to 11.5 for 22 hours. Lime has been in use for a number of years for the disinfection and odor suppression of solid wastes.

Fecal coliform bacteria are used to indicate the potential presence of pathogens in class B biosolids (Ryan 1994). Bacteria are important pathogens with regard to biosolids and land application because the potential exists for regrowth following treatment (Yanko, 198
The density of fecal coliforms in sewage are estimated to be $10^3$/ml (Yanko, 2000). The EPA Part 503 regulations require that fecal coliform density may not exceed one thousand most probable number (MPN) or colony forming units (CFU) per gram of total solids (dry weight basis) if the sludge is to qualify as Class A sludge, or must be less than two million MPN or CFU per gram total solids (EPA 1999) if the sludge is to qualify as Class B sludge. *Salmonella* is a microorganism that is of concern in biosolids. Yanko (1988) found that *Salmonella* was frequently found in biosolids in a survey of biosolids in the United States. The EPA Part 503 regulations require that treated sewage sludge must be monitored for fecal coliforms or *Salmonella* species and have a *Salmonella* density of less than three most probable number (MPN) per four grams total solids (dry weight basis) to qualify as a Class A sludge (EPA 2000).

Protozoan parasites include *Giardia* and *Cryptosporidium*, which cause acute gastrointestinal distress resulting in severe diarrhea and may lead to death in immunocompromised individuals (Garcia, 1997). *Cryptosporidium parvum* is commonly found in surface water, is highly resistant to conventional methods of water treatment, and therefore may be a better candidate for the monitoring of human parasites in biosolids for treatment effectiveness.

Helminth ova are currently utilized to indicate effective treatment of biosolids for land application. (Yanko 1988) (WHO 1986). Current EPA 503 regulations require biosolids to be screened for *A. lumbricoides*; however, there
are little data available to indicate the effects of lime stabilization on the viability of these pathogens.

**Materials and Methods**

**Lime Treatments**

Lime treatments were performed independently in 50-mL volumes of Ca\((OH)_2\) solution for inactivation of bacteria, protozoa and helminth. *Cryptosporidium parvum* and *Giardia lamblia* were inoculated to achieve a final concentration of $10^5$ oocysts ($+/\!-5.1\%$) and $5 \times 10^5$ cysts ($+/\!-3.9\%$) per ml, respectively. Aliquots of 3,000 *Ascaris lumbricoides* ova were resuspended in the matrix for both the control and the test samples. Samples were continually mixed on a stir plate and the initial pH and temperature were recorded for control and test beakers. The pH of the test beakers was simultaneously adjusted to 12.0 using an 8% aqueous slurry comprised of calcium hydroxide and deionized water. Approximately 0.4 - 0.5 mL of calcium hydroxide slurry was required to elevate the pH to 12.0, corresponding to a lime dose of approximately 80 g/kg total solids. The pH was maintained at 12.0 for 2 hours at which time, 0.1 N HCl was added drop by drop until a pH value of 11.5 was achieved and maintained for the duration of the experiment. Experiments were performed at room temperature with temperature and pH readings recorded hourly for control and test beakers. The viability of bacteria was evaluated at time points 0.1, 2, 12 and 24 hours. The infectivity of protozoan pathogens was evaluated at 0.1, 2, 12, 24, 48, and 72 hours. Survival of helminth ova was evaluated at 24, 48 and 72 hours. Following
lime stabilization, test beakers were neutralized with 0.1 N HCl and aliquots were removed from designated control and test beakers for enumeration.

**Bacteria**

**Fecal Coliforms**

Fecal coliform densities in RO water samples obtained following lime stabilization experiments were evaluated using a most probable number assay (MPN) according to method 1680, established by the EPA for fecal coliform detection in biosolids by multiple tube fermentation (EPA, 1998). The MPN assay is an estimation of bacterial density and employs culture specific media combined with elevated temperature to isolate and enumerate fecal coliforms. A presumptive step using lauryl tryptose broth (LTB) (Difco) as the selective enrichment medium and a completed step using *E. coli* (EC) (Difco) media permit the recovery and isolation of fecal coliforms. Ten-fold serial dilutions were created and inoculated into 5 test tubes containing sterile LTB and a Durham fermentation tube to indicate gas production. Tubes were incubated for 48 hours at 35°C and observed at both 24 and 48 hours for the presence of presumptive growth indicated by gas or acid production in the fermentation tubes. Failure to produce gas or acid in the LTB media within 48 hours was recorded as a negative presumptive test. LTB presumptive positive tubes were transferred to fermentation tubes containing sterile EC media. EC fermentation tubes were incubated in a water bath at 44.5°C for 24 hours. Gas production in EC broth in 24 hours was considered a positive fecal coliform reaction. Failure to produce gas was a negative reaction and indicated fecal coliform bacteria were not present.
Results of the MPN procedure were reported in terms of MPN/g total solids calculated from the number of positive EC culture tubes. Positive control cultures consisting of \textit{E. coli} were included in each assay to ensure negative results were not from inhibition.

\textit{Salmonella} sp.

\textit{Salmonella} densities following lime stabilization experiments were evaluated using a most probable number assay (MPN) according to method 1682 established by the EPA for \textit{Salmonella} detection in biosolids by multiple tube fermentation (EPA 1998). Sample dilutions were created using phosphate buffered saline. Enrichment was accomplished using selenite brilliant green sulfa (SBG) broth followed by isolation on xylose-lysine deoxycholate agar (XLD). Positive samples were confirmed with triple sugar iron agar (TSI), lysine iron agar (LIA), and urease broth followed by positive serological typing using polyvalent antisera. Results of the MPN procedure were reported in terms of MPN/g total solids. Positive control cultures consisting of \textit{Salmonella typhimurium} and negative control cultures consisting of \textit{E. coli} were included in each assay to ensure negative results were not from inhibition.

\textbf{Protozoa}

\textit{Cryptosporidium parvum}

\textit{C. parvum} oocysts, Moredun (MD) isolate (Okhuysen, 2002); propagated in immunosuppressed mice (Yang et al 1996) were used throughout this study.
Fecal pellets were collected from infected animals and homogenized in 10 ml of water. Oocysts were purified by sedimenting on a step gradient of 15%-25% (w/v) Nycodenz (N, N'-bis (2,3 dihydroxypropyl acetamido-2, 4,6-tri-iodoisophthalamide) (Sigma; St. Louis, Missouri) in water (Widmer et al 1998), brought to 50 ml in water, and pelleted by centrifugation at 4000 x g for 15 min. Purified oocysts were resuspended in 1 ml of water. Following lime stabilization, oocysts were neutralized to pH 7 with 0.1 N HCl, transferred to 50 ml conical polypropylene centrifuge tubes, and precipitated by centrifugation at 4000 x g for 15 min. Pellets were resuspended in 2 mL of RO water and the oocyst concentration determined with a hemocytometer in duplicate. In some experiments, the presence of Ca(OH)2 crystals interfered with obtaining accurate oocyst counts, making it necessary to immunofluorescently label the oocysts (Merifluor Cryptosporidium/Giardia, Meridian Bioscience Inc., Cincinnati, Ohio). Oocysts were then counted by epifluorescent microscopy.

Animal Infectivity

Neonatal CD-1 mice 1-3 days of age (Charles River Laboratories, Wilmington, Mass.) were orally infected with oocysts suspended in 5-μl water using a pipette (Tzipori, 1998). Infected and uninfected control mice were sacrificed on day 7 post-infection, the intestine removed and homogenized in 300-μl phosphate buffered saline in a microcentrifuge tube. A portion of 10 μl of intestinal slurry from each mouse was spread in each well of 10-well Teflon coated microscope slides and the samples air-dried. Oocysts were stained by

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immunofluorescence and detected by epifluorescence microscopy. Samples with a minimum of 1 immunofluorescently labeled oocyst were scored as positive. Positive control mice were infected with oocysts from the same stock as used for the treatments. Oocysts for the negative controls were heat-inactivated at 70°C for 20 min (Fayer, 1994).

*Giardia lamblia*

*Giardia lamblia* cysts of strain H3 purified from experimentally infected gerbils were obtained from Waterborne, Inc., New Orleans, Louisiana. Cysts were purified on sucrose and Percoll density gradients and shipped in PBS supplemented with penicillin, streptomycin (500 U/ml each) and gentamycin (50g/ml).

To assess the infectivity of the cysts, Mongolian gerbils (4-wk-old, Charles River, Wilmington, Massachusetts) were each orally infected with a 20 μl suspension of 1000 cysts (first experiment) or 500 cysts (second experiment) (Widmer et al, 2002). Groups of 8 animals were used for each treatment. Fecal pellets were collected from the gerbils on day 6, 8, 10, 12, and 14 post-infection. The presence of *G. lamblia* cysts in the feces was monitored by immunofluorescence using the Merifluor *Cryptosporidium/Giardia* kit (Meridian Diagnostics, Cincinnati, Ohio) as described (Widmer et al, 2002). Fecal samples were scored as positive if a minimum of 1 immunolabelled cyst was detected.

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**Helminth**

*Ascaris lumbricoides*

*A. lumbricoides* ova were purchased from Tropical Biologicals (Guaynabo, Puerto Rico). Viable ova were concentrated from fecal samples collected from infected humans in the Dominican Republic and shipped in a suspension of 2% formaldehyde to inhibit microbial growth. The suspension was stored at 20°-23°C and aerated daily by removing the lid to exchange the air inside, replacing the lid and shaking four-five times. Storage of ova for more than two weeks required decanting the supernatant and replacing with fresh 2% formaldehyde every two or three weeks. Viability of ova was assessed prior to use in experiments following the EPA Part 503 method. Lime treatment and neutralization was performed as described for *C. parvum*. The entire test and control volumes were centrifuged at 1000-x g for 10 minutes. The supernatant fluid was decanted and the pellet resuspended in 10 mL of RO water. 1 ml sample aliquots were screened using Sedgwick-Rafter counting chambers under 10x magnification. Viability was determined by observing motile larvae in embryonated ova by microscopic evaluation under 40x magnification. One hundred ova were evaluated for each time point.
**Data Analysis**

Statistical analysis of lime stabilization trials to evaluate treatment effectiveness for bacteria was conducted using a general linear model within the SPSS statistical software package. Percent viability was calculated for helminth ova test and control samples and the difference was used to determine overall treatment effectiveness. Infectious dose values for *C. parvum* were calculated according to Reed and Muench (1938) and using the logit method of Finch et al. (1993) and are shown in Figure 2.

**Results**

**Bacteria**

The results of three trials conducted with fecal coliforms (*E. coli*) and *Salmonella* sp. spiked into a limed water matrix at room temperature are presented in Figure 1. In two trials, following 0.1 hours of liming *E. coli* concentrations were below detectable levels (<1 MPN/ gram total solids). In one trial, the concentration of fecal coliforms following initial liming (0.1 hours) was 6.0 x 10^5 MPN/g total solids and was below detectable levels following 2 hours of liming at pH 12. This data point was considered an outlier and was not included in statistical analysis. In all trials, *Salmonella* sp. concentrations were below detectable levels (<3 MPN/4 grams total solids).
Cryptosporidium parvum

The average reduction (n=3) in oocyst concentration was 74.2% (±18.8%) for the controls and 81.7% (±29.9%) for the treatments (2 hr and 24 hr treatments pooled). Oocyst concentrations in control and treated samples dropped significantly during the experiment. The reduction in oocyst concentration was not related to the treatment, since the difference between mock treatment and the actual treatment was statistically not significant (p=0.75, paired t-test), nor was it related to the duration of the exposure (2 hr vs. 24 hr, p=0.41, t-test). The reason for the loss in oocysts was not investigated, but is likely to have resulted from the adherence of oocysts to glass and plastic surfaces and incomplete recovery during centrifugation.

Single-dose experiments

To assess the effect of liming on C. parvum oocysts, single-dose experiments were performed with oocysts exposed to lime solution for 0-72 hours. Immunofluorescent analysis of the 7-day post-infection mouse intestinal homogenates demonstrated that the treatments did not inactivate the oocysts. All samples, including those treated for 72 hours, contained infectious oocysts.

Dose-response experiments

The infectivity of oocysts treated with lime for 2 hr and 24 hr was compared with that of mock-treated control oocysts using dose-response
experiments. Groups of 8 neonatal mice were infected with 6 oocyst doses ranging from $10^2$ to $10^4$. The infection was assessed 7 days post-infection as previously described. A positive control group was infected with 5000 oocysts per mouse pup and a negative control group received the same number of heat-inactivated oocysts. The cumulative method and the logit method (Finch, 1993) both showed that lime stabilization for 24 hr did not inactivate the oocysts (Figure 2). To the contrary, the treatment increased the infectivity of the oocysts by more than 4-fold. Whereas the calculated ID$_{50}$ for the untreated oocysts was 1180, the ID$_{50}$ for the treated oocysts was only 263 oocysts. The same effect was observed after a 2-hr exposure; the ID$_{50}$ for the control oocysts was 986 and for the treated sample was 128 oocysts, a more than 7-fold reduction in ID$_{50}$.

*Giardia lamblia*

Single-dose experiments in gerbils were performed to assess the inactivation of *G. lamblia* cysts exposed to lime solution for 0 hr, 24 hr, 48 hr and 72 hr. In the first liming experiment, cysts were suspended in water. A second experiment was performed using sludge. In contrast to *C. parvum*, cysts limed were completely inactivated during a 48 hr or 72 hr treatment. Following a 24-hr exposure, infectious cysts were still detected, as demonstrated by the presence on day 10 post-infection of 2 positive animals in a group of 8. None of the eight animals infected with cysts exposed to lime for 48 hr and 72 hr excreted cysts between day 6 and day 14 post-infection, consistent with complete inactivation. All positive control animals inoculated with untreated cysts became infected, whereas 2 animals infected with heat-inactivated cysts (negative control)
remained negative until day 14 post-infection, when the experiment was terminated. Mock treatments, during which cysts were subjected to the same conditions as the treated cysts, except that no lime was added to the cyst suspension, slightly reduced cyst infectivity at 24 hr, 48 hr and 72 hr as compared to the positive control group. The proportion of positive gerbils in these mock control groups was 8/8, 7/8, 5/8 and 7/8 for 0 hr, 24 hr, 48 hr, and 72 hr mock treatment, respectively.

In a second experiment liming was performed in sludge instead of water. This experiment confirmed the inactivating effect of lime. Lime treatments for 24 hr, 48 hr, and 72 hr resulted in a complete cyst inactivation; whereas the 0-hr treated and all the mock treated treatment groups infected all animals (8/8).

_Ascaris lumbricoides_

The results of two trials conducted with _Ascaris lumbricoides_ ova spiked into a limed water matrix at room temperature are presented in Figure 3. In both trials _A. lumbricoides_ ova in test samples remained viable following 72 hours of liming at room temperature. There was no significant difference between viability of control and test samples at all time points (p value= 0.52).

**Discussion**

Very few data are currently available indicating the persistence of pathogens under lime stabilization conditions. The goal of this study was to
evaluate the persistence of fecal coliforms, *Salmonella* sp., protozoa and helminth under lime stabilization conditions. Organisms were evaluated in an RO water matrix, due to problems with recovery efficiency from solid matrices (Bean and Brabants 2001). The water matrix represents a best-case scenario since it has been documented that organic material and particulate matter enhance the survivability of microorganisms (Kato et al., 2001 and Straub et al., 1993).

The results indicate that lime stabilization effectively inactivated fecal coliforms and *Salmonella* in a water matrix at room temperature (28°C) when 8% calcium hydroxide slurry created with RO water was added to achieve a pH of 12.

Fecal coliforms are currently used as the microbiological parameter evaluated for Class B certification. Therefore, fecal coliforms are the only parameter used to assess treatment effectiveness. These experiments demonstrate that fecal coliforms cannot be used as an indicator of treatment effectiveness for all pathogens. Fecal coliforms were inactivated immediately upon addition of lime in an RO water matrix at room temperature. The data demonstrate that lime stabilization is effective for inactivation of fecal coliform bacteria when evaluated in limed water.

Indicator organisms for Class A certification include *Salmonella*. *Salmonella* was evaluated and results revealed similar inactivation to fecal
coliforms, thus indicating that bacterial organisms in these experiments behave similarly and are effectively inactivated when exposed to lime in a water matrix.

*Ascaris lumbricoides* is the current parasite indicator for treatment effectiveness to obtain Class A certification although this organism is not consistently present in biosolids samples due to varying geographic distribution (Bean and Brabants, 2001) and inefficient recovery methods (Bean and Brabants, 2000). Previous work performed in our laboratory and in this study indicate that while *Ascaris* is resistant to liming, its scarcity in sludge and low recovery efficiencies prevent it from being a good indicator organism. For these reasons, protozoa, which are ubiquitous in the environment and recovered as frequently as *A. lumbricoides*, were included in this investigation. The results demonstrate viability of *A. lumbricoides* ova following 72 hours of liming. In comparison, *C. parvum* and *G. lamblia*, evaluated under the same conditions, demonstrated enhanced infectivity as a result of exposure to lime.

**Conclusions**

These results demonstrate that liming is effective for reduction of bacteria when evaluated in an RO water matrix at room temperature. The results also demonstrate the ineffectiveness of liming to inactivate *Ascaris lumbricoides* and *Cryptosporidium parvum*, which is of particular concern with regard to land application of biosolids. The results warrant further investigation to determine if inactivation of the organisms evaluated is similar in a solid matrix where they may be less susceptible to alkaline inactivation. This research presents data in

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what should be an on-going investigation to evaluate methods of sludge or
biosolids preparation in an effort to minimize public health threats associated with
land application of biosolids, maximize potential benefits of biosolids and move
our society towards sustainability.
Figure 1: Fecal Coliforms and *Salmonella* sp. Inactivation in Three Trials Conducted in Limed RO Water at Room Temperature
Figure 2: Dose-Response Curves for Cryptosporidium parvum
Dose-response curves were obtained for the inactivation of C. parvum with lime using the cumulative method of Reed and Muench (1938) (left) and the logit method of Finch et al. (1993) (right). The 50% cumulative infection and 0 logit used for determining the ID50 values are shown with dashed horizontal lines. Note the increase in oocyst infectivity (lower ID50) in response to the treatment.
Figure 3: *Ascaris lumbricoides* Inactivation in Two Trials Conducted in Limed RO Water at Room Temperature (28°C)

*A. lumbricoides* ova seeded remained viable following 72 hours of lime stabilization at 28°C.
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CHAPTER FIVE

EVALUATION OF Cryptosporidium parvum, Giardia lamblia and Ascaris lumbricoides Survival Under Heat Inactivation Conditions

INTRODUCTION

Composting is a process that converts organic wastes into a soil amendment product. Organic wastes are partially decomposed in the process by bacteria, worms, and other living organisms resulting in a product, which can be used as fertilizer or a soil conditioner. Composted materials are used to provide nutrients to the soil and replenish depleted organic matter lost through farming. During the composting process, biosolids are stabilized, odors are reduced and high temperature is thought to kill pathogens (Corbitt, 1990). The inactivation of Cryptosporidium parvum, Giardia lamblia and Ascaris lumbricoides was evaluated under temperature conditions of 55°C and 70°C in this study. All composting high temperature inactivation experiments were performed using a biosolids extract in order to maintain consistency and use the same sample matrix for all experiments. This composting protocol was kindly provided by Bill Yanko and Shawn Thompson of the Los Angeles County Sanitation District, Los Angeles, Ca. Thermal death times were achieved for all parasites using heat inactivation at 70°C. No animal infectivity was performed on protozoa since viability assays demonstrated that cysts and oocysts were non-viable by the end of the 60-minute experiments.
The organic components of sewage sludge are biologically decomposed during composting and the resulting product is drier and more stable than dewatered, raw or digested sewage sludge. Pathogens should be below detectable levels as a result of heating during the composting process (Krogmann, 2001). In aerated static pile composting, a bulking agent such as wood chips is added to dewatered biosolids and piled onto a concrete composting pad. The mixture is mechanically aerated during a 26-day period to oxygenate the pile and support aerobic biological activity and to reduce the heat and moisture content of the compost pile mixture. After this 26-day period, the mixture is cured or dried. The resulting product is screened to remove wood chips before the compost moves to distribution.

During composting, bacteria are responsible for 80-90% of the biological degradation of the organic matter found in sewage sludge (Krogmann, 2001). Actinomycetes and fungi are also involved in biological degradation. Bacteria survive up to temperatures of 75°C and have reduced degradation rates at higher temperatures. Factors that influence microbial degradation during composting include moisture content, oxygen content, temperature, carbon/nitrogen ratio and pH. The optimum pH range for composting is between 7 and 8 (WEF, 1995).

Because most microbial decomposition occurs in liquid films on the surface of particles, moisture is essential to the decomposition process. The minimum moisture content ranges between 12% and 25% and varies depending on the coarseness of the feedstock and the composting technology used (Krogmann, 2001). Oxygen supply is essential and aeration serves to dry the compost as well as control temperature.
optimum temperature during the high-rate decomposition is 55°C. At temperatures over 60°C, the diversity of microorganisms is reduced and at 75-80°C no biological activity was detected (Strom, 1985). Temperature requirements to reduce pathogens below detectable levels are 3 days above 55°C in aerated static pile composting systems. Biodegradable wastes usually contain enough macronutrients including carbon to sustain the composting process with the exception of nitrogen. Carbon/nitrogen ratios (C/N) should be between 20:1 and 30:1 when composting begins and varying the composition of the initial feedstock can control this.

Materials and Methods

All composting high temperature inactivation experiments were performed using a biosolids extract in order to maintain consistency and use the same sample matrix for all experiments. Bill Yanko and Shawn Thompson of the Los Angeles County Sanitation District kindly provided this protocol.

Biosolids Extract

Four hundred grams of an aerobically digested sludge was suspended in 1 liter of RO water and centrifuged (30 minutes at 10,000 x g) to remove the solids fraction. The supernatant fluid was filtered through a series of membrane filters of decreasing pore size to 0.45 μm. The extract was then heated to 70°C for 30 minutes to inactivate any organisms penetrating the filter and refrigerated for use in future experiments.
Inactivation Experiments

Nine hundred ul aliquots of biosolids extract was dispensed into microfuge tubes (one per time point) and heated to temperature (55°C or 70°C) by floating in the water bath for 30 minutes prior to inoculation. Microorganisms were added to each tube to achieve a final concentration of $10^3$ *Giardia lamblia* cysts, $10^3$ *Cryptosporidium parvum* oocysts, and 90-100 *Ascaris lumbricoides* ova per tube. Inoculated tubes were gently mixed and then floated in the heated water bath. At selected time intervals, one tube was removed from the water bath, cooled and analyzed for viability. For the 55°C composting experiments, viability assays performed for *Cryptosporidium parvum* included excystation only. Viability for *C. parvum* from composting experiments performed at 70°C included both excystation and vital staining (DAPI/PI). Protocols for both of these viability assays are included as appendices. Light microscopy was performed to identify broken, intact and/or empty cysts of *Giardia lamblia* as an initial screening of viability. Vital staining (FDA/PI) was then performed only on those samples that contained intact cysts. Viability of *Ascaris lumbricoides* was determined by microscopic evaluation of ova. Untreated oocysts/cysts/ova were used as a positive control and negative controls included the extract alone, which contained no organisms, and heat inactivated oocysts/cysts (65°C for 30 minutes).

Results

55°C Inactivation Experiments

Two experiments were run at 55°C to assess the inactivation of helminth ova and protozoan cysts/oocysts. In the first experiment, *Ascaris lumbricoides* ova and
Cryptosporidium parvum oocysts were tested over a 60-minute period, which included samples taken at 9 time points, beginning 5 minutes after the sample was heated at 55°C and ending after one hour at this temperature (Table 1). The second experiment was performed at the same temperature and with the same biosolids extract, but time points were limited to 15, 30, 45 and 60 minutes.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>% Viability Ascaris ova</th>
<th>% Viability Cryptosporidium oocysts</th>
<th>% Intact Giardia cysts</th>
<th>% Viability Giardia cysts</th>
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<tr>
<td>Time point (minutes) Run 1 Run 2</td>
<td>Run 1 Run 2 (Run 2 only)</td>
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<td>Run 1 Run 2</td>
<td>Run 2 (Run 2 only)</td>
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<td>89</td>
<td>73</td>
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Table 1: Heat inactivation of Ascaris lumbricoides ova, Cryptosporidium parvum oocysts and Giardia lamblia cysts at 55°C in Biosolids Extract
Helminth ova

The first experiment, trial 1, to assess inactivation or loss of viability over 60 minutes of time in biosolids extract at 55°C showed that Ascaris viability was not reduced at this temperature. The ova were subject to this temperature and one sample was removed from the water bath and incubated at room temperature for 30 days before being evaluated for viability microscopically. A second run with fewer time point pulls also showed that after 60 minutes at 55°C, 73% of the ova were viable (Figure 1). The ova controls performed prior to starting these experiments were 88% viable (trial 1) and 75% viable (trial 2).

Figure 1

Figure 1: Inactivation of Ascaris lumbricoides ova at 55 degrees Celsius
Cryptosporidium parvum oocysts

Both trials 1 and 2 demonstrate that viability (assessed by excystation) is reduced after 60 minutes. In trials 1 and 2, 78% and 75% of the oocysts were viable respectively at the start of the experiment (viability assessed by excystation). When comparing the loss of viability after 60 minutes, trial 1 demonstrated that viability dropped from 78% to 28% and trial 2 demonstrated that viability dropped from 75% to 42% (Figure 2).

Figure 2

![Graph showing inactivation of Cryptosporidium parvum at 55 degrees Celsius]

Figure 2: Inactivation of Cryptosporidium parvum at 55 degrees Celsius
Giardia lamblia cysts

Giardia lamblia cysts were assessed first by light microscopy to determine intactness before performing additional viability testing. If all cysts appeared damaged by light microscopy, no additional viability testing was performed. If intact cysts were present, as was seen in the four test time points (15, 30, 45 and 60 minutes), vital staining using FDA/PI followed microscopy and 100 cysts were counted. When comparing the loss of viability after 60 minutes, viability dropped from 89% at the start of the trial (untreated control) to 59% after 60 minutes at 55°C (Figure 3).

Figure 3

Figure 3: Inactivation of Giardia lamblia at 55 degrees Celsius
70°C Inactivation Experiments

Three experiments were conducted at 70°C to assess the inactivation of *A. lumbricoides* ova, *C. parvum* oocysts and *G. lamblia* cysts. Four time-points were sampled for each parasite over a 60-minute period (Table 2). Vital staining and excystation were performed on *C. parvum* oocysts. *G. lamblia* cysts were initially screened by light microscopy to determine intactness. If intact cysts were identified, vital staining was then performed on *G. lamblia*. *Ascaris* ova viability was assessed by microscopy.

<table>
<thead>
<tr>
<th>Time Point (minutes)</th>
<th>% Viability <em>Ascaris</em> N=3</th>
<th>% <em>C. parvum</em> Excystation N=3</th>
<th>% <em>C. parvum</em> viable DAPI/PI N=3</th>
<th>% <em>G. lamblia</em> intact N=3</th>
<th>% <em>G. lamblia</em> viable FDA/PI N=3</th>
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Table 2: Average Percent Heat inactivation of *Ascaris lumbricoides* ova, *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts at 70°C in Biosolids Extract

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Helminth ova

Three separate trials were performed and the mean of the three runs is shown in Table 2. Ova were subjected to 70°C and one sample was taken from the water bath at each time point and incubated at room temperature for 30 days before being evaluated microscopically for viability. After 60 minutes at 70°C, no viable helminth ova were found (Figure 4).

Figure 4: Inactivation of Ascaris lumbricoides ova at 70 degrees Celsius
Cryptosporidium parvum oocysts

After 15 minutes at 70°C, 9% of the oocysts were viable when assessed by excystation (Figure 5) and no viable oocysts were seen with the use of vital staining. At all time points thereafter, either excystation or vital staining demonstrated no viable oocysts.

Figure 5

Figure 5: Inactivation of Cryptosporidium parvum oocysts at 70 degrees Celsius
**Giardia lamblia cysts**

After 15 minutes at 70°C, 7% of 100 cysts appeared intact and 6% stained as viable using FDA/PI. At all time points thereafter, no viable cysts were demonstrated by vital staining (Figures 6 and 7).

**Figure 6**

Figure 6: Inactivation of *Giardia lamblia* cysts at 70 degrees Celsius assessed by Microscopy
Figure 7: Inactivation of *Giardia lamblia* at 70 degrees Celsius assessed by Vital Staining.
Discussion

Pathogens are destroyed during composting due to thermal killing, antibiotic action or by the decomposing organisms and their products (Golueke et al., 1958). This study was designed to determine whether pathogens could survive thermophilic temperatures present during composting. Thermal death times were achieved for all parasites using heat inactivation at 70°C. Comparative data on the effects of thermal treatment on ova and oocysts of parasites indicate that the infectivity is reduced by thermal treatment (Moce-Llinina et al., 2002). When sewage sludge is composted to achieve 70°C for a minimum of one hour, helminth ova, protozoan cysts and oocysts should be destroyed although when organisms associate with particulates in a complex sample matrix like biosolids; some protection from heat may be afforded. Once the oocysts, cysts and helminth ova are destroyed however, there is no threat of regrowth with parasites as has been demonstrated with bacteria.

No animal infectivity was performed on protozoa since viability assays demonstrated that cysts and oocysts were non-viable by the end of the 60-minute experiments. Process controls for composting to include monitoring the temperature of piles can ensure that pathogens are inactivated. Composting as a method of treating biosolids is effective in controlling odors, which makes acceptance of land application more tolerable for the general public. One disadvantage of composting is the threat of bioaerosol creation. It is difficult to control this potential health threat, especially to workers in the composting facility and neighboring communities. The major areas of concern in bioaerosol creation are fungi; specifically Aspergillus fumigatus, and
endotoxins and risk assessment has not been conducted for bioaerosols in composted biosolids (Milner et al., 1994).

Class A treatment methodologies result in a “virtually” pathogen free product. These methodologies typically achieve a high temperature as part of the process as compared to Class B processes, which do not. A Class A product will have a reduction in pathogens, which is greater than the reduction achieved in Class B products. For this reason, there are a growing number of sewage treatment plants moving to Class A processes in order to produce a product which the public will be more likely to accept as safe.

There are many factors including the production of heat that contribute to the breakdown of organic material during the process of composting. To test biosolids samples for the possible presence of any potential pathogen is impossible due to the limitations of methods of detection and the cost of testing. There are emerging pathogens of concern for which no methods of detection even exist. Therefore, process controls may be a more effective way of ensuring that the biosolids product meets certain predetermined standards before being labeled a “Class A” product. If controls can ensure that the process successfully achieved the Class A product by meeting time, temperature, pH, etc., this could be a measure of quality assurance for the production of Class A biosolids by composting.
REFERENCES


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University of New Hampshire

Helminth Ova in Biosolids by Flotation

March 22, 2001

Prepared by: Christine Bean and Jacqueline Brabants

Administrator: Dr. Aaron Margolin
I. Procedural Section:

A. Scope & Applicability: This test method describes the detection, identification, enumeration and determination of viability of *Ascaris* ova in water, wastewater, sludge and compost. These pathogenic intestinal helminths occur in domestic animals and humans. The environment may become contaminated through direct deposition of human or animal feces or through sewage and wastewater discharges to receiving waters. Ingestion of water containing infective *Ascaris* ova may cause disease.

B. Summary of Method: This method is used to concentrate pathogenic *Ascaris* ova from wastewater, sludge, and compost. Samples are processed by blending with buffered water containing a surfactant. The blend is screened to remove large particulates. The solids in the screened portion are allowed to settle out and the supernate is decanted. The sediment is subjected to density gradient centrifugation using magnesium sulfate (sp. 1.20). This flotation procedure yields a layer likely to contain *Ascaris* and some other parasitic ova if present in the sample. Small particulates are removed by a second screening on a small mesh size screen (38μ). Proteinaceous material is removed using an acid-alcohol/ethyl acetate extraction step. The resulting concentrate is incubated at 26 °C until control *Ascaris* eggs are fully embryonated. The concentrate is then microscopically examined for the categories of *Ascaris* ova on a Sedgwick-Rafter counting chamber.

C. Definitions:

1. Helminth Ova: Literally translates into *worms eggs*. Includes Nematodes (roundworms), Cestodes (tapeworms), and Trematodes (flukes). This procedure includes three Nematodes: *Ascaris*, *Trichuris*, and *Toxocara*, and one Cestode: *Hymenolepis*.
2. Class A Biosolids: contain an ova density of <1 viable ova/ 4g of total solids on a dry weight basis.
3. *Ascaris lumbricoides*: A large roundworm having very thick transparent shelled eggs covered by a mammillated albuminoid outer layer. (55-75 um long X 35-50 um wide)
4. *Trichuris trichiura*: A whip-worm which has a thick shelled, barrel shaped egg that is yellow-brown with prominent, clear, mucoid plugs at the ends. (50-55 um long X 22-24 um wide)
5. *Toxocara canis* and *cati*: Small roundworm which has brownish eggs almost spherical with surficial pits, and are unembryonated when laid. (75-85 um long X 60-75 um wide). *Important because humans are often accidental hosts which result in visceral larval migrans.*
6. *Hymenolepis nana* and *diminuta*: Are the smallest of the tapeworms. *H.nana* has a spherical to subspherical egg with a thin shell with two thickenings at opposite poles. (30-47 um X 20-60 um). *H.diminuta* is larger with a thick shelled yellow-brown egg (70-85 um long X 60-80 um.

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Important because it is the most common tapeworm in the United States.

D. Safety Warnings:

1. The Analyst must know and observe normal safety procedures and proper handling should be followed closely when working with wastewater. Mouth-pipetting is prohibited.

E. Cautions: Field and laboratory staff collecting and analyzing environmental samples are under some risk of exposure to pathogenic microorganisms. Staff should apply safety procedures used for pathogens to handle all samples.

F. Inferences:

1. Sludge samples should not be frozen during transport or storage since freezing could be detrimental to the viability of ova present in the samples. Sludge samples should be stored at refrigerator temperature.
2. Numerous transfers of the sample to new vessels may decrease the recovery of ova.
3. Under microscopic examination, interfering debris such as: pollen, starch, plant hairs, and vegetable material may interfere with reading of the sample (Figure 1).

G. Personnel Qualifications:

1. Laboratory Analyst/Technician: should have a B.S. and/or at least 1 year training in working with wastewater. Technicians should be well versed in safety as well as proper handling of wastewater samples both semisolid and fluid. The analyst must also demonstrate acceptable performance during an on-site evaluation.

H. Apparatus & Materials:

1. Centrifuge, Table Top, that can sustain forces of at least 660 x g with a swinging bucket rotor to hold 15, 50, 100 and 250 ml centrifuge glass or plastic conical bottles.
2. Centrifuge tubes 250 ml, 100 ml
3. 15 ml and 50 ml Teflon tubes
4. Analytical balance capable to 10 mg.
5. Thermometers 0EC to 50EC, checked against a NIST thermometer
6. Automatic Pipette, with filter
7. Blender and Blender Jar
8. Incubator capable of maintaining temp. 26EC
9. Laboratory vacuum source and vacuum apparatus (2 L flask or larger) stopper to fit vacuum flask, fitted with a glass or metal tubing as a connector for 1/4 inch tygon tubing.
10. Binocular light microscope with 10X, 40X, 100X objectives
11. Gloves
12. 2L Pyrex Beakers
13. 48 and 400 Mesh Tyler Stainless Steel Sieve
14. Autoclave
15. Large Plastic Funnel
16. Wooden applicator sticks
17. Pasteur Pipettes and bulb
18. Hydrometer
19. Slides and Cover slips
20. Sedgwick-Rafter cell
21. Teflon Spatula
22. Large test tube rack to accommodate 100 or 250 ml centrifuge tubes
23. Small test tube rack to accommodate 15 ml and 50 ml centrifuge tubes
24. Number A0" stoppers
25. Wash bottles (500 ml) label AWater.@
26. Spray bottles 2 (16 fl oz.) label one AWater,@ and one A7X Limbro"

I. Reagents
1. Dulbecco=s Phosphate Buffered Water (Sigma # D5652)
2. 7X Limbro (Sigma #)
3. Magnesium Sulfate sp. gr. 1.20 (Fisher # Z68-500)
   Check sp.gr with hydrometer: to low add powder to high add water.
4. Acid/Alcohol Solution: V/V
   0.1 N Sulfuric Acid H2SO4 (Fisher # A300-500)
   35% Ethyl alcohol (Fisher # A407-500)
5. 0.1 M Sulfuric Acid (Fisher # A300-500)
6. Fresh AAscaris ova for positive control purified from AAscaris infected human fecal material purchased from Tropical Biologicals Puerto Rico

J. Instrumentation and Methods of Calibration:
1. Incubator at 26EC, Thermometer in the bath checked against NIST thermometer.
2. Analytical Balance, NIST Weights (1,10,100 grams)

K. Sample Collection:
1. Solid Samples should be collected in sterile bags. Liquid samples should be collected in clean, leak proof, sterile screw cap containers.
2. Part 503 suggests that a geometric mean of a minimum of 7 grab samples taken over a 14 day period is adequate for sampling of biosolids.
3. Collect 1 liter of compost, wastewater, or sludge in accordance with Practice D 1066, Specification D 1192, and Practices D 3370, as applicable.

L. Handling & Preservation:
1. Sample transport to laboratories should be on ice and refrigerated. Do not freeze samples. Samples not analyzed promptly should be stored at 0-4 degrees C and analyzed within 24 hrs.
2. Always keep copies of all data sheets from analysis of the sample in your log book. Be sure to sign each copy.

M. Sample Preparation and Analysis:
1. Initial Preparation
   A. For dry or thick samples, weigh 50 grams and place in 450 ml RO water in a beaker and let soak overnight at 4-10 EC.  
   B. Transfer to a blender and blend at high for one minute.
2. For liquid samples measure 450 ml sample blend at high speed for one minute.
3. Pour homogenized sample into one liter beaker and using wash bottle, thoroughly rinse blender container into beaker. Add 200 ml 1% 7X Limbro
4. Allow sample to settle overnight at 4-10 EC. Stir occasionally with a wooden applicator, as needed to ensure that material floating on the surface settles. Additional 1% 7X Limbro may be added and the mixture stirred if necessary.
5. Vacuum aspirate supernate to just above the layer of solids. Transfer sediment to blender and add 200 ml RO water. Blend again for one minute.
6. Transfer blended sample to beaker and rinse blender. Add 200 ml 1% 7X Limbro. Allow to settle for overnight at 4-10 EC.
7. Vacuum aspirate supernate to just above the layer of solids.
8. Add 200 ml 1% 7X Limbro and stir for 5 minutes on a magnetic stirrer.
9. Strain sample through 50- mesh tyler sieve placed in a funnel over a tall beaker and add 100 ml 1% 7X Limbro. Wash sample through sieve with a spray of 1% 7X Limbro from a spray bottle. Allow sample to settle overnight at 4C.
10. Vacuum supernate to just above layer of solids. Mix sediment and distribute equally to 250 ml bottles. Wash sediment from beaker into bottles with RO water from a wash bottle.
12. Centrifuge for 3 minutes at 1250 rpm (400g) at 4EC with no brake. Pour off supernate to above the level of sediment.
13. Resuspend the pellet in MgSO<sub>4</sub> solution in a1:1 ratio to yield a layer of ova. Centrifuge immediately for 3 minutes at 1250 rpm. DO NOT USE BRAKE.
14. Pour supernate into 250 ml centrifuge bottles and dilute to at least 2 the concentration with RO water. Allow to settle overnight while ova float to the surface. Do not disrupt flotation during this time.
15. Carefully decant supernate which contains ova if present and centrifuge in 250 ml centrifuge bottle(s).
16. Centrifuge the tubes for 3 minutes at 1400 rpm with no brake. Discard supernate.
17. Aspirate supernate and resuspend pellet in 4 ml 0.1N H₂SO₄.-
18. Add 4 drops of surfactant. Cap and mix the contents thoroughly by shaking several times.
19. Insert plastic filtering device into top of 15 ml centrifuge tube. Pour fecal suspension through filtering device into the centrifuge tube.
20. Discard filtering device. Bring volume to tube up to 15 ml with RO water and centrifuge at 500 xg (1800-2000 rpm) for 2 minutes.
21. Decant supernatant and retain the sediment. Resuspend the sediment in 9ml of RO water.
22. Add approximately 3ml ethyl acetate. Cap the tube and shake for 30 seconds inverting the tube while shaking. CAUTION: pressure may build up with the tube during shaking. Carefully release the pressure by opening the cap on the centrifuge tube away from your person under the fume hood.
23. Centrifuge the tube at 500 xg (1800-2000 rpm) for 2 minutes.
   Examination of the tube after centrifugation should reveal four distinct layers from the top:
   1. A layer consisting of ethyl acetate
   2. A plug of fecal debris
   3. A discolored aqueous layer
   4. A sediment layer containing the parasites
24. Hold the tube in a vertical position. Free the plug of debris by ringing with a wooden cotton swab. Decant the upper layers leaving the sediment. DO NOT TURN THE TUBE UPRIGHT UNTIL THE SIDES OF THE TUBE HAVE BEEN CLEANED WITH THE COTTON TIP SWABS.
25. Resuspend the sediment in 1 ml RO water. Place suspension on Sedgwick Rafter counting chamber. Read for presence of Ascaris ova. If none are present, report no viable helminth ova seen. If Ascaris ova are recovered, proceed with step 26
26. Mark liquid level on each tube and incubate along with control vials containing Ascaris ova mixed with 4 ml 0.1 N H₂SO₄ at 26EC for 3-4 weeks. Check liquid level every 1-2 days and add RO water up to the initial liquid level line as needed.
27. Examine the concentrates microscopically using a Sedgwick-Rafter cell to enumerate the detected ova. Classify ova as either unembryonated, embryonated to the first, second or third larval stage. (see attached figures 1-6)
28. Appendix A. shows pictures of the three ova mentioned in this procedure and some pseudo parasites and pitfalls that might be seen during examination. These pictures should be used routinely to aid in examining samples.
O. Calculations:
1. Calculate % Total Solids using the % moisture result.

\[ \text{% Total Solids} = 100\% - \text{% moisture} \]

2. Calculate categories of ova/g dry weight in the following manner

\[ \text{Ova/g dry wt} = \frac{(\text{NO}) \times (\text{CV}) \times (\text{FV})}{(\text{SP}) \times (\text{TS})} \]

Where:
- NO = Number of ova
- CV = Chamber volume (=1 ml)
- FV = Final Volume in ml
- SP = Sample processed in ml or grams
- TS = % Total Solids

P. Records Management:
1. All records for all sludges are kept in a log book by date.

2. Report the results as the total number of *Ascaris* ova, number of unembryonated *Ascaris* ova, number of 1st, 2nd, or 3rd stage larva; reported as number of *Ascaris* ova and number of various larval *Ascaris* ova per gram dry weight.

Q. Waste Management: All laboratory waste is sterilized prior to disposal.

II. Quality Control and Quality Assurance
A. Quality Control:
1. Calibration:
   a. Balance is calibrated with NIST weights (1, 10, 100 grams) every month.
   b. Thermometer is calibrated with NIST thermometer every six months.
   c. Check temperatures in incubator daily and record in log book.
   d. Centrifuge is calibrated according to procedures outlined in the Lab Administration text

2. Obtain reference parasites from qualified outside sources. Tropical Biologicals for *Ascaris* ova and Dr. Widmer Giovanni at Tufts Veterinary School for *Cryptosporidium parvum* and *Giardia lamblia*

3. Run one positive control with each batch of samples.
B. Quality Assurance:
   1. The minimum requirements of a formal quality assurance (QA) program consist of an initial demonstration of laboratory capability through the analysis of positive and negative control samples and blanks, and analysis of positive and negative control samples and blanks as tests of continued performance.

III. References:


Appendix A Identification of Helminth Ova

<table>
<thead>
<tr>
<th>Helminth</th>
<th>Ova Size</th>
<th>Shell</th>
<th>Color</th>
<th>Char.</th>
<th>Worm</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ascaris lumbricoides</em></td>
<td>55-75 um L 35-50 um W</td>
<td>Thick Shell</td>
<td>Transparent</td>
<td>Outer Bumpy layer</td>
<td>Large Round</td>
</tr>
<tr>
<td><em>Trichuris trichiura</em></td>
<td>50-55 um L 22-24 um W</td>
<td>Thick Shell</td>
<td>Yellow</td>
<td>Barrels shaped</td>
<td>Whip Worm</td>
</tr>
<tr>
<td><em>Toxocara canis</em></td>
<td>75-85 um L 60-75 um W</td>
<td>Shelled spherical</td>
<td>Brownish</td>
<td>surface pits</td>
<td>Small Round</td>
</tr>
<tr>
<td><em>Toxocara cati</em></td>
<td>75-85 um L 60-75 um W</td>
<td>Shelled spherical</td>
<td>Brownish</td>
<td>surface pits</td>
<td>Small Round</td>
</tr>
<tr>
<td><em>Hymenolepis nana</em></td>
<td>20-60 um L 30-47 um W</td>
<td>Thin Shell spherical</td>
<td>Yellow Brown</td>
<td>Thickenings at ends</td>
<td>Tape Worm Smallest</td>
</tr>
<tr>
<td><em>Hymenolepis diminuta</em></td>
<td>70-85 um L 60-80 um W</td>
<td>Thick Shell spherical</td>
<td>Yellow Brown</td>
<td>Larger than nana</td>
<td>Tape Worm Smallest</td>
</tr>
</tbody>
</table>
Appendix B

Procedure for Cryptosporidium Excystation:
(Robertson et al Parasitology 1993 (196) pp. 13-19)

Reagents: *Reagents should be made freshly (<30 minutes before use)
-Bile Solution: 1% bovine bile in Hank’s minimal essential medium (HMEM)
-Sodium Hydrogen Carbonate Solution: 0.44% sodium hydrogen carbonate in RO water

Procedure:
1. Add 200 µl of bile solution and 50 µl of sodium hydrogen carbonate solution to 100 µl of purified oocysts
2. Mix reactants gently and incubate at 37C for 4 hours

Enumeration of excystation percentages:
For each enumeration or time point, at least 100 oocysts should be counted and this should be performed in duplicate. The percentage excystation is calculated as follows:

\[
\text{Percentage excystation} = \frac{\text{No. of ghosts (totally excysted)} + \text{No. of partly excysted oocysts}}{\text{Total no. of oocysts counted}} \times 100
\]

Count at a magnification of x400 by using differential interference microscopy.
Dynal® Immunomagnetic Separation (Dynabeads GC-Combo)

April 7, 2000

Prepared by: Christine L. Bean and Jacqueline J. Brabants

Administrator: Dr. Aaron B. Margolin    Lab Manager: Nicola A. Ballester
I. Procedural Section:

A. Scope & Applicability: This method is used for rapid, selective separation of *Giardia* cysts and *Cryptosporidium* oocysts from water sample concentrates using immunomagnetic separation (IMS). The method replaces flotation techniques currently used for separating cysts and oocysts from other debris in water sample concentrates.

B. Summary of Method: Dynabeads anti-*Cryptosporidium* and Dynabeads anti-*Giardia* are incubated with the water sample concentrate along with SL-Buffer. The antibodies coated on the beads will selectively bind cysts and/or oocysts within the water sample concentrate and form a complex. The Dynabeads-organism complexes are separated using a Dynal® magnetic particle concentrator (Dynal® MFC) and subsequently the cysts and oocysts are dissociated from the beads. The resultant suspension for screening will be clean and of a small volume (approximately 50 μl).

C. Definitions:

1. *Giardia*: A genus containing flagellate protozoan organisms, including *Giardia lamblia*. The organism exists in a trophozoite (9-21 μm in length and 5-15 μm in width) and a cyst form and targets the intestinal tract manifesting as diarrhea, cramping and greasy stools.

2. *Cryptosporidia*: Small obligate, intracellular parasites that infect the intestinal tract of a wide range of mammals and range in size from 2-6μm. The species infecting humans and cattle is referred to as *Cryptosporidium parvum*. The principal route of infection is the fecal-oral transmission and infective oocysts are excreted in stool. Clinical manifestations include explosive, self-limiting diarrhea in immunocompetent individuals.

D. Safety Warnings:

1. The Analyst must know and observe normal safety procedures and proper handling should be followed closely when working with protozoan parasite samples. Mouth-pipetting is prohibited.

2. Standard laboratory protective clothing must be worn.

3. 0.02% sodium azide is used as a preservative in this product. Flush with large volumes of water to prevent build-up of azide in plumbing.

E. Cautions: Field and laboratory staff collecting and analyzing environmental samples are under some risk of exposure to pathogenic microorganisms. Staff should apply safety procedures used for pathogens to handle all samples.
F. Inferences:
1. Numerous transfers of the sample to new vessels may decrease the recovery of ova.
2. Under microscopic examination, interfering debris such as pollen, starch, plant hairs, and vegetable material may interfere with reading of the sample (Figure 1).
3. Cysts and oocysts must be washed twice in PBS/Tween followed by once in demineralized water before seeding experiments are conducted.
4. Kit and reagents must be brought to room temperature before use.
5. Vortex only for dissociation and not after the capture processes.
6. The use of vacuum aspirators has been shown to significantly reduce the recovery.
7. Resuspend the Dynabeads before use to obtain a homogenous suspension of beads in suspension. Shake SL-Buffer A and B before use.
8. Precautions should be taken to prevent bacterial contamination of opened vials.

G. Personnel Qualifications:
1. Laboratory Analyst/Technician: should have a B.S. and/or at least 1 year training in working with wastewater. Technicians should be well versed in safety as well as proper handling of wastewater samples both semisolid and fluid. The analyst must also demonstrate acceptable performance during an on-site evaluation.
2. Any laboratory skilled, equipped, and/or certified for *Giardia* and *Cryptosporidium* testing on water and environmental samples may use Dynabeads® GC-Combo. The user must be skilled in using conventional techniques and interpreting results. A user who is proficient in recognition of *Giardia* cysts and *Cryptosporidium* oocysts must perform screening of slides for enumeration of separated oocysts.

H. Apparatus & Materials:
1. Automatic Pipette, with filter
2. Binocular light microscope with 10X, 40X, 100X objectives
3. Oil Immersion
4. Gloves
5. Autoclave
6. Slides and Cover slips
7. Dynal Spot-on well microscopy slides
8. Sedgwick-Rafter cell
9. Small test tube rack to accommodate Dynal® tubes
10. Microfuge tube rack
11. 1.5 ml microfuge tubes
12. Dynal® Magnetic particle concentrator MPC
13. Dynal® L10 tubes
14. Dynal® rotating mixer
15. Vortex
16. (1) 15 ml conical tube
17. Micropipette (10-1000μl)
18. Fluorescence microscope
19. Demineralized cyst/oocysts free water

I. Reagents
1. Hydrochloric acid
2. Sodium hydroxide solution
3. Methanol
4. Fluorescein isothiocyanate (FITC) conjugated anti-Cryptosporidium
   and anti-Giardia monoclonal antibody.
5. 4’6 diamidino-2-phenyl indole (DAPI)
6. DABCO/glycerol mounting medium
7. Dulbecco’s Phosphate Buffered Water (Sigma # D5652)
8. Giardia and Cryptosporidium positive control
9. Dynabeads® anti-Cryptosporidium and Dynabeads® anti-Giardia
   These two reagents are uniform, monodisperse, and paramagnetic,
   microscopic beads with purified antibodies against Cryptosporidium
   oocysts and Giardia cysts covalently bound to the surface. The beads
   are supplied as a suspension in phosphate buffered saline (PBS), pH
   7.4 with 0.1% bovine serum albumin (BSA).
10. 10X SL-Buffer A (clear, colorless solution) and 10X SL-Buffer B
    (magenta solution). This is a two component buffer system which has
    been specifically designed to enable efficient separation of both
    Giardia
    cysts and Cryptosporidium oocysts to occur from a wide range of
    water types. Buffers are stable when stored unopened at 2-8°C until
    the expiration date stated.

J. Instrumentation and Methods of Calibration:
1. Centrifuge is calibrated according to procedures outlined in the Lab
   Administration text.

K. Sample Collection:
1. The water sample concentrates can be of any treated water or
untreated (Raw) water which is intended for potable supply

2. Water sample concentrates should be prepared by standard filtration and centrifugation methods.

3. The quantity of particulate matter in each sample should be such that the packet pellet volume is 5% or less.

4. Following centrifugation at a minimum of 1050g for 10 min carefully remove all the supernatant. Add reagent grade water so that the final volume does not contain more that 0.5 ml packet pellet (measured following centrifugation) per 5 ml. If final volume is greater than 5 ml the sample must be subdivided with each aliquot not exceeding 5 ml.

5. If the sample is suspended in eluting detergents or preserving solutions then it should be resuspended in water.

L. Handling & Preservation:
   1. Samples not analyzed promptly should be stored at 0-4 degrees C and analyzed within 24 hrs.
   2. Always keep copies of all data sheets from analysis of the sample in your log book. Be sure to sign each copy.

M. Sample Preparation and Analysis:
   1. Obtain a flat-sided Dynal® (L10) tube.
   3. Immediately transfer 5 ml of water sample to the Dynal® (L10) tube containing the buffer.
   4. Vortex Dynabeads® anti-Cryptosporidium to fully Resuspend.
   5. Add 50 µl of Dynabeads® anti-Cryptosporidium to the Dynal® tube containing the sample.
   6. Vortex Dynabeads® anti-Giardia to fully Resuspend.
   7. Add 50µl to the Dynal® (L10) tube containing the sample.
   8. Affix the Dynal® (L10) tube to a Dynal® rotating mixer and rotate 15-20 rpm for 1 hour at room temperature.
   9. Following incubation remove tube from mixer and place in a Dynal® MPC-1 with the flat side towards the magnet
   10. Without moving the tube from the Dynal® MPC-1, place magnet side downwards and gently rock tube for 2 minutes.
   11. Return tube to the upright position and decant supernatant while tube is in the concentrater.
   12. Remove the tube from the concentrater and add 500µl of 1X SL buffer A and mix gently. DO NOT VORTEX!
   13. Transfer the liquid to a 1.5 ml microcentrifuge tube.
   14. Place the microcentrifuge tube into a Dynal® MPC-S
   15. Gently rock the tube for 1 minute with one rock per second
   16. Remove supernatant from the tube while held in the MPC-S. DO NOT
SHAKE TUBE!

17. Remove magnetic strip from Dynal® MPC-S
18. Add 50µl of 0.1 N HCL to the microcentrifuge tube and vortex thoroughly for 10 seconds.
19. Place tube in MPC-S without magnet and allow to stand 10 minutes at room temperature.
20. Vortex thoroughly for 10 seconds.
21. Ensure all sample is at the base of the tube and place in MPC-S.
22. Allow tubes to stand undisturbed for 10 seconds.
23. Prepare Dynal® spot-on slide and air dry.
24. Scan slide with light microscope.
25. Appendix A. shows pictures of the two protozoans mentioned in this procedure and some pseudo parasites and pitfalls that might be seen during examination. These pictures should be used routinely to aid in examining samples.

P. Records Management:
1. All records for all samples are kept in a log book by date.
2. Report the results as the total number of Cryptosporidium oocysts and Giardia cysts seen during examination.

Q. Waste Management: All laboratory waste is sterilized prior to disposal.

II. Quality Control and Quality Assurance
A. Quality Control:
1. Calibration: Centrifuge is calibrated according to procedures outlined in the Lab Administration text
2. Obtain reference parasites from qualified outside sources. Dr. Widmer Giovanni at Tufts Veterinary School for Cryptosporidium parvum and Giardia lamblia
3. Run one positive control and one negative control with each batch of samples.

B. Quality Assurance:
1. The minimum requirements of a formal quality assurance (QA) program consist of an initial demonstration of laboratory capability through the analysis of positive and negative control samples and blanks, and analysis of positive and negative control samples and blanks as tests of continued performance.
III. References:

APPENDIX D

University of New Hampshire

Merifluor® Cryptosporidium/Giardia

October 22, 2003

Prepared by: Christine L. Bean and Van Pham

Administrator: Dr. Aaron Margolin
I. Procedural Section

A. **Scope & Applicability:** This is an in vitro direct immunofluorescent detection method for identification of *Cryptosporidium* oocysts and *Giardia* cysts in biosolid samples.

B. **Summary of Method:** Merifluor is using the method of direct immunofluorescence. The Detection reagent contains labeled monoclonal antibodies directed against cell wall antigens of *Cryptosporidium* oocysts and *Giardia* cysts. Once the sample from Dynal is treated with the Detection reagent and a counterstain, the monoclonal antibodies attach to *Cryptosporidium/Giardia* antigens present in the specimen. The slides are rinsed with buffer to wash off the unbound antibodies. A cover slip is applied with a drop of mounting medium, and the slide is examined under fluorescent microscopy for *Cryptosporidium* oocysts with an apple green color. The background material in the specimen is stained dull orange to red color.

C. **Definitions:**
1. *Cryptosporidia:* Protozoan, obligate intracellular parasites that causes severe diarrhea in humans worldwide. *Cryptosporidium parvum* is the species that infecting humans and cattle. *C. parvum* causes potentially life-threatening disease in people with AIDS or immunocompromise. This disease is transmitted via the fecal-oral route.
2. *Giardia:* Flagellate protozoan organisms that include *Giardia lamblia.* The disease condition cause by this organism is diarrhea, camping and greasy stools.

D. **Safety Warning:**
1. The analyst must know and observe normal safety procedures and proper handling should be followed closely when working with protozoan parasite samples. Mouth-pipetting is prohibited.
2. Standard laboratory protective clothing must be worn and avoid skin contact with reagents
3. 0.02% sodium azide is used as a preservative in this product. Flush with large volumes of water to prevent build-up of azide in plumbing.

E. **Cautions:**
Field and laboratory staff collecting and analyzing environmental samples are under some risk of exposure to pathogenic microorganisms. Staff should apply safety procedures used for pathogens to handle all samples.

F. **Inferences:**
1. False negative results could be due to vigorous wash procedure or insufficient specimen drying time.
2. Reagent kits must be brought to room temperature before use.
3. Protect the Detecting reagent and Counterstain from exposure to light.
4. Mix all the reagents well before use.
5. Do not scratch the slide when mixing reagents.
6. Keep the slide moist when incubating.
7. Use reagents before outdate.

G. Personnel Qualifications:

1. Laboratory Analyst/Technician: should have a B.S. and/or at least 1 year training in working with wastewater. Technicians should be well versed in safety as well as proper handling of wastewater samples both semisolid and fluid. The analyst must also demonstrate acceptable performance during an on-site evaluation.

2. Any laboratory skilled, equipped, and/or certified for Giardia and Cryptosporidium testing on water and environmental samples may use Dynabeads® GC-Combo. The user must be skilled in using conventional techniques and interpreting results. A user who is proficient in recognition of Giardia cysts and Cryptosporidium oocysts must perform screening of slides for enumeration of separated oocysts.

H. Apparatus & Material:

- Applicators
- Wash bottles
- Humidity chamber
- Microscope slide coverslips
- Gloves
- Autoclave
- Fluorescent microscope

I. Reagent:

- FITC labeled anti-Cryptosporidium and anti-Giardia monoclonal antibodies containing a protein stabilizer and 0.1% sodium azide.
- Eriochrome Black solution
- Buffer
- Buffered glycerol containing formalin, an antiquencher and 0.05% sodium azide.

J. Instrumentation and Methods of Calibration:
Fluorescent microscope in the Microbiology Department is used for evaluating test results. Please see Robert Mooney to set up the microscope.
K. Sample Collection:
1. The water sample concentrates can be of any treated water or untreated water which intended for potable supply.
2. Water sample concentrates should be prepared by standard filtration and centrifugation methods.

L. Handling & Preservation:
1. Samples not analyzed should be stored at 0-4° C.
2. Always keep copies of all data sheets from analysis of the sample in the log book.

M. Sample Preparation and Analysis:
1. Transfer sample to a treated slide well and spread it evenly entire well. Do not scratch the treated surface of the slide.
2. Add a drop of Positive Control to a separate treated slide well and spread it evenly over the entire well.
3. Add a drop of Negative Control to a separate treated slide well and spread it evenly throughout entire well.
4. Allow the slides to air dry at room temperature.
5. Place one drop of Detection Reagent in each well.
6. Place one drop of Counterstain in each well.
7. Mix the reagents with an applicator stick and spread over the entire well.
8. Incubate the slides in a humidified chamber for 30 minutes at room temperature. Notes: Protect from light.
9. Rinse the slides with a gentle stream of 1X Wash Buffer until excess Detection Reagent and Counterstain is removed. Note: Do not submerge the slides during rinsing.
10. Remove excess buffer by tapping the long edge of the slide on a clean paper towel. Note: Do not allow slide to dry.
11. Add one drop of Mounting Medium to each well and apply a coverslip.
12. Seal the slide with nail polish and allow to air dry.
13. Scan each well thoroughly using fluorescent microscope.

N. Records Management:
1. All results of all samples are kept in a log book by date.

O. Waste Management: All laboratory waste is sterilized prior to disposal.

II. Quality Control and Quality Assurance

A. Quality control:
1. Calibration: Centrifuge is calibrated according to procedures outlined in the Lab Administration text.
2. Obtain reference parasites from qualified outside sources. Dr. Widmer Giovanni at Tufts Veterinary School for Cryptosporidium parvum and Giardia lamblia.
3. Run one positive control and one negative control with each batch of samples.

B. Quality Assurance:
1. The minimum requirements of a formal quality assurance (QA) program consist of an initial demonstration of laboratory capability through the analysis of positive and negative control samples and blanks, and analysis of positive and negative control samples and blanks as tests of continued performance.
APPENDIX E

Procedure for Viability Assay of Cryptosporidium using Fluorogenic Vital Dyes:
(Campbell et al. AEM 1992 (58) pp. 3488-3493)

Reagents: *Prepare and store at 4°C in the dark
- DAPI: Working solution of DAPI is 2 mg/ml in absolute methanol
- PI: Working solution of PI is 1 mg/ml in 0.1 M PBS, pH 7.2

Procedure:
1. Add 10 µl of DAPI working solution and 10 µl of PI working solution to 100 µl of purified oocysts suspension.
2. Mix reactants and incubate at 37°C for 2 hours.
3. Wash oocysts twice in Hank’s Balanced Salt Solution before viewing by epifluorescence microscopy.

Microscopy:
For each sample time point read, ten µl aliquots of oocysts suspension is viewed under both DIC (Nomarski) optics and epifluorescence with and Olympus BH2 microscope equipped with a UV filter block (3.50-nm excitation, 450-nm emission) for DAPI and a green filter block (500-nm excitation, 630-nm emission) for PI. Proportions of ruptured (ghost), PI-positive (PI+), DAPI-negative PI-negative (DAPI-PI-) and DAPI-positive PI-negative (DAPI+PI-) is quantified by enumerating 100 oocysts in duplicate for each time point sample.

Identification:
1. Ghost oocysts: ruptured oocysts, which can be identified under Nomarski optics
2. PI+: oocysts fluoresce bright red under the green filter block (red fluorescence varies in intensity depending on where the sporozoite nuclei are located)
3. DAPI+PI-: No inclusion of PI and nuclei of sporozoites fluoresce sky blue under UV filter block
4. DAPI-PI-: No inclusion of PI, not ghosts, shows a rim of fluorescence or an absence of fluorescence under the UV filter block.
APPENDIX F

Procedure to Viability Assay of *Giardia lamblia* using Fluorogenic Vital Dyes: (Schupp et al AEM 1987 (53) pp. 704-707

**Reagents:** * Prepare and store at 4°C in the dark

- Fluorescein diacetate (FDA): Stock solution of FDA is 10 mg FDA in 1 ml acetone
  Working solution is 0.04 ml stock mixed with 10 ml Dulbecco PBS at pH 7

- Propidium iodide (PI): Stock solution of PI is 0.5 mg PI mixed with 50 ml Dulbecco PBS pH 7

**Procedure:**
1. 4 μg of FDA per 10^6 cysts or 3 μg of PI per 10^6 cysts
2. Cysts are stained for 5 minutes
3. Observe microscopically with Olympus BH-2 epifluorescence microscope at excitation wavelengths of 455 to 490 nm for FDA and 545 to 546 nm for PI.

**Identification:**

FDA+ (intense green) Viable cysts

PI+ (bright orange at 450-490 nm with excitation barrier filter combination) (bright red at 545 to 546 nm) Nonviable cysts

FDA-PI- (appear black- nonstaining cysts) interpreted as a subpopulation of FDA+ cysts

**Principle of dyes:**

**FDA:** An intact lipid bilayer slows leakage of the fluorochrome from within intact cells while injured cells cannot retain or accumulate the fluorochrome. In viable *G. lamblia* cysts, both the lipid membrane of the cell and the cyst wall may serve as barriers to the diffusion of fluorescein, whereas damaged or nonviable cysts may not accumulate fluorescein due to the presence of rupture membranes.

**PI:** has not been shown to transverse intact cell membranes; therefore, only cells with disrupted or broken membranes are counterstained by PI. The fluorochrome is specific for DS nucleic acids.

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The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved your request for a time extension for this protocol. Approval is granted until the "Next Review Date" indicated above. You will be asked to submit a project report with regard to the involvement of animals before that date. If your project is still active, you may apply for extension of IACUC approval through this office.

The appropriate use and care of animals in your project is an ongoing process for which you hold primary responsibility. Changes in your protocol must be submitted to the IACUC for review and approval prior to their implementation. If you have questions or concerns about your project or this approval, please feel free to contact the Regulatory Compliance Office at 862-2003 or 862-3536.

Please note: Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

Please refer to the IACUC # above in all correspondence related to this project. The IACUC wishes you success with your research.

For the IACUC,

Dr. Giovanni Widmer, Tufts
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For the IACUC,

[Signature]
John A. Litvak, Ph.D.
Chair

cc: File

Dr. Giovanni Widmer, Tufts