Adapting USEPA Protocol for the Detection and Enumeration of *Giardia* and *Cryptosporidium* in an Agricultural Setting

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Table of Contents

Abstract

An ecological water treatment system that utilizes a series of plants and water tanks is being tested at the Ohio State University Waterman Dairy as an alternate method for agriculture waste management. *Giardia* and *Cryptosporidium* are two genuera of parasites that can be found in most surface water supplies due to runoff from agricultural facilities, sewage treatment plants, or wildlife areas such as forests. As a result, the level of these two parasites in the water supply is a concern in regards to public health. The purpose of this study, initially, was to evaluate viability of the United States Environmental Protection Agency (USEPA) Method 1623 commonly used for detection of *Giardia* and *Cryptosporidium* in surface water with an untraditional sample source—agricultural waste. An additional purpose was to evaluate the level of *Giardia* and *Cryptosporidium* cysts and oocysts entering the ecological treatment system and the level exiting the system, thus determining the efficiency of the system for reduction of parasitic load.

Method limitations were established by enumerating stock organisms, running spiked distilled water controls, and finally using experimental samples from the agricultural waste entering the water treatment facility. The USEPA Method 1623 was used to evaluate the level of *Giardia* and *Cryptosporidium* in the samples. Eluted samples were concentrated, stained, and observed via immufluorescence. Modifications to the published protocol were made to account for the difference of this project's sample type (agricultural waste verses surface water) and to account for not using immunomagnetic separation (IMS) for the concentration of the sample.

The major finding from this project is the inability of a capsule filtration unit recommended in the the USEPA Method 1623 to evaluate the parasitic load in agricultural waste samples. The particle size and load, despite sieving of the sample, overtaxes the filter used in the

sample processing system. This makes elution and recovery of the cysts and oocysts near impossible. An accidental finding occurred in regards to cysts and oocyst integrity and the ability to detect their presence efficiently with immunofluorescent staining.

Alternate methods will need to be investigated in order to properly detect and quantify *Giardia* cyst and *Cryptosporidium* oocyst levels for the ecological water treatment system processing agricultural waste as this capsule filtration testing system is ineffective because of particle size and load in the waste. In addition, further research into cyst and oocyst integrity verses the ability to detect with immunofluorescent staining will need to be pursued.

1. Background

The organisms found within the *Giardia* genus are protozoan parasites (USEPA, 2000) located in the duodenum and jejunum (Baron, 1996). They occur in the trophozoite and cyst form within the host. The trophozoites average about 12 µm to 15 µm in length and have a pear shape. There are two nuclei at the anterior end and four flagella that mediate propulsion (Baron, 1996). The trophozoite attaches to the small intestinal epithelium by means of an adhesive disc and then reproduces by binary fission (Baron, 1996). Some of these encyst into oval shaped cysts about 6 µm to 12 µm in length that contain two to four nuclei and diagonal fibrils (Baron, 1996). Most of the infective cysts that are passed via feces contain two potential trophozoites and when these are passed, the infection can be perpetuated (Bowman et al., 2003).

Cryptosporidium is the genus of ten named species of protozoan parasites that are capable of infecting a variety of animals including fish, amphibia, reptiles, birds, and mammals (USDA APWL, 2004). The parasite grows only inside cells of the host animal and most commonly this is within the digestive tract. The stage of the protozoan's lifecycle that infects the

host when ingested is known as an oocyst. This oocyst has a very tough outer "shell" that surrounds four individual parasites called sporozoites. When the oocyst is internalized, the tough shell opens and the sporozoites enter the cells that line the lower small intestine to initiate their development. The products of the protozoa asexual development are known as meronts. These meronts contain merozoites which begin to develop into micro and macrogametes so that the microgamete can fertilize the macrogamete producing more oocysts. When the oocyst is mature it breaks free from the host cell and can either be thin-walled or thick-walled. The thin-walled oocysts begin another cycle of the infection within the host and the thick-walled oocysts pass out of the animal through the feces which is the mode of transmission by which the infection can be passed to other animals (USDA AWPL, 2004).

As a result of water run-off being contaminated by feces, *Giardia* and *Cryptosporidium* are found in most surface water supplies (Schijven et al., 2004). Varying levels of these parasites can be found in water sources that are in proximity to agricultural facilities, sewage treatment plants, or wildlife areas such as forests resulting from the parasites spreading via cysts and oocysts that are shed from the host in their feces (USEPA, 2000). Infections from the ingestion of the parasites' cysts or oocysts can cause infections known as Giardiasis or Cryptosporidiosis in both animals and humans. The symptoms of both diseases are similar, ranging from diarrhea and dehydration to vomiting and lethargy (Baron, 1996; USDA AWPL, 2004). Unless the host is immunocompromised, these diseases are usually self-limiting and the body can eliminate them in a time frame of four days to several months for *Giardia* (USEPA, 2000) and within a few weeks for *Cryptosporidium* (Kneen and Lemley, 2004). Those that have compromised immune systems such as individuals with HIV, AIDS, or cancer or individuals with other conditions such as

diabetes, alcoholism, or pregnancy may experience more serious complications from an infection (Kneen and Lemley, 2004).

Giardia is less resistant to environmental factors such as temperature increases and heat-thaw cycles (USEPA, 2000) than *Cryptosporidium*. *Cryptosporidium* is essentially unaffected by conventional disinfectants that are used in traditional water treatments, therefore, the parasite must be physically removed from the water source using methods such as utilization of a filter (Juranek, 2004). In order to prevent these infections, the USEPA has established guidelines for both *Giardia* and *Cryptosporidium* removal from public water supplies being 99.9% and 99% elimination respectively (Kneen and Lemley, 2004; USEPA, 2000).

As a result of the health threat that these parasites pose, special water filtration is key to preventing outbreaks of infections such as the one that occurred in Milwaukee, Wisconsin in 1993 where it was devastatingly shown that conventional filtration and chemical treatment is inadequate in removing cysts or oocysts. As a result of *Cryptosporidium* in the water supply, about 400,000 individuals became sick and more than 100 died. Though water from the tap is presumed safe, the Center for Disease Control (CDC) recommends boiling water for a minute, running it through a fine filter, or using bottled water to protect immunocompromised individuals from infections such as this one that occurred in Milwaukee (Rutz, 1996). Knowledge of the inadequacies of conventional filtration and chemical treatment has catalyzed exploration of alternative methods for effective water treatment. One of these systems is known as an Ecological Treatment System (ETS) which utilizes plants and animals that can naturally remove the impurities of water thus enabling less chemical treatments. It is unknown, however, whether the ETS removes parasitic cysts and oocysts from the source. In addition, though these harmful components are not specifically known to be in greater concentration in agricultural areas, it is

necessary to determine the levels of cysts and oocysts that may escape this method of water treatment so that agricultural waste cannot be blamed for an outbreak of infection if this system is used to process waste products.

No prior research has been done on this system in regards to the levels of parasites present before and after treatment. In a Burlington, Vermont trial that ran from December 1995 to May 1996, the Advanced Ecologically Engineered System (AEES) designed by Todd and Jospehson was tested for efficiency in handling components of waste that are of concern in water treatments by running 80,000 gallons of municipal waste per day through the system (Todd et al., 2003). When analyzed, it was found that it not only met, but also exceeded the design parameters for carbonaceous biochemical oxygen demand ($CBOD_5$), total suspended solids (TSS), total Kjeldahl nitrogen (TKN), ammonia (NH_3), nitrate (NO_3^-) and total nitrogen (TN) as well as fecal coliform bacteria (Todd et al., 2003) which is why it is now under investigation for agricultural purposes and to determine its ability to eliminate other infectious particles such as *Giardia* cysts and *Cryptosporidium* oocysts.

In addition to the lack of research on this system in regards to parasite elimination, this ETS has never been tested on agricultural waste which has a more concentrated level of all biological waste products including parasites. Because the agriculture waste is pumped into the system with less extra water, it is not nearly as diluted by other wastewater sources like municipal waste is. Therefore, it is necessary to thoroughly test this system with the agricultural waste products to see how well the plants chosen and tank system established can handle this specific waste.

2. Research Plan

The initial steps of this research are to determine whether the USEPA Method 1623 (USEPA, 2001) and the Filta-Max® testing system are adequate for recovery and elution of cysts and oocysts present in the ETS. Method modifications are likely to occur as a result of the difference in sample material.

If this method proves acceptable for use in this situation, the outflow levels of *Giardia* and *Cryptosporidium* from the ETS can be compared to the inflow levels of the parasites. If it is found that the levels decrease through the ETS, subsequent samplings can be taken from different locations between the tanks in series so a more specific place where the elimination occurs can be identified.

3. Significance

If it is found that this ecological design is effective, it can help to eliminate the large lagoons that are at many farming facilities. Not only do lagoons occupy a sizeable amount of property on the farm, they also can emit an unpleasant odor that is a nuisance to those living in the country not accustomed to farming smells. As the lagoons are removed and replaced with an ETS, it can provide a potential fresh water supply from what would instead sit only to slowly decompose and continue to fill up. Through this system, though it would occupy the same area, the water that is used to move the waste could be continuously renewed. The clean, recycled water could be reused as wash water for cleaning the barn floors or other farm purposes and would minimize the amount of water the farm would require from outside sources. To add more value to this system, the plants that are utilized for the filtration can be selected so that they are

used in other ways such as vegetables to provide food or woody plants that can be added into the animal bedding.

With a broader perspective in mind, this system could have positive implications for developing countries. If the ETS does provide a clean water source, it could offer fresh water for many areas that have infected surface water. One of the reasons people become ill when they travel to developing countries is because of the parasites that are present in the water. In addition, many health problems such as malnutrition are a result of parasitic infections from fecal contamination of water and food. Using the ETS could prevent the illnesses that accompany drinking contaminated water. In addition, utilization of the ETS could assist in proper sanitation and waste management for specifically agricultural waste, but also expanded to human waste as well. Thirdly, the ETS could provide another means of food for these countries that have little nutrition. If the proper plants are incorporated into the tanks, fruits and vegetables could be grown for the community in which the ETS is established.

4. Objectives

The objectives of this research project are to:

- Determine if USEPA Method 1623 and the Filta-Max® system are adequate methods to recover and elute *Giardia* cysts and *Cryptosporidium* oocysts from a filtered sample of water and manure sludge.
- Determine the level of initial and final *Giardia* cysts and *Cryptosporidium* oocysts while utilizing the ETS designed by Todd and Josephson referred to as an Advanced Ecologically Engineered System (AEES).

5. Materials and Methods

The foundation for the entire experimental procedure was from the USEPA Method 1623 (USEPA, 2001).

5.1 Stock organism enumeration

5.1.1 First iteration and second iteration

The stock suspension of *Giardia lamblia* received was 375,000 organisms per ml (Waterbourne™, Inc., New Orleans, LA). The desired final concentration for a spiking suspension was between 8,000 to 12,000 organisms per milliliter (80 to 120 organisms per 10 µl). Initially, the intermediate step of diluting the suspension to 20 to 50 organisms per large hemacytometer square was eliminated and the concentration of 80 to 120 organisms per 10 µl was the dilution made. A 1:50 (suspension:total volume) dilution with the stock suspension of *Giardia* in 0.01% Phosphate Buffered Saline with Tween20 (0.01% PBST) (0.137 M NaCl; 0.00268 M KCl; 0.0081 M Na_2HPO_4, anhydrous; 0.00147 M KH_2PO_4; pH 7.4; filter sterilize or autoclave; Tween20 for a 0.01% solution) was made. From this dilution, 10 µl was loaded onto a hemacytometer for enumeration. The average number of six independent hemacytometer chambers of 10 µl was calculated.

A similar dilution scheme was employed for the *Cryptosporidium parvum* stock suspension (Waterbourne™, Inc., New Orleans, LA). The original concentration of this suspension was 625,000 organisms per milliliter. A 3:200 dilution of stock *Cryptospordium* in 0.01% PBST was made. Six independent hemacytometer chambers of 10 µl were counted and the average enumeration from those counts was calculated.

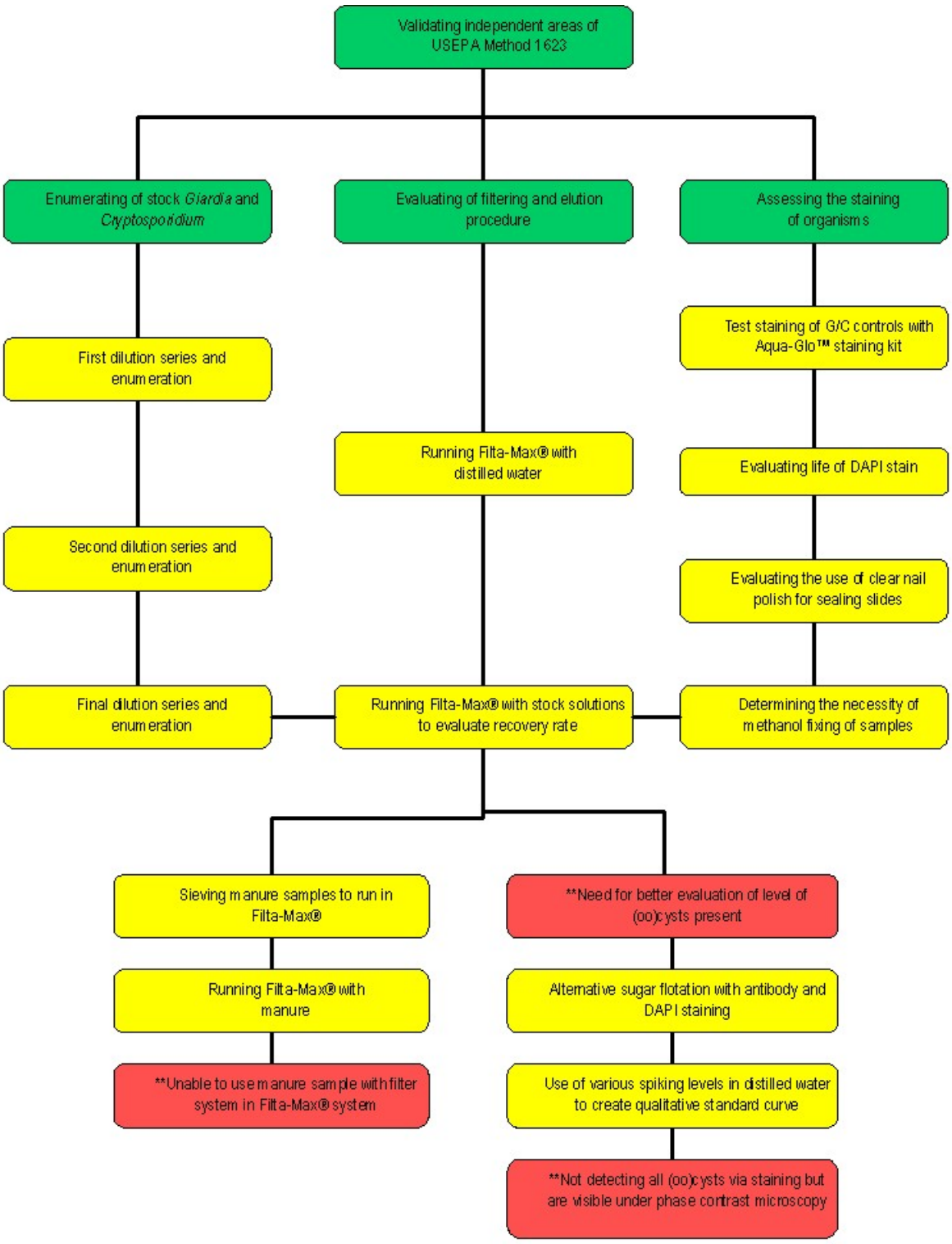

Figure 1. Research flow chart to outline and summarize the order of events for validation and adaptations on USEPA Method 1623. Green boxes denote main objectives. Yellow boxes denote steps executed in process of validation. Red boxes denote problems or pitfalls of USEPA Method 1623 with animal agriculture waste.

5.1.2 Third iteration

The intermediate dilution step was used in this dilution scheme. In order to have 20 to 50 organisms per large hemacytometer square approximately 1,800 to 4,500 organisms needed to be in 10 µl so that approximately 180 to 450 organisms would be in the counting field and approximately 20 to 50 would be in each counting square as the counting area is approximately 10% of the entire 10 µl loaded into the hemacytometer chamber. For *Giardia*, no dilution was made as 3,750 cysts/10 µl was within the target range. For *Cryptosporidium*, a 1:2 dilution was made to yield approximately 3,125 oocysts/10 µl. Samples of 10 µl of both suspensions were counted four times in independent hemacytometer chambers.

The following equation from USEPA Method 1623 (USEPA, 2001) was used to back-calculate the original concentration of the stock suspensions organisms:

$$\frac{\text{number of organisms counted}}{\text{number of mm}^2\text{ counted}} \times \frac{10}{1\text{mm}} \times \frac{\text{dilution factor}}{1} \times \frac{1000\text{ mm}^3}{1\text{mL}} = \text{number of organisms/mL}$$

Once it was determined that there was adequate accuracy for the dilution and counting of organisms, the spiking dilution was made from the first dilution. The *Giardia* suspension was diluted 1:35 in 0.01% PBST and the *Cryptosporidium* suspension was diluted 1:30 in 0.01% PBST. To determine the accuracy of these suspensions for spiking, 10 µl was loaded into hemacytometer chambers for enumeration five times.

5.2 Staining slides

5.2.1 Control slide staining (12)

Two sets of 10 µl of provided positive control from Aqua-Glo™ G/C Direct, FL, staining kit (Waterborne™, New Orleans, LA) were dispensed onto circle well slides for drying.

Two 10 µl samples of expired positive controls from Meriflour staining kit were also dispensed onto circle well slides for drying. For the negative control, 50 µl of sterile Phosphate Buffered Saline (sPBS) (0.137 M NaCl; 0.00268 M KCl; 0.0081 M Na_2HPO_4, anhydrous; 0.00147 M KH_2PO_4; pH 7.4; filter sterilize or autoclave) were dispensed onto a circle well slide for drying. Once the slides were completely dry, 1 drop of the G/C antibody reagent from the staining kit was added to each well of positive and negative control samples. The slides were placed into a humid chamber for 40 minutes in the dark at room temperature. After incubation the slides were soaked in a 1x saline solution for 3 minutes. Excess saline was tapped off onto a paper towel and 50 µl of diluted 4',6-diamidino-2-phenylindole (DAPI) staining solution (prepared per USEPA Method 1623; USEPA, 2001) was added to each well for 2 minutes. One drop of sPBS was added to each well and then was allowed to drip onto a paper towel for elimination of excess sPBS and DAPI stain. One drop of provided counter-stain was added to each well for 2 minutes. All slides were then rinsed for 1 minute with sPBS. Slides were left to dry by placing them on a slant under a heat lamp with paper towels underneath. Once dry, 1 drop of the mounting medium was placed in the center of each well and a cover-slip was added. Identification using an ultraviolet (UV) microscope was used. Identification was based on visualization of organisms that fluoresced green. Distinguishing between *Giardia* and *Cryptosporidium* was done based upon size and shape. Slides were stored in a humid chamber at 4 °C for up to one week for further analysis.

5.2.2 Evaluating life of DAPI stain

Per USEPA Method 1623, DAPI should be prepared fresh on each test day (USEPA, 2001). It was desired to see if it would be as effective for staining if stored at 4 °C in the dark for a few days. Known positive samples were prepared for staining in the same manner as above.

DAPI used was from 2 days prior. All other staining and identification processes were identical. Later, DAPI was tested for 7 day longevity. All other staining and identification processes were identical.

5.2.3 Evaluating the use of clear nail polish for sealing

Per USEPA Method 1623, clear nail polish should be used as a last step in the preparation of slides for identification (USEPA, 2001). During the first staining procedure, it was not utilized to seal the edges of the cover-slip. On another staining trial, clear nail polish was used to determine if it was necessary to seal the slides. All other staining and identification processes were identical.

5.2.4 Necessity of methanol fixing samples on slides

During a trial using a high number of cysts and oocysts for spiking, two different slide preparations were made with the variable of methanol fixation. USEPA Method 1623 suggests a methanol fixing step to occur after samples on slides have dried and before actual staining begins (USEPA, 2001). It states to add 50 µl of absolute methanol to each well that has been dried for staining and allow it to dry for 3 to 5 minutes. Directions from the Aqua-Glo™ staining kit states that a methanol fixation step may be completed as recommended by USEPA Method 1623 (Waterborne™, 2005; USEPA, 2001), but also states that it is not necessary for the reagent in this kit to bind well with cysts and oocysts.

Two control slides were prepared, one with methanol fixation per USEPA Method 1623 (USEPA, 2001) and one without. In addition, two of the test slides from the spiked sample run through the Filta-Max® process were prepared, one with methanol fixation and one without.

5.3 Filta-Max®

5.3.1 Running Filta-Max® uninfected

A pump was utilized so that the required pressure per square inch (PSI) for pushing water through a Filta-Max® filter cartridge could be achieved. Ten liters of distilled water were run through the pump and filter system at approximately 2 L/minute. Once a full ten liters were filtered, the cartridge was processed using the Filta-Max® elution directions provided by the company (IDEXX, 2002).

5.3.2 Running Filta-Max® infected

When confidence was established in running the Filta-Max® without pathogens, quantitative analysis was begun to establish a lower limit on the range for detection of oocysts and cysts with the Filta-Max® and capsule filter.

Enough of *Giardia* and *Cryptosporidium* dilutions were added to 10 L of distilled water so that approximately 300 *Giardia* cysts and 300 *Cryptosporidium* oocysts were present in the sample. As appropriate, increasing levels of cysts and oocysts were added to 10 L so that cysts and oocysts could be visualized on the final slides using the staining procedure previously verified.

5.4 Sieving process for manure samples

A sieving process was established as the Filta-Max® filter would not handle the size of particles that are present in a manure sample. From the manure manifold of the ETS, 1 L was sampled. This was rinsed through a series of sieves (500 µm, 250 µm, 150 µm, and 36 µm) to eliminate large particles but still allow passage of *Giardia* and *Cryptosporidium*. A total of 9 L

of distilled water were used to rinse the sieves and sample so that the dilution was 1:10 for 10 L to run through the Filta-Max® system.

5.5 Alternate sugar floatation with antibody and DAPI staining

Sugar floatation and centrifugation techniques were used in combination with addition of the staining reagents to the concentrated pellet directly rather than once a sample was on the slide. About 1 g of known *Giardia*-positive feces was mixed with approximately 14 ml of water to make a homogenous mixture. This sample was centrifuged at 1,000 rpm for five minutes. The supernatant was discarded and 30 drops of the G/C antibody from the Aqua-Glo™ staining kit was added and vortexed to mix well with the pellet. After an incubation period at room temperature in the dark for 60 minutes, 500 µl of DAPI was added to the sample tube and incubated for another 10 minutes. A sugar floatation solution with specific gravity of 1.18 was added and mixed well with the antibody-tagged pellet. Another 5 minutes of centrifugation at 1,000 rpm was used. The solution was then topped-off drop-wise to a bulging meniscus, a cover-slip was added, and allowed to sit for 10 minutes. The cover-slip was removed and placed on a slide for reading with UV microscopy.

Another sample of approximately 1 g of known *Giardia*-positive feces was mixed with 10 L of distilled water for running through the Filta-Max® system. Once the eluate was collected at the end of the run, the supernatant above the pellet was reduced to approximately 5 ml. The pellet was vortexed to resuspend and then transferred to a 15 ml centrifuge tube. The volume was raised to 15 ml with distilled water and centrifuged at 1,000 rpm to repellet. The water supernatant was removed and the above procedure for staining with sugar floatation was used, substituting 10 drops of G/C antibody rather than 30 drops as before.

5.6 Creation of a qualitative standard curve

Samples of the diluted stock cyst and oocyst suspensions were added to 10 L of distilled water. The first sample was spiked with approximately 100 *Giardia* cysts and approximately *Cryptosporidium* oocysts each. Another sample was made with approximately 1,000 *Giardia* cysts and *Cryptosporidium* oocysts each. The final sample was made with approximately 10,000 *Giardia* cysts and *Cryptosporidium* oocysts each. The 10 L samples were processed with the Filta-Max® system and samples were stained using the alternate sugar floatation and staining procedure.

6. Results and Discussion

6.1 Stock organism enumeration

The relative standard deviation for enumeration of the stock organisms as set by USEPA Method 1623 is less than or equal to 19% for *Giardia* and less than or equal to 16% for *Cryptosporidium* (USEPA, 2001). When it was apparent the back-calculation from the counts on the hemacytometer were not within the parameters, new dilutions and enumerations were made. Once the enumerations and back-calculations demonstrated an accurate concentration of cysts and oocysts as compared to shipping concentration and consistency in concentration between independent enumerations, the dilutions were used for spiking and the dilution scheme was repeated for subsequent spiking trials.

The intermediate step of diluting to 20-50 organisms per large hemacytometer square was necessary for manual enumeration so that enough organisms were present to provide an accurate enumeration.

6.2 Staining slides

6.2.1 Evaluating life of DAPI stain

Preparing 50 ml of diluted DAPI each test day was wasteful when only a few slides were prepared with 50 µl used for each well on the slide. The positive control and the experimental slides stained with 2 day old DAPI exhibited completely stained cysts and oocysts present just the same as slides that were stained with DAPI prepared freshly on the test day. DAPI one week old provided similar results as the 2 day old DAPI. The experimental slides with 7 day old DAPI exhibited completely stained and identifiable cysts and oocysts comparable to the freshly prepared DAPI sample slides. The USEPA method does state that DAPI should be diluted fresh each day, however, with the comparison of fresh and older DAPI it is apparent that even DAPI stored at 4 °C for up to one week can offer just as efficient staining and identification as freshly prepared DAPI.

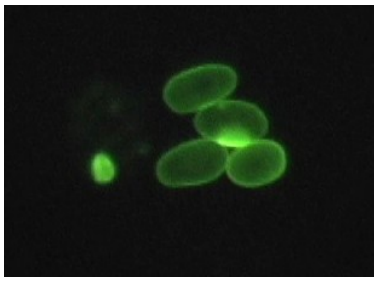

Figure 2. Direct immunofluorescence stain of positive control with Aqua-Glo™ G/C Direct, FL, staining kit viewed with UV microscopy. Small organism on left is *Cryptosporidium* oocyst and organisms on right are *Giardia* cysts.

6.2.2 Evaluating the use of clear nail polish for sealing

When the edges of the coverslips were sealed to the slide with clear nail polish after mounting media and a coverslip were applied to the stained slides, clarity of the slides was compromised. The organisms were still present and obviously stained, but compared to the unsealed sample slides the cyst and oocyst shape and edges were harder to identify. When the

slides were observed closer, it was apparent that some of the nail polish had traveled under the coverslip, through the mounting media, and into the well that contained the stained sample. The effect of the nail polish might not have effected the cysts and oocysts directly, but the clarity of visualization was affected. Because of this, use of the clear nail polish was discontinued. Slides still had the mounting media and coverslip applied, but they were left unsealed. The slides were either read the day they were prepared or stored at 4 °C in the dark and read the following day.

6.2.3 Necessicity of methanol fixing samples on slides

The slides that were fixed with methanol for both the test samples and the positive controls looked very similar to those that were not fixed with methanol. No significant differences were observed between the different procedures. Because no observable difference between methanol fixed and non-methanol fixed samples were observed, this step in the staining procedure was discontinued. In addition, because the specific staining kit used states that it is unnecessary to use the methanol, it was acceptable to remove this step.

6.3 Running Filta-Max® infected

Recovery rates of the spiked distilled water samples were not acceptable and so pitfalls and problems in the system or procedure were assessed. Problems that were encountered when eluting back and concentrating the cysts and oocysts spiked in distilled water samples which needed to be worked around in order to increase recovery rate and sensitivity included:

1. Not using immunomagnetic separation (IMS) and therefore not concentrating the eluate to a minimal volume for sampling.
2. Counting through background debris
3. Potentially not washing the filter vigorously enough.

In order to solve these problems the eluate was reduced to as small an amount as possible without disturbing the pellet resting at the bottom of the sample tube after centrifugation. This was safe to do so because the cysts and oocysts theoretically were trapped in the pellet. In addition, larger samples of the eluate (20µl) were used for staining in an effort to have a more representative sample taken of the concentrated eluate. Also, the filter was agitated more upon washing it with the Filta-Max® system.

When a diluted manure sample was used for testing, the Filta-Max® system proved inadequate. As the 10 L were filtered through the capsule filter, the particulate load damaged the filter and caused the remaining sample to rush through the filter housing. No *Giardia* or *Cryptosporidium* were eluted back and this could have been due to a truly negative sample of manure, a positive result that was below the system limitations, or no organisms being caught in the filter as a result of the damage cause by the particulate load.

Though there may be ways around the elution and concentration steps without using IMS, the first step of filtering through the capsule filter is a limiting factor. It is possible that the manure sample could be sieved through another screen of approximately 20 or 25 µm, but then the chance exists that the cysts and oocysts may get caught up in this small opening even though they are smaller. The matrix that may be created by the fecal material may inhibit adequate passage through the sieves as well. A sample of 0.5 L from the manure manifold into 9.5 L of distilled water or 1 L of manure into 19 L of distilled water for a dilution of 1:20 may create a dilution that is able to be filtered through the Filta-Max® filter, however, consideration must be given to how large of a manure sample must be taken in order to be representative of the ETS manifold.

6.4 Alternate sugar flotation with antibody and DAPI staining

The *Giardia* cysts present in the sugar flotation stained sample that was not run through the Filta-Max® system were too numerous to count. They were very well stained and it was easy to visualize the green fluorescence.

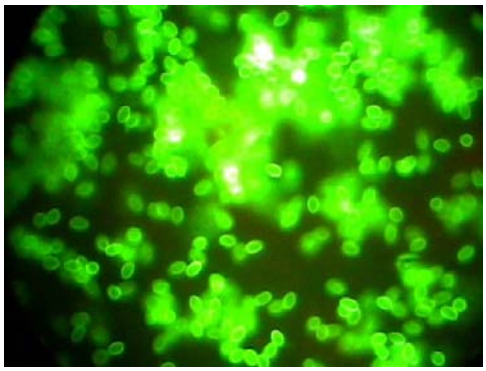

Figure 3. Direct immunofluorescence stain with alternate sugar flotation of known-positive *Giardia* sample viewed with UV microscopy. Organisms are *Giardia* cysts.

When a sample of the same size and from the same source was run through the Filta-Max® and stained with the sugar flotation and DAPI staining, *Giardia* cysts were visualized clearly but not to the extent in volume that they were when the sample was not run through the Filta-Max®.

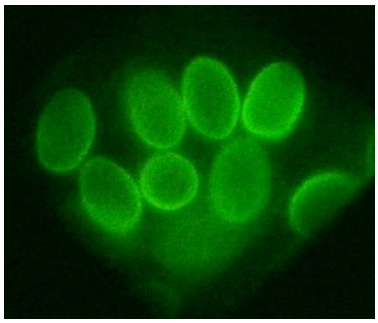

Figure 4. Direct immunofluorescence stain with alternate sugar flotation of known-positive *Giardia* sample and processed with Filta-Max® system viewed with UV microscopy. Organisms are *Giardia* cysts.

This alteration to the method appears to work effectively if adequate amounts of the G/C antibody reagent are used. The difference in the amount of *Giardia* cysts present on the slide between the unprocessed and processed samples can be attributed to the recovery rate associated with using the Filta-Max® equipment for filtering and eluting the samples.

A qualitative standard curve could be created from this alternate staining method by running different levels of spiked samples and recording organism/field for a set number of microscope fields. This could provide a general idea for limitations of cyst and oocyst recovery using the Filta-Max® and also generate reference ranges for comparison of infected experimental samples.

6.5 Creation of a qualitative standard curve

After four attempts of trying to create a standard curve with three different spiking concentrations, results were still much below what was anticipated based upon the amount of cysts and oocysts spiked into the samples. Cysts and oocysts were only consistently seen on the highest concentration slides and occasionally a few were seen on the middle concentration slides. Because the sensitivity of this method did not prove to be greater than the conventional method, the lower limits were not able to be qualitatively determined either.

An accidental finding did occur when a slide from the fourth stained trial was placed under phase contrast microscopy. The amount of *Giardia* cysts and *Cryptosporidium* oocysts visible were too numerous to count. However, when the same slide was viewed with UV microscopy, significantly less were visualized stained. This raised a discrepancy between the sugar flotation and staining working for the known *Giardia*-positive fecal sample and the sugar flotation and staining for water spiked with just the cyst and oocyst stock suspensions.

7. Conclusion

The method that was utilized during this experiment is one that has been validated for detecting *Giardia* cysts and *Cryptosporidium* oocysts in surface water. The USEPA also states that in order for this testing to be used on other water sources, the laboratory must prove that it meets specific acceptance criteria (USEPA, 2001). Applying this testing system to agricultural waste is a very different application than what it was traditionally designed for and so many areas needed validation. As a result of this, small steps were taken in each area in the attempt to determine the effectiveness on a whole.

Due to particulate size and load of the manure samples using the Filta-Max® system with USEPA Method 1623 will not be adequate. Equipment constraints also factored into the overall effectiveness because IMS was not available to use in the procedure. The unavailability of this equipment did not eliminate the necessity to concentrate the eluate to as small a volume as possible for sampling accuracy, and so other methods were attempted to accomplish the same goal such as applying the sugar floatation technique in conjunction with the DAPI staining to create a qualitative standard curve.

A question that must be asked is whether testing the affluent and effluent of the system for parasites is completely necessary. In many studies (Santin et al., 2004; Starkey et al., 2005; Trout, et al., 2005) Giardiasis and Cryptosporidiosis are essentially isolated to the calf and population. The waste that is entering this ETS at the OSU Waterman Dairy is from the free stall barns and the incidence of *Giardia* and *Cryptosporidium* is much less, if not non-existent, as compared to the levels that could be found in the calf barns. This suggests that the effluent of the system is most likely just as limited in parasite numbers as the affluent is simply because of the minute amount potentially entering the system and less due to settling of particles through the

numerous water tanks present. However, this does not change that an adequate method to test this assumption needs to be established.

8. Areas for Further Research

Visualization of the stock *Giardia* cysts and *Cryptosporidium* oocysts stained and floated in the sugar solution under phase contrast microscopy but not in the UV microscopy as compared to consistent UV visualization of the known *Giardia*-positive fecal sample in the sugar flotation with staining and of the positive control suspension provided in the staining kit raises the question as to what was different between the samples that affected monoclonal antibody tagging and fluorescent staining. One option could be that the sugar affected the binding of the antibody to surface proteins, however, that would not explain the success in staining the known *Giardia*-positive sample with this method. Another possible is the integrity of the cysts and oocysts in the stock suspensions. The provided positive control was fixed in 1.0% formalin in PBS, whereas the stock suspensions were not fixed. The stock suspensions were a few months old. They were no longer guaranteed infective by the company, however, the staining technique relies upon surface proteins on the cysts and oocysts which could or could not be associated with infectivity. If they are unassociated with infectivity, they should still be able to be tagged for immunofluorescence detection. The fact that the fresh *Giardia*-positive sample that was processed with the alternative sugar floatation and antibody/DAPI staining worked exceedingly well, and the same procedure with the stock suspensions did not, supports the question as to why this system does not work for "older" cysts and oocysts.

In the environment *Giardia* cysts and *Cryptosporidium* oocysts may be present in water supplies for extended periods of times. The USEPA states that *Giardia* specifically can survive

in water temperatures less than 10 °C and a small fraction can even survive a freeze-thaw cycle (USEPA, 2000). *Cryptosproidium* oocysts are generally sturdier than *Giardia* cysts and can survive harsher conditions. If the cysts and oocysts have been there too long, will the USEPA Method 1623 still detect them? Another question to ask is: Just because they are undetected, does that mean they are noninfectious? The USEPA states themselves that whether cysts are detected relies largely upon what methods are used to collect and analyze the water samples in question (USEPA, 2000). If only a few cysts or oocysts are required to initiate an infection, a sensitive detection method must be in place.

To investigate this further, a study can be initiated using fresh stock suspensions to inoculate negative fecal samples. These samples then can be allowed to sit (and exposed to environmental elements) for various lengths of time and then processed with this method. A comparison between viualization of cysts and oocysts under UV microscopy and phase contrast microscopy in addition to the duration of environmental exposure from this trial could offer insight into why the fresh *Giardia*-positive sample was unmistakably positive when floated in the sugar solution and stained with DAPI, but only some of the stock cysts and oocysts were visible under UV microscopy with DAPI staining when they were present under phase contrast microscopy.

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