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Ingestion of Waterborne Protozoan Parasites by *Daphnia*

by

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Abstract

The drinking water industry is currently facing a serious crisis from waterborne protozoan parasites. These parasites have been found to contaminate a large number of water sources in North America and the most recent methods of detecting *Giardia* and *Cryptosporidium* in water are semi-quantitative, inaccurate, and can result in false negative results. This study examines a possible alternative to the standard methods for detecting waterborne protozoa. Our data show that the planktonic filter feeder *Daphnia* are able to ingest waterborne *Giardia* cysts and *Cryptosporidium* oocysts. It was demonstrated that individual *Daphnia* ingest cysts and oocysts in a similar, non-selective manner as they would a natural algal food source. Furthermore, we have shown that populations of individual *Daphnia* ingest waterborne *Giardia* cysts and when these populations are sampled in large numbers ingested cysts can be detected in their tissues. Due to the wide distribution of *Daphnia* in reservoirs, lakes and ponds world wide, this has major implications for the drinking water industry. It would be possible to test thousands of Litres of water for the presence of *Giardia* and *Cryptosporidium* by collecting a few million *Daphnia* using nets- a practice that is simple and cheap.

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Dedication

To: my beloved Desiree,

and my parents.

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Abbreviations

Ag	Antigen
ASTM	American Standard for Testing & Materials
CMP	Colloidal Magnetite Particles
DABCO	1,4-diazabicyclo[2,2,2]octane
DAPI	4,6-Diamidine-2-Phenylindole
FITC	Fluorescein Isothicyanate
GALD	Greatest Axial Linear Dimension
ICR	Information Collection Rule
IFA	Immunofluorescent Antibody Assay
Ig	Immunoglobulin
ILL	Incipient Limiting Level
IMS	Immuno Magnetic Separation
Mab	Monoclonal Antibody
NTU	Nominal Turbidity Units
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
Re	Reynold's Number
RFU	Relative Fluorescent Units
SDS	Sodium Dodecyl Sulfate
USEPA	United States Environmental Protection Agency

1. Introduction

The purpose of this chapter is to provide the reader with an understanding of the problems associated with detecting waterborne protozoan parasites which was the primary impetus for this study. Following this, the general objectives of this thesis will be introduced, which will be lead into a review of *Daphnia* biology pertinent to the fulfilment of these objectives. Lastly, the specific aims of the study will be outlined in full.

1.1. Detection of Protozoan Parasites

1.1.1. Introduction

Giardia and *Cryptosporidium* are parasitic protozoa that can cause gastroenteritis in humans [31; 35]. Both are members of the Kingdom Protozoa, being single celled organisms sharing both prokaryotic and eukaryotic characteristics. *Cryptosporidium* are coccidian, intracellular parasite that infects the intestinal and respiratory tracts of mammals, birds, and reptiles and has no effective drug treatment [15]. *Giardia* are flagellated parasites of the Class Zoomastigophora that have been found in the intestinal tracts of all classes of vertebrates [58]. Both *Giardia* and *Cryptosporidium* have an encysted, environmentally resistant, infectious life stage, referred to as cysts and oocysts respectively. These stages allow them to be passed from host to the environment and on to another host via a fecal to oral route [60]. The infectious dose for these parasites using healthy human volunteers is relatively low (10 cysts for *Giardia* and 132 oocysts for *Cryptosporidium*) [66; 20].

The detection of *Giardia* cysts and *Cryptosporidium* oocysts has become a very important procedure for water treatment facilities that supply drinking water to the

public. The increased incidence of outbreaks, such as those in Milwaukee, USA [49], Creston, Canada [35], and Swindon, UK [67], has placed much pressure on the world's water industries to ensure safe drinkable water. The United States Environmental Protection Agency (USEPA) has been a leader in funding and developing new methods and technologies for detecting waterborne protozoa. The Information Collection Rule (ICR) method was developed by them for use in water treatment facilities in the United States shortly after the Milwaukee outbreak. It was designed to collect standardized data on the occurrence of protozoa by implementing a single protocol for use in all facilities. It's major advantage over most commonly used techniques, such as the zinc sulfate method, was the use of an immunofluorescent assay (IFA) that could detect 12 times more *Giardia* cysts, and could also detect *Cryptosporidium* oocysts [46]. The ICR method and all current methods follow three basic steps: 1) concentration using filters, surfactants, and centrifuges, 2) purification by density gradient centrifugation, and 3) identification by IFA and microscopy.

Many problems were associated with the ICR method, including highly variable recoveries, and false-negative and positive results [14]. A more accurate procedure was needed and so Method 1622 was created by careful analysis of every step in the filtering and IFA process, optimizing old and new technologies. The new method was designed to be easily implemented in laboratories currently using the ICR method. Round-robin testing of Method 1622 in the United States have showed considerable improvements over the ICR method, but still only recovered 43% of spiked oocysts [12].

In this section I will review the literature of the last fifteen years that gave rise to the ICR method and Method 1622. The individual procedures and important issues involved with the three basic steps described above will be discussed.

1.1.2. Filtration and Concentration

For most detection protocols, large volumes of water are concentrated to maximize the number of parasites present in the sample. Until Method 1622 the most accepted tool for concentrating *Giardia* cysts and *Cryptosporidium* oocysts was a yarn wound, 1 μm (nominal) porosity cartridge filter [33; 45; 61]. The ICR method uses a 10in. yarn-wound polypropylene cartridges but several types of filters are available with different yarn material. LeChevallier et. al. [45] compared the oocyst and cyst trapping ability of yarn filters composed of nylon, rayon, polypropylene, and cotton. They found that while some were better than others, all allowed some cysts and oocysts to pass through. This inability to trap 100% of cysts and oocysts could have been a major factor in the poor performance of the ICR method and other protocols that use yarn wound filters to concentrate water samples have been shown to have inconsistent overall parasite recoveries, ranging from 0% to 100% [13].

Although yarn wound filters have been very popular for handling large volumes of sample, there are other filters such as flat bed membrane filters that can be used for filtering water samples from specific types of environmental water sources. Flat bed filters have been shown to have high recoveries of oocysts and cysts from good quality water, and are suitable for small volume samples with low turbidity [33]. One study using a flat bed 1 μm Costar™ disk filter recovered 89.3% to 101.3% of *Cryptosporidium* oocysts from de-ionized water [23]. Clancy et. al. [13], reported a 96% recovery of

Cryptosporidium oocysts from reagent water using Costar disk filters. The inability to filter large volumes of water without clogging was most likely the reason that flat bed filters were not included in the ICR method.

The USEPA directed their efforts into finding a filter that could reproduce the capture efficiency of a flat bed filter, with the volume handling capacity of the yarn wound cartridge filter. The filter that has been chosen for validation in Method 1622 is the Gelman Envirocheck™ capsule. It is a cartridge filter that houses 1300 cm^2 of folded polysulfone membrane with a 1μm nominal porosity [11]. Fricker et. al. [23] found the recovery for the Gelman filter to be 69.7% for oocysts and 83.5% for cysts. It was also chosen for its ease of use in the field and the laboratory.

1.1.3. Elution

A. Buffers - Nearly all methods described in the literature for removing captured cysts and oocysts involve washing with a solution containing a surfactant of some sort at a neutral pH. This is because it has been theorized that electrical properties of cysts and oocysts make them difficult to remove from filters [45]. While pH has been shown not to significantly influence recoveries of cysts and oocysts from filters [45], researchers have found that oocysts demonstrate marked hydrophobic properties under certain conditions. Drozd and Schwartzbrod [19] found that when pH was varied to extreme values, surface hydrophobic potential increased due to the conductivity of the medium. The surface hydrophobicity of oocysts increases with the ionic strength of the medium as well as with the pH. This is further supported by tests comparing the effects of surfactants on parasite recoveries. The effects of Tween 80 (0.1%) on the recovery of *Cryptosporidium* oocysts was tested by Musial and his colleagues who found that the filter eluate containing

Tween 80 recovered significantly higher oocysts than when the buffer did not contain Tween 80. The ICR method adopted this and used a 0.1% Tween 80/sodium dodecyl sulfate (SDS) buffer with a pH of 7.4.

B. Washing Techniques - Regardless of the elution buffer used, the method by which the filter is washed will depend on what type of filter it is. Yarn wound filters are traditionally processed using an intense hand-washing method and the efficiency of recovery often depends on the individual washer's technique. Automated equipment, called stomachers have replaced the hand washing procedure in the ICR method to eliminate some variation associated with individual hand washing performance. However, one study has indicated that hand washing recovered an average of 14% more *Giardia* cysts and 1% more *Cryptosporidium* oocysts than stomacher homogenizing [45].

During both the hand washing and stomacher methods for yarn wound filters the yarn must be cut from the support core and sectioned into several pieces before being placed into the chosen eluting solution for extraction. Reported recoveries using this method were 12 % for cysts and 8% recovery of oocysts [61]. Overall, recovery rates for the yarn filters vary, depending on the material of the filter, elution solutions, and methods used to homogenize the filter [33].

The elution method is different for flat bed membrane filters. Nieminski et. al. [61] used a method where the flat bed filter was placed on an inclined plate and rinsed with water containing 0.01% Tween 80 and 1% sodium dodecyl sulfate (SDS) three times, and then scraped with a squeegee between rinses. They found a 49% overall recovery of cysts and a 9% recovery for oocysts.

The Gelman Envirocheck filter used in Method 1622 requires an entirely different elution method than the other types of filters. The folded membrane filter is left inside the capsule, which is filled with the elution buffer. The elution buffer for this technique contains Laureth 12, an industrial surfactant. The capsule is then shaken at 600rpm on a wrist action shaker for 5min. This is performed twice for each capsule, with the eluted solutions combined. This method is preferable to that of the yarn wound cartridge filters for two reasons: first, it limits the exposure of the technician to possibly infectious material, and second, the time of filter processing is dramatically reduced.

1.1.4. Preservation

Before the development of Method 1622, it was common practice to preserve concentrated samples, usually with 4-10% formaldehyde in PBS. The purpose of the preservative was to prevent the growth of algae and protozoa during storage which could interfere with the microscopic enumeration of the cysts and oocysts [33]. This was done by centrifuging the filtrate using a swinging bucket rotor at speeds between 800 and 1000 x g, sufficient to pellet cysts and oocysts out of suspension. Pellets were then re-suspended in a solution of formaldehyde, usually equal to the volume of the concentrated pellet [69]. The American Standard for Testing and Materials (ASTM) method as well as the ICR method used a 10% formaldehyde solution [61]. A preservation step was not included in Method 1622 because it was designed to collect information on cysts and oocysts viability.

1.1.5. Clarification

There are many types of particulate material present in most natural water sources, including inorganic and organic sediments, phytoplankton and zooplankton. It is

necessary to remove as many of these other particles as possible because they can interfere with identification and enumeration of cysts and oocysts. Many different methods and materials have been tested for clarifying cysts and oocysts from sample debris and have contributed significantly to the development of procedures used in the ICR and even Method 1622. Purification methods have traditionally involved the use of a density gradient floatation procedure [80]. Suspensions of cysts and oocysts are layered over a solution with a specific gravity greater than their own (usually a specific gravity of 1.10). During centrifugation, particles that have greater specific gravities than the solution pellet out, while the cysts, oocysts and lighter material remain at the interface. It has been speculated that oocysts and cysts may become absorbed by heavier debris in the sample that are spun down during centrifugation. This was tested by Musial et. al. [60] who tested the effects of surfactants and sonication on cyst and oocyst recoveries. Adding 1% SDS to the floatation solution increased the floatation recovery of oocysts from 38% to 97.8%. Sonicated samples also showed higher recoveries than un-sonicated samples. The largest recoveries were seen when both surfactants and sonication were used together [60].

Regardless of the density gradient solution used, the layering of the sample with the density gradient is a delicate procedure. The two methods have been used and are known as the 'overlay' and 'underlay' techniques [33]. The overlay approach places the selective concentration media (i.e. percoll-sucrose) into a centrifuge tube and then carefully layers the sample onto the top without disrupting the interface. Conversely, the underlay method places the sample into the centrifuge tube first and injects the selective concentration media underneath the sample, again without disrupting the interface.

Hibler [33] prefers the underlay method, as he finds it is often easier to inject the media instead of the solution, due to the small bore size of the needle and the possibility of clogging by the debris within the sample. This technique is now a standard practice and has been incorporated into Method 1622 and the ICR Method. Hibler [33] also recommends beginning the centrifugation immediately after the underlay/overlay due to the tendency for particles to settle at the interface and form a trap for the cysts and oocysts and cause them to spin to the bottom.

Rose et. al. [75] compared *Giardia* cyst recoveries using several solutions including, potassium citrate (40% solution, 1.16 g/ml), Zinc Sulfate (1.18 g/ml), and percoll-sucrose (1.29 g/ml). They found that the potassium citrate solution recovered 76%, compared to 77% for the percoll-sucrose solution and 40% for the Zinc Sulfate. Percoll-sucrose was the floatation solution chosen for the ICR method, but recent technological advances have made significant improvements over the floatation method by increasing cysts and oocyst recovery and reducing variability. Bifulco and Schaefer [4] developed an immunomagnetic separation technique using colloidal magnetite particles (CMP) of 40nm average size coated with immunoglobulin G (IgG) specific for *Giardia* cyst antigens (Ag). When a sample containing *Giardia* cysts is mixed with the CMP solution, the cysts are coated with the CMPs, which can then be harvested with a strong magnet and separated from the rest of the sample debris. Bifulco and Schaefer (1993) have reported that the recovery of cysts from water samples with turbidities ranging from 70 to 6400 nominal turbidity units (NTU) was not below 60%, with the highest recovery occurring at turbidity of 568 NTU. There are a couple of new systems now on the market from Dynal and ImmuCell. These beads are similar to those

developed for *Giardia*, but contain specific IgGs anti-*Cryptosporidium* antibodies only. These products have been included in Method 1622, and have achieved high, reproducible recoveries that meet USEPA requirements [12].

1.1.6. Staining and Microscopy

Visualization of cysts and oocysts amongst all of the debris inherent in environmental water samples is a daunting task for a microscope technician. *Giardia* cysts tend to be oval in shape, 11-14 μm in length, and 7-11μm in width [79], while *Cryptosporidium* oocysts are spherical or slightly ovoid and have a diameter of 4-6 μm [46]. It is very difficult to identify cysts and oocyst just by shape alone under bright field observation, so phase microscopy has been adopted to visualize the internal structures of oocysts and cysts. *Giardia* cysts typically contain 4 nuclei within a single trophozoite, while *Cryptosporidium* oocysts contain four sporozoites that contain one nucleus each. *Giardia* cysts have internal structures such as median bodies and axonemes, which can also be visualized under phase contrast microscopy. Unfortunately, even with these unique structures, *Giardia* and *Cryptosporidium* do not always appear distinct from the other debris within a water sample. After all of the careful concentration and purification of a water sample, the most difficult part is determining what in the sample is parasite and what is just debris or algae. That is why the use of dyes and stains have become necessary for identifying cysts and oocysts using microscopy.

One of the first stains used to help identify *Giardia* cysts was Lugol's iodine. In a protocol called Zinc Sulfate ($ZnSO_4$) Floatation Analysis that was the precursor to the ICR, purified samples were stained with Lugol's iodine and observed microscopically [46]. *Giardia* cysts stain darkly with Lugol's Iodine, while *Cryptosporidium* oocysts do

not [87]. This method only recovered 5.9% of *Giardia* cysts and could recover no *Cryptosporidium* oocysts [46].

The most significant advance in the detection methods of cysts and oocysts is known as immunofluorescent assay (IFA) staining that was developed in by Sauch [76] for labeling *Giardia* cysts. This technique has since been adapted for use on *Cryptosporidium* oocysts. The method uses mammalian antibodies, produced against *Cryptosporidium* oocysts or *Giardia* cysts specifically, that are linked to a fluorescent molecule such as fluorescein isothiocyanate (FITC) that fluoresces under ultraviolet illumination. The selective differential staining of cysts and oocysts allow them to be more easily identified among sample debris, shown by a 12 times increase in cyst recovery over the zinc sulfate method [46]. The IFA has been the backbone of all nearly methods developed since, including the ICR method and Method 1622.

The Sauch method used polyclonal antibodies to stain *Giardia* cysts for viewing under epifluorescent microscopy but more since then monoclonal Ab (Mab) have become popular as there is less cross-reactivity with debris associated with the Mab stains [83]. There are two staining techniques available for Mab, known as direct and indirect. Direct IFA uses an Ab specific for cysts or oocysts with a fluorescent molecule such as FITC conjugated to it. Indirect IFA uses two antibodies, one specific for antigens on cysts and oocysts and the other specific for epitopes on the first antibody. After the first antibody has bound with the cyst or oocyst epitopes, a second batch of antibodies conjugated to FITC bind to epitopes on the first antibody. The indirect method allows for higher intensity of staining because more than one conjugated Ab-FITC molecule can bind to the specific Ab. While increased intensity allows for easier microscopic visualization,

direct methods have demonstrated more cross-reactivity than indirect stains [83]. Cross reactivity refers to the binding of stain to objects other than cysts and oocysts and is a major problem in natural water analysis for *Giardia* and *Cryptosporidium*.

Ideally, the IFA method only labels cysts and or oocysts. There are, however, many types of algae present in natural water sources that can interfere with epifluorescent identification of cysts and oocysts that are IFA stained. Rodgers et. al. [74] outlined the various types of algae, their shapes and sizes, as well as the degree of autofluorescence they have under epifluorescent examination. They found two species of algae out of fifty four tested that bound the Hydrofluor-Combo stain (Meridian Diagnostics, Cincinnati, Ohio) and fluoresced a bright apple green similar to labeled cysts and oocysts. One was *Navicula minima* (a diatom) and the other was *Synechoccus elongatus* (a cyanobacterium). To alleviate cross-reactivity with the cyst/oocyst specific Ab stain, Rodgers and his colleagues used non-specific immunoglobulins (Igs) to block the algal cells from binding the *Giardia* and *Cryptosporidium* specific IgG from the stain without interfering with it's ability to bind the cysts and oocysts. They found that this significantly decreased the amount of cross-reactivity and background staining. Both the ICR method and Method 1622 use Bovine Serum Albumin to block non-specific binding.

Although the IFA staining can show the microscopist which objects may be cysts or oocysts, there is still the important aspect of whether that parasite possesses the ability to infect a person who drinks the water it was found in. Being able to determine the viability of cysts and oocysts within a sample is important information for accurate risk assessment. Animal infectivity models and *in vitro* excystation protocols have traditionally been used as measurements of cyst and oocyst viability but they are not

considered practical for use in the water industry facilities due to the cost requirements [18]. There have however, been several studies showing strong correlation between *in vitro* excystation and *in vivo* infectivity protocols and the exclusion of certain dyes from cysts and oocysts. Both *Cryptosporidium* oocysts and *Giardia* cysts have a resistant wall that will exclude some dyes if it is undamaged. Hudson et. al. [34] showed that boronic acid derivative fluorescent dyes (FluoraBoras™) could be used as indicators of *Giardia* cysts viability and showed good correlation with excystation protocols. Campbell et. al. [9] also showed that *Cryptosporidium parvum* oocyst inclusion of 4', 6-diamidino-2-phenylindole (DAPI), which is a vital dye specific for the nucleotide bases adenine and thymine (AT), in conjunction with exclusion of propidium iodide (PI), show correlation with excystation protocols. It must be mentioned however, that exclusion or inclusion of dyes only indicates outer wall permeability and is only correlated with cyst and oocyst viability. To demonstrate this, Jenkins et. al. [37] showed that by using sodium hyperchlorite, which permeabilizes *Cryptosporidium* oocysts, there was no difference between DAPI and PI staining patterns for formaldehyde killed oocysts and the permeabilized oocysts. Permeabilized oocysts that displayed non-viable DAPI and PI patterns of staining, were still able to excyst.

Although DAPI and PI stains show a strong correlation with parasite viability and have been incorporated into Method 1622, these stains have been found to be ineffective in determining the viability of cysts and oocysts inactivated by chemical disinfectants such as chlorine and ozone [77; 41]. This is important for drinking water facilities that test finished water for the effectiveness of sterilization procedures. Nucleic acid stains from Molecular Probes have now been tested on chemically treated cysts and oocysts.

Taghi-Kilani et. al. [82] found that *Giardia* cysts that were inactivated by chlorine and ozone were stained by SYTO-9 and Live/Dead BacLight stains. Belosevic et. al. [2] found that *Cryptosporidium* oocysts inactivated by chlorine, chlorine dioxide and ozone showed viability staining that was consistent with animal infectivity protocols; oocysts that stained viable were still able to infect gerbils while non-viable oocysts did not. These stains hold promise for water industry applications and can be easily incorporated into Method 1622, in place of DAPI and PI.

1.1.7. Method 1622

Method 1622 was developed to replace the ICR method which had significant losses from all steps of the protocol [61]. A recent article by Clancy et. al. [11] detailed the development and validation of Method 1622. Two laboratories were involved in the work: Clancy Environmental Consultant in the United States and Thames Water Utilities Laboratories in England. Every step was meticulously evaluated for oocyst loss and new technologies were tested that were commercially available. Table 1 compares the ICR method and Method 1622.

Once optimization was completed and the authors had decided on which technologies were most accurate, a validation study was performed in the two laboratories. Fresh oocysts were spiked into four natural water matrices with different turbidities and biological content and analyzed using the optimized method. The average recoveries for the different matrices ranged from 21.6% to 52%, with a combined average of 35.3%+/-13%. No false positives or negatives were reported and the Minimum Detection Limit was calculated to be 4oocysts/L.

Table 1. Comparison of Method 1622 and the Information Collection Rule (ICR) method.

	Information Collection Rule	Method 1622
Concentration:		
Volume of water sampled	100L (raw water) 1000L (treated water)	10L
Filter type	Yarn wound cartridge	Folded membrane cartridge
Elution buffer	0.1% Tween 80/0.1% SDS; pH 7.4	1% Laureth-12; pH 7.4
Elution method	Stomacher or hand-washing.	Wrist action shaker.
Volume after elution	3L	275mL
Purification:		
Method used	Density floatation with Percoll-Sucrose.	Immunomagnetic separation (IMS)
Volume after purification	5mL	100µL
IFA:		
Monoclonal Ab staining	Indirect	Direct
Viability staining	None	DAPI
Microscopy:		
Examination method	Kohler illumination; epifluorescence (FITC)	Kohler illumination; epifluorescence (FITC and DAPI)
Total volume of original water sample analyzed	≤5L (Clancy et. al., 1999)	10L

1.1.8. New Techniques

A. Polymerase Chain Reaction.

Polymerase chain reaction is a process that takes small segments of single stranded (denatured) DNA, along with a specific probe to act as a primer, and amplifies that DNA piece using an enzymatic reaction. The method allows for very small amounts of a specific sequence of DNA to be detectable through the amplification reaction. Although not a new technique, it has only recently been applied to the problem of detecting *Giardia* and *Cryptosporidium* in water samples [38; 85; 81; 65; 71; 72; 73]. During the procedure, disrupted oocysts or cysts are subjected to an automated series of reactions with DNA Polymerase I, an enzyme which amplifies any DNA bound by the primer until the DNA levels reach a detectable amount (10^6 fold amplification) [36].

Development of an accurate primer is a very important aspect for PCR detection of both *Cryptosporidium* and *Giardia*. Rochelle, et. al. [73] compared eight pairs of published primers specific for either *Giardia lamblia* or *Cryptosporidium parvum* to determine their effectiveness with water samples. They found that for *Cryptosporidium*, the LAX primers demonstrated the greatest sensitivity, while for *Giardia*, the ABB primers detected a single cyst within a sample. They also stated that none of the primers they tested were ideal for both sensitivity, and specificity, and that none of them are compatible with multiplex analysis of *Cryptosporidium* and *Giardia* together.

One of the drawbacks for PCR testing of protozoan parasites within environmental water samples is the pre-concentration step. Aside from the inherent error involved with the procedure, the concentration of organic matter and dissolved solids within a concentrated sample can interfere with the activity of enzymes in the PCR

procedure [36]. Therefore it is very important that the water sample be purified well prior to the enzymatic reaction series. Qi-Deng, et. al. [65] showed that IMS clarification that was developed for use with Method 1622 can also be used for the purification of *Cryptosporidium parvum* oocysts from feces, for use with PCR. They achieved a sensitivity as low as 100 oocysts (100x greater than IFA) with this technique. Although they claim that their procedure is 100x more sensitive than IFA identification, pre-concentration and purification steps are not perfect and add error from cyst and oocyst loss.

A study done by Wagner-Weining and Kimming [85] demonstrated that viable *C. Parvum* can also be detected by PCR. The procedure involved excystation of the oocysts prior to PCR analysis, so that the analysis was performed only on the sporozoite DNA from viable oocysts. This method seemed to work well but did not take into consideration the effects of filtration, and purification on the viability of the oocysts, which could lead to an underestimation of viability. Also, the applicability of the method to regular water industry testing was not discussed.

B. Flow Cytometry

The use of Flow Cytometry to detect *Cryptosporidium* oocysts and *Giardia* cysts is a relatively new development that has been spearheaded by researchers in Australia. The technique itself is not new, but it has special properties that lend themselves well to detecting waterborne objects. The Flow Cytometry machine is able to count and sort particles of specific size and fluorescence, as well as separate them from other debris within the sample. This is done by vibrating streams of sample at frequencies that generate extremely small droplets. As the droplets pass through lasers and fluorescent

lights, the machine counts the particles and can separate droplets containing the desired size and fluorescent properties. With respect to the detection of cysts and oocysts, Flow Cytometry can detect cysts and oocysts that are stained with IFA and viability stains and sort them onto a slide for microscopic evaluation [36]. As with PCR, the major problem with this Flow Cytometry is that it requires some form of pre-concentration, such as filtration, that can add significant error to the method.

1.1.9. Summary

In summary, the detection of *Giardia* cysts and *Cryptosporidium* oocysts is complicated, often highly inaccurate, and only semi-quantitative. The consequences of disease outbreaks have been observed and the price can be very high. Method 1622 has made significant improvements by eliminating much of the variation and false negatives associated with the ICR method. It has not, however, significantly improved parasite recovery, leaving the door open for underestimations of parasite levels and the chance of false-negative results. New technologies are offering promise, but are not addressing all aspect of parasite detection, specifically in the area of sample concentration.

1.2. Objectives of This Study

Current detection methods offer little confidence in their results, whether it be from loss during filtration, and purification, or from microscopist error due to non-specific binding of IFA stains. The new methods being developed, such as IMS, PCR and Flow Cytometry, show that the focus of research is on purification and identification of cysts and oocysts from concentrated samples. On the other hand, there have been no significant improvements in the techniques for concentrating water samples. Filters are inherently limited by their own design, which is to trap small particles and are considered to be the area of greatest parasite loss [75]. Presently, no filter has been designed that will readily release all trapped material and as a result parasite recoveries will never be better than their recovery from filters.

This thesis proposes a possible solution to this problem. Recent publications have shown that oysters, as well as rotifers, have the ability to ingest *Cryptosporidium parvum* oocysts [21; 22]. The oysters acted as natural filters to remove oocysts from suspension in aquaria, and concentrate them in their hemolymph, gills, GI tract, and feces [27]. The ability to concentrate suspended particles may not be restricted to oysters alone; other filter feeding organisms are present in reservoirs and rivers that are used to supply drinking water. One such group of organisms are members of the genus *Daphnia*. *Daphnia* are tiny planktonic fresh water crustaceans that filter primarily on nanoplanktonic algae, with a smaller proportion including bacteria, detritus and bluegreen algae. They are found in many lakes and reservoirs that are used to supply drinking water, and are easily sampled in large numbers by towing fine nets through the water

column. The goal of this thesis is to test the suitability of *Daphnia* as a natural filter in detecting waterborne protozoa.

1.3. Biology of *Daphnia*

1.3.1. Basic *Daphnia* Biology.

Species of the Genus *Daphnia* are classified under the Order Cladocera, Class Branchiopoda, Sub-phylum Crustacea. There are more than 50 species distributed world wide in habitats ranging from small seasonal ponds to large lakes and reservoirs; they make up a major component of the worlds fresh-water zooplankton [32]. *Daphnia* possess typical cladoceran morphology – their body is made up of a cephalon, and a thorax and abdomen that have fused into a single trunk bearing five pairs of thoracic appendages and a flexed, claw-like caudal ramus. Between the pairs of thoracic appendages is a ventral food groove where filtered food is transported anteriorly to the oral cavity. The body and appendages below the cephalon are encased within a folded carapace with a gap running posteriorly on the ventral side, giving it a bivalve-like appearance. It is through this gap that currents of water are driven by the thoracic appendage to maintain gas exchange and a food supply for the animal [6].

The head region of *Daphnia* possesses a single compound eye for light detection and phototaxis, and a pair of antennae modified as swimming paddles. The oral opening is also located on the cephalon and is flanked by a pair of mandibles used for processing food [6]. The digestive tract of *Daphnia* is composed of a chitin lined stomodeal foregut or esophagus, a mesenteron (midgut) with a pair of blind-ended caeca, and a short proctodeal hindgut that is also lined with chiton. The midgut region has no chiton and is a region where nutrient absorption and digestion occurs [78]. Because very little degradation of food occurs via the mandibles, digestion is dependent on enzymes such as proteases, lipases, and amylases produced in the midgut [32].

Reproduction in *Daphnia* predominantly occurs asexually via cyclical parthenogenesis. Populations are typically made up of female individuals that begin to reproduce asexually after the fourth or fifth juvenile instar. Eggs are deposited into a brood pouch under their carapace where they hatch and develop until they are released just before the female's next moult. Neonatal *Daphnia* are tiny versions of the female parent, however, during times of overcrowding and stress, male progeny may be produced. These males will be necessary for fertilisation and development of ephipia, which are resistant to drought, freezing, and enzymatic digestion. These ephipia play an important role in colonising new habitats or old ones after a time of stress [32].

1.3.2 Feeding at Low Reynold's Numbers.

A. Introduction - Filter feeding is the dominant process of primary consumption in freshwater and marine environments [25]. This method of feeding was once thought to be simply a matter of trapping suspended particles using hairy "filters" that let water pass through but not the particles. Unfortunately it is not that simple. Very tiny organisms function in a world of low Reynolds number. In other words they live and feed in a world that is sticky and viscous where suspension feeding has been compared to capturing crumbs in olive oil using forks travelling less than a ½ mm/s (a situation with a Reynolds number of 10^{-2}) [40]. Setae and setules on the feeding appendages of planktonic suspension feeders such as the Cladocera and the Copepoda have Reynolds numbers that are in the range of 0.1 to $5x10^{-5}$ [10]. This makes sieving food from suspension a difficult or impossible task.

How is it then that these tiny creatures can be so successful at capturing suspended material from their sticky and thick environment? To answer this, we need a

basic understanding of fluid dynamics at very low Reynolds numbers. This will be discussed in some detail below, with specific focus on the fluid dynamics of arrays of setae and setules present on the thoracic feeding appendages of *Daphnia*.

B. Low Reynolds Mechanics - When a solid object moves in an aqueous environment the molecules on it's surface interact directly with the molecules of the fluid. The fluid molecules make inelastic collisions with the object's molecules and stick to, rather than slide across, each other. As the fluid moves relative to the object, the fluid particles adhered to it's surface are forced to move with the object instead of with the fluid. These bound particles of fluid, depending on it's viscosity, are exerting attractive forces on other particles of fluid, slowing them down as they shear by each other. As a result, very viscous fluids, or fluids with strong internal attractive forces, have high drag and are more difficult to move through. The viscous forces oppose the forces of inertia exerted by the object. How a fluid moves around an object is determined by two forces- inertia and viscosity. When the inertial forces are much greater than the viscous forces, flow around the object tends to be turbulent. If on the other hand, the inertial forces are low with respect to the viscous forces, the fluid will streamline over the object in laminar flow [40].

To predict whether fluid flow will be laminar or turbulent, a ratio of inertia to viscosity forces can be determined for the particular object and the fluid conditions. When this ratio is larger than a critical number, fluid flow past an object switches from laminar to turbulent. The size of the object, it's velocity, the viscosity of the fluid and the density of the fluid can be combined to determine this ratio, commonly known as the Reynolds number (Re). These variables are expressed as:

$$Re = \rho \ell U / \mu \qquad (1),$$

where ρ is the water pressure, μ is the viscosity of the fluid, U is the velocity of the fluid with respect to the object, and ℓ is the greatest length of the object in the direction of flow [84].

Re is a useful tool in estimating how a fluid will behave near a solid body under various conditions. When Re is high, inertial forces dominate and turbulences tend to persist as fluid flows past an object. On the other hand, at low values of Re, disturbances tend to be countered by the viscous resistance to shear deformations. As a result, fluid motion around objects with low Re is laminar [40].

It was once thought that tiny, branched hairs acted as nets or rakes to capture suspended food particles from fluid flowing through them. We now know that depending on the Reynolds numbers at which these hairs perform, and how they are arrayed with respect to one another, fluid will either flow around or through the array. At very low Reynolds numbers, a "boundary layer" of fluid exists around the surface of any solid object that is moving with respect to the fluid. At the point where the fluid comes into contact with the solid object, the velocity of the fluid is zero. As the distance from the surface increases, the velocity of the fluid flowing by will increase until it reaches free-stream velocity. The distance from the surface where the fluid has reached 99% of free-stream velocity is the thickness of the boundary layer (δ) [84]. The relative thickness of the boundary layer around a cylinder depends solely on Reynolds number of the cylinder [84] and has been roughly estimated by Koehl [40] using the following equation:

$$\delta = o[d / (Re)^{1/2}] \qquad (2),$$

where d is the diameter of the cylinder. So the density of the fluid, its velocity, and the size of the object will alter the thickness of the boundary layer. This is important when one is considering how tiny aquatic organisms such *Daphnia* obtain food particles suspended in an aqueous environment. These small crustaceans have highly specialised thoracic appendages that are used for swimming and feeding. Tiny hairs, or setae, line these appendages and are used to capture food particles or propel the organism through it's aqueous environment. The setae also have smaller branches called setules that create a "meshwork" with the neighbouring setae. Having a boundary layer of slower moving fluid makes the faster free flowing fluid move around it, creating a physical barrier surrounding the object. If δ is greater than half the distance between two setae or setules moving in parallel against the flow of fluid, it can be predicted that the fluid will not flow between the setae [40]. This would make the appendage with its hairy setae and setules act more like a paddle than a rake [10; 47]. This has been demonstrated to be the case using scaled up models of *Daphnia* feeding appendages in glycerine, assuming the principle of dynamic similarity [25].

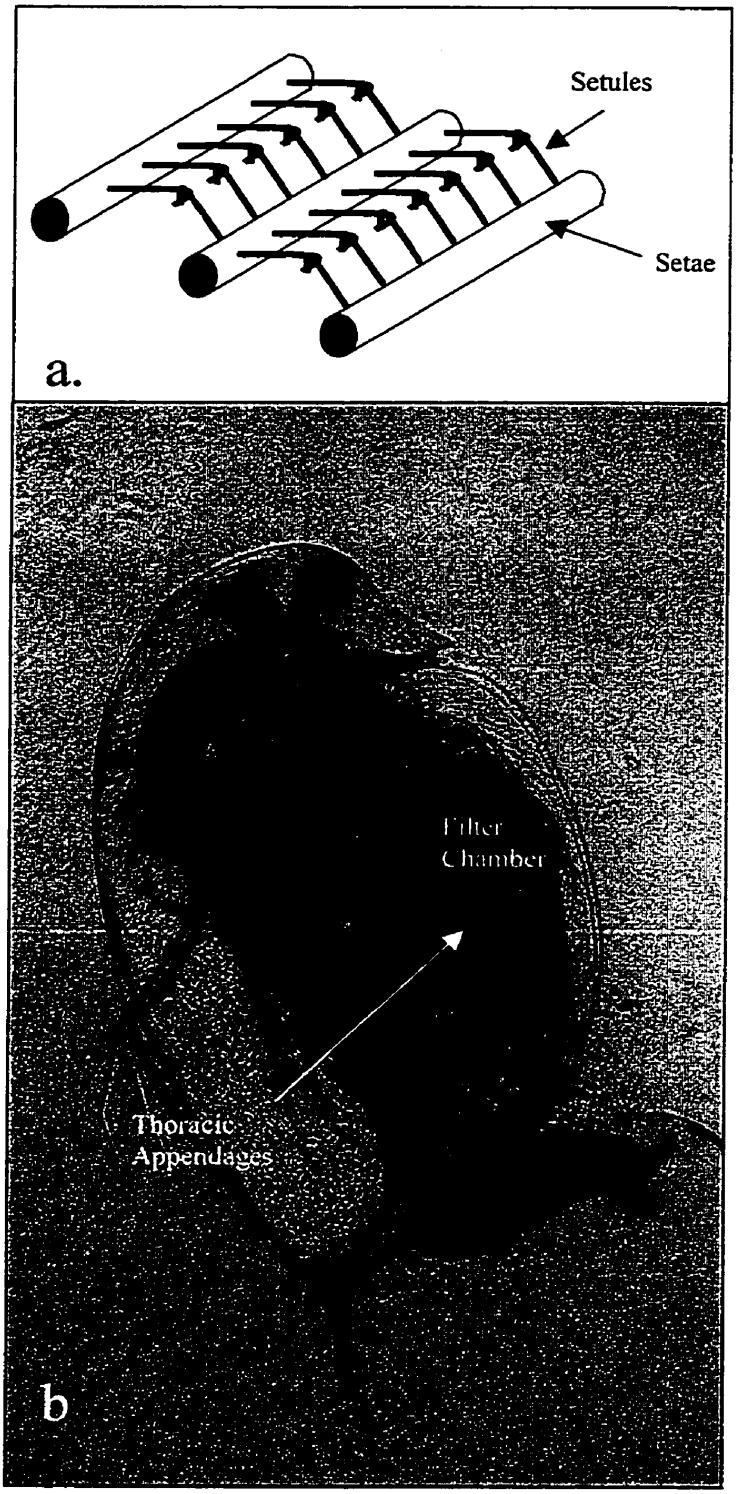
While the thickness of a boundary layer is dictated solely by the Reynolds number it is functioning at, the "leakiness" of an array of setae has another influence to consider. An array of setae and setules is not acting in free-flow systems, but is closely associated with the body of the organism as well as other appendages nearby. It has been shown that the leakiness of an array of hairs can be increased by it's proximity to a surface. This distance can be relatively large; for cylinders at $Re=10^{-4}$, surfaces that are 200,000 cylinder diameters away can influence the fluid's drag on the cylinder. If an appendage with setae arrays operating at $Re \leq 10^{-2}$ is moving along or toward a body's wall the

leakiness of that array will be increased. Also the array will not be affected by changes in velocity and small changes in the spacing between hairs will have a much larger effect on leakiness close to surfaces than in unbounded fluid. When Re values are between 10^{-2} and 1, changes in velocity have a large influence on leakiness while surfaces have very little if any effect [47]. It can be seen from this that such things as behaviour, growth and maturity, and the phenotypic plasticity of setae and setule spacing can have significant influence on the efficiency of particle capture.

C. *Daphnia* Feeding - As mentioned above, *Daphnia* feed with comb-like structures protruding from the third and fourth pairs of the thoracic appendages. What makes these meshes functional as sieves are the fine arrays of setae and setules (see Figure 1a). The even spacing between setules, commonly known as "mesh size," can range from 0.2 to 1 μm depending on the species and the size of the individual daphnid. This creates a flow situation for the arrays that have Reynolds numbers in the order of 10^{-3}. Estimates of boundary layers for this situation show that δ is larger than half the distance between setules and so it would seem impossible that these structures could be used as filters [43]. So how does *Daphnia* capture food particles with a mesh that acts like a paddle instead of a filter? The answer can be found by looking at how *Daphnia* use all five of their thoracic limbs together, not just the filter-bearing ones.

The bivalve-like carapace of *Daphnia* creates an enclosed area around the feeding appendages that is referred to as a "filter chamber". Within this chamber are five pairs of thoracic appendages that run in series down the ventral side of the animal's body (see Figure 1b.) The screens on the third and fourth limbs separate the filter chamber from the space between the paired limbs. As the animal feeds, all five appendages move forward

Figure 1. Structures involved in *Daphnia* feeding. a) Schematic cross section of a daphnia filter screen. Adapted from Lampert, 1987. b) Photomicrograph of a 2mm long female *Daphnia pulex* highlighting structures involved in filtering feeding.


Setules
Setae
a.
Filter
Chamber
Thoracic
Appendages
b

and laterally creating a larger medial space between them. Water rushes into the empty space through the gap in the carapace walls. All five limbs then move posteriorly and medially trapping a small amount of water between them. By pressing the limbs into it's body, the animal increases the pressure within the medial space forcing the water out through the filters [43]. Once particles are captured on the screen, they are moved to the ventral food groove where they are transported anteriorly to the mouth and then ingested [43].

1.3.3. *Daphnia* Feeding Rates

A. Introduction – *Daphnia* feeding can be described using two measurable rates: the ingestion rate and the filtering rate. Filtering rate measures the volume of water that is processed over a period of time by an individual animal, and is typically described in units of mL/h. Ingestion rate is the amount of food that is consumed by an individual daphnid per unit time. It is typically measured *in vitro* as cell number, dry weight, carbon mass, or particle volume per unit time. [43]. The two rates are intimately related to one another by the food concentration. We can easily calculate the filtering rate by multiplying the ingestion rate by the food concentration. Likewise, ingestion rate can be calculated by dividing the filtering rate by the food concentration.

The concentration of food available to a daphnid has a major impact on the rates of both filtering and ingestion. To optimize food consumption at low food concentrations, *Daphnia* must filter water at a maximum rate. The ingestion rate will depend primarily on the concentration of food available. As food concentrations increase, the filtering rate will remain maximized and the ingestion rate will increase until the point were there is more food available than the animal can ingest. This point is referred to as the Incipient

Limiting Level (ILL). After this point, the *Daphnia* no longer needs to filter as much volume to attain the maximum ingestion rate and so filtering rate decreases. The result of this interplay between maximum ingestion and filtering rates is two distinct responses to increasing food concentration. Ingestion rate increases more or less linearly with food concentration until the ILL, after which it is constant and maximum. The filtering rate function is the opposite [43].

These rate functions are typical for *Daphnia* and have been useful predictive tools for limnologists and population biologists. The ingestion rate function is the more useful of the two and has been described mathematically in two ways: rectilinearly and curvilinearly. The rectilinear representation is a two-part function where ingestion rate increases linearly up to the ILL and then is constant as food concentration continues to increase. The rectilinear method for fitting data is quite simple. A single concentration is visually determined and set as the ILL and data below this point and above are grouped separately and linearly regressed using the least-squares method. This method assumes that there is no "interference" between food particles and the feeding mechanisms until the ILL. This "interference" could be a result of feeding appendage saturation by food particles or gut filling that is faster than food excretion. If indeed these factors do influence ingestion rate, the rectilinear method may not be an accurate representation of the transition zone around the ILL. A curvilinear function, which is quasi-hyperbolic, would more accurately represent this type of functional relationship [59]. For curvilinear fitting, there are two commonly used equations: an Ivlev or Mechaelis-Menton equation. The Ivlev function takes the form:

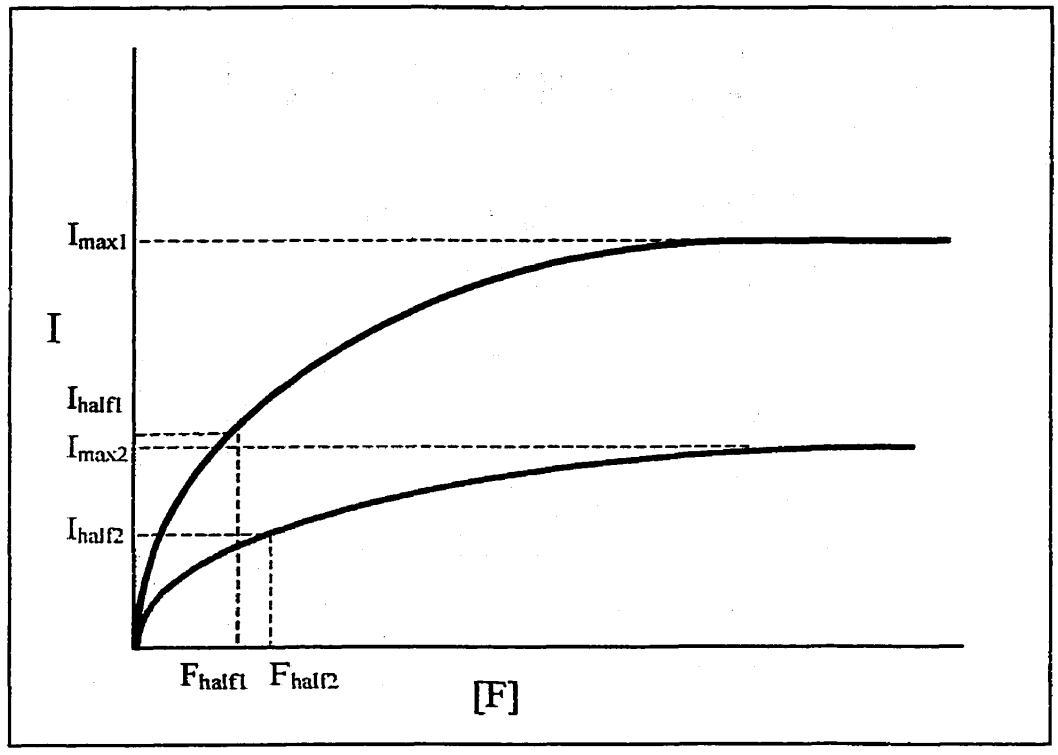
$$I = I_{max} [1 - e^{-\delta(p)}] \qquad (3),$$

where I is the ingestion rate, I_{max} is the maximum rate of ingestion, p is the food concentration, and δ is a constant. The other common curvilinear description of *Daphnia* ingestion rate is the Michaelis-Menton enzyme kinetic function. It is described by the following equation:

$$I_o = I_{max}[\mathrm{F}] / F_{half} + [\mathrm{F}] \qquad (4).$$

Ingestion rate (I_o) at each concentration of food ([F]) can be determined using the maximum ingestion rate (I_{max}), and the half saturation constant, where ingestion is half the maximum rate (F_{half}) (See Figure 2). Both the Ivlev and the Michaelis-Menton functions look similar when plotted, but when Mullin (1975) compared the mean square variances for both curvilinear fits, the Ivlev model came out the lowest. They were however, not statistically different using the F-test. There is no consensus as of yet regarding which function best represents these responses to food concentration.

Figure 2. Graphical representation of two Michaelis-Menton functions with different F_{half} and I_{max} values. The y-axis represents the ingestion rate (I) while the x-axis displays food concentration ([F]).


I_{max1}
I
I_{half1}
I_{max2}
I_{half2}
F_{half1}
F_{half2}
[F]

B. Food Size and Type - While rectilinear and curvilinear equations may be helpful in determining the general shape of ingestion rates as a function of food concentration, there are many physical and environmental factors that influence the size and exact shape of the response. For instance, the ILL marks the food concentration after which there will no longer be an increase in ingestion rate; the animal has maximized its ability to consume food. This is primarily based on how much volume of food the animal can physically ingest, not how much carbon or dry weight the food contains [64]. This means that the food type and size can have a strong influence on maximum ingestion rates. If the animal is capable of compressing the food particle with it's mandibles into a smaller volume the maximum ingestion rate may be higher than other food types of similar volume that cannot be broken down [43]. Also, the ILL at which maximum ingestion rate is achieved can be influenced by the body size of the animal. As the size of a daphnid increases, the ILL also increases. [28].

C. Body Size - The body size of any organism is one of the most important factors affecting biological function. Body size is directly related to many biological functions including food ingestion rates, body temperature regulation, basal metabolism, oxygen diffusion, and locomotion just to name a few. The reasons for this may stem from the relationship between body size and metabolism. Most relationships with body size take the form of:

$$Y = aW^b \qquad (5),$$

where Y is a biological characteristic, such as basal metabolism, W is the measurement of the organisms body size, and a and b are empirically derived constants. Generally, for metabolic rates in the animal kingdom $b = ¾$ [63]. Given this standard relationship, and

knowing the pivotal role metabolism plays in animal physiology, any physiological rate that varies linearly with metabolism also will vary with body size. This includes such things as respiration, locomotion, urine flow, blood flow, and of course ingestion.

Daphnia are no exception to this rule. A commonly observed phenomenon is the differences in feeding rates between differently sized individuals. This has been extensively studied in many different species of *Daphnia* and the relationship can be described by Equation 1. with b generally ranging between 2 and 3 [43]. The reason that b varies, and is not ¾ like metabolic relationships, is due to the many environmental factors that influence feeding behavior of *Daphnia*. Factors such as temperature, food size and concentration, diel rhythms, and starvation influence the value of b in the body size-feeding rate function [43]. Any factor that increases the feeding rates of larger animals more so than smaller animals will increase the value of b and vice versa. One such factor is the size of food the animals are feeding on. As food particle size increases, so too does the value of b in the body size, feeding rate function. This means that large animals are more efficient than small daphnia at feeding on larger particles, and small animals handle finer particles more efficiently than large animals [43].

Other environmental factors also have strong influences on the value of b. For instance, b decreases when food concentrations are above the ILL. Also, temperature increases cause the value of b to decrease according to the following relationship:

$$b = 2.43 - 0.044\mathrm{T} \qquad (6),$$

where T is the temperature the *Daphnia* are feeding at [43]. Also, increasing temperatures or high food concentration reduces the coefficient of variation between

body size and feeding rate. For high food concentrations, this increased variability can be eliminated by a starvation period prior to feeding [43].

D. Temperature - *Daphnia* are freshwater organisms, and as such their environments can be subject to relatively large changes in temperature over a short period of time. We know that temperature has a strong influence on metabolic processes, so it is of no surprise that it influences the feeding rates of *Daphnia* as well. Grazing experiments on *Daphnia* have shown that the temperature of the water in which *Daphnia* live influences their feeding rate in an optimal fashion. Studies have shown that as temperature increases, so too does the maximum ingestion and filtering rates up to an optimal foraging temperature, after which the rates fall [43]. This optimal temperature may depend on the temperature at which the *Daphnia* populations were cultured. For instance, animals that were pre-incubated at 12°C had optimal filtering rates at 14°C whereas *Daphnia* pre-incubated at 20°C had maximum rates at 20°C [8; 39]. There is also evidence that when *Daphnia* are cultured for several generations at a given temperature, they may overcome the filtering limitations imposed at higher temperatures, showing no optimal ingestion rate between 7°C and 25°C [43].

E. Diel Rhythms and Light - One interesting temporal feature of *Daphnia* feeding behavior is a 24h pattern of changing feeding rates, especially under *in situ* conditions. This pattern has been extensively observed and appears to follow a generalized pattern of low filtering rates during daylight hours, a drastic increase shortly after nightfall, which then falls after midnight and then peaks again just before sunrise [29]. This change in feeding corresponds with the diel vertical migration of *Daphnia* in natural lakes and ponds. It was also observed that *b* values for *in situ Daphnia pulex* filtering rates were

increased from daylight values of 0.52 to 2.00 during the night [28]. This means that larger *Daphnia* show more drastic changes in filtering rates between dark and light conditions than do small *Daphnia*.

The mechanism for this change in feeding pattern appears to be light induced. When *Daphnia* in *in situ* grazing chambers were darkened during the day and illuminated during the night, filtering patterns assumed nighttime highs during the day and corresponding lows at night. But the question arises: why do *Daphnia* lower their filtering rate during the day, and increase them at night? One possible reason is forwarded by Haney (1985) who suggests that as *Daphnia* migrate upwards in the water column at night they can take advantage of patches of increased food concentration nearer the surface by increasing feeding rates, feeding opportunistically during their migration. At sunrise *Daphnia* migrate down into waters that contain less food and so ingestion rates will be lower during the day. There is also some suggestion that there could be an influence from water temperature changes which may coincide with light and dark phases [43]. As already discussed, increasing the temperature will increase or decrease filtering rate depending on the animals optimum feeding temperature.

1.4. Conclusions and Specific Aims

Consideration of the stated problems and the general study objectives raises some questions that this research will attempt to address. Firstly, do *Daphnia* ingest waterborne cysts and oocysts? Evidence using natural food sources suggests that *Daphnia* are generalist filter feeders but we should not assume that this is true for parasitic protozoa. Secondly, if *Daphnia* do ingest cysts and oocysts, do they feed on the parasites at similar rates as they do natural food sources such as algae? If *Daphnia* somehow select for, or against, the parasites while feeding, the ingestion rates of the parasites will be different from the rates of ingestion of a natural algal food source. Lastly and most importantly, can ingested parasites be detected in populations of *Daphnia*? If populations of *Daphnia* can be sampled and their intestinal contents examined for the presence of cysts or oocysts, this would provide an inexpensive and potentially highly sensitive detection method.

The specific aims of this study are as follows:

1. To test whether *Daphnia pulex* ingest suspended *Giardia* cysts and *Cryptosporidium* oocysts.
2. To test whether *Daphnia* graze on cysts and oocysts non-selectively. This will be done by determining and comparing individual grazing rate functions for a natural algal food source as well as *Giardia* cysts and *Cryptosporidium* oocysts.
3. To test whether populations of *Daphnia* can be used to detect waterborne *Giardia* cysts.

2. Selection and Ingestion of *Giardia* Cysts and *Cryptosporidium* Oocysts by *Daphnia pulex.*

2.1. Introduction

Water quality is a serious public health issue due, in part, to the increased incidence of waterborne associated outbreaks of protozoan parasites such as *Giardia* and *Cryptosporidium.* In 1993, an estimated 403,000 individuals were affected by *Cryptosporidium* from a single drinking water source in Milwaukee (Wisconsin, U.S.A.) and water related outbreaks of Giardiasis have been documented in Creston (British Columbia, Canada), Banff (Alberta, Canada), and Edmonton (Alberta, Canada) [56; 35; 49]. Currently, the techniques for detecting cysts and oocysts are semi-quantitative, inaccurate and require highly skilled technicians [83; 87]. Recent tests of the most current method (USEPA Method 1622) showed that it recovers only 43% of spiked cysts and oocysts [12]. While this is a significant improvement over previous methods, it certainly does not guarantee that parasites will be detected if present in drinking water sources.

A great deal of effort is being directed at improving detection techniques whereas little work is being done to help understand the ecology of waterborne protozoan parasites. *Giardia* and *Cryptosporidium* have been found to be highly prevalent in both Canada, and the Unites States, with between 43% and 100% of natural water testing positive for one or both parasites [70; 44; 62; 86]. Very little is known about how aquatic organisms that inhabit drinking water sources may interact with waterborne cysts and oocysts. Most aquatic freshwater systems have organisms that filter-feed on suspended particulate material, and their impact on suspended populations of cysts and oocysts is

not understood. Some recent publications have shown that oysters and clams can filter-feed suspended *Cryptosporidium* oocysts *in vitro* as well as *in situ*, concentrating the parasites in feces and digestive tract and the researchers were able to detect the parasites within the tissues and feces [21; 27]. More recently, rotifers have been shown to ingest *Cryptosporidium* oocysts in suspension and to pass them through their digestive tracts visibly intact[22].

This raises some interesting questions about how other, more prevalent, filter feeding animals might affect resident populations of cysts and oocysts. One such filter-feeding organism which is common in ponds, lakes, and reservoirs world-wide is the tiny crustacean *Daphnia* [52; 54; 53; 55]. Members of the genus *Daphnia* are small zooplankton that feed on algae, bacteria, fungi, protozoa and organic debris [32]. Populations of these crustaceans are highly efficient grazers and have been shown to filter entire water bodies in a single day [50]. This makes it likely that some portion of a *Daphnia* population would encounter waterborne cysts or oocysts if that water source has been contaminated.

To date, no studies have shown that *Daphnia* do, or do not, ingest waterborne *Giardia* cysts or *Cryptosporidium* oocysts. This investigation had two purposes: first to establish that ingestion of waterborne cysts and oocysts by *Daphnia* does occur; and second, to compare ingestion rate functions to determine if *Daphnia* feeding behavior is different for *Giardia* cysts and *Cryptosporidium* oocysts, than it is for natural algal food source, in this case *Chlamydomonas*.

2.2. Material and Methods

2.2.1. Culture techniques

The *Daphnia pulex* used in these experiments were isolated from the Glenmore Reservoir, a small meso-oligotrophic drinking water reservoir [88], which supplies drinking water to approximately half of the households in the city of Calgary, Canada. They were cultured at room temperature in 4L beakers of water from the Glenmore Reservoir that was filtered through a 0.2μm nominal porosity cartridge filter. A mixture of edible algae containing primarily *Monoraphidium minutum*, was grown in a goldfish aquarium that had half of it's volume exchanged weekly with fresh deionized water. The beakers of *Daphnia* received 100mL of this mixture daily and had half of the population removed weekly to maintain rapid growth and reproduction.

Culture techniques for *Chlamydomonas reinhardtii* Dang. used in the feeding experiments are detailed in Appendix I.

2.2.2. Staining

Giardia lamblia cysts were obtained from Waterborne™ Inc. (New Orleans) while *Cryptosporidium parvum* oocysts were kindly supplied by Dr. Merle Olson from the University of Calgary. Both cysts and oocysts were preserved in 5% formalin in Phosphate Buffered Saline (PBS) and stored at 4°C. Immunofluorescent Antibody (IFA) staining of cysts and oocysts for fluorescent microscopy was done using Giardi-a-Glo™ and Crypt-a-Glo™ from Waterborne™ Inc. (New Orleans). Cysts/oocysts were centrifuged at 5000rpm for 10min using a Hermle Labnet Z-18M microcentrifuge. The supernatant was removed, the cysts/oocysts were resuspended in 10μL of diluted IFA (1:20 in PBS), and then incubated in the dark at 37°C for 40min. One wash was performed and the cysts/oocysts were re-suspended in 2mL of filtered reservoir water. The concentrations of the stained cysts/oocysts were determined using hemacytometer

counts and the solution was stored in the dark at 4°C for no more than 2 days before discarding.

Fluorescent staining of cysts/oocysts used in feeding experiments was performed with the non-specific vital stain Propidium Iodide (PI). The staining protocol was similar to the IFA method described above with a few notable differences. The concentration of stain used was quite high, 500µg/L, and cysts/oocysts were incubated for 20min at room temperature in the dark. Solutions of PI stained cysts/oocysts were stored at 4°C in the dark, and stored for 2-3 months before discarding.

2.2.3. Microscopy

To visualize ingested cysts/oocysts within *Daphnia pulex*, 5-10 females were placed in 15mL polypropylene tubes with high concentrations (>100,000/mL) of IFA stained cysts/oocysts and allowed to graze for one hour. During the feeding period tubes were placed on a tissue culture wheel turning at approximately 1rpm in the dark and at room temperature. After one hour had elapsed the *Daphnia* were removed and killed in boiling water, rinsed three times with 100mL of deionized tap water and then wet mounted on a microscope slide. Individual *Daphnia* were observed under epifluorescence using a Nikon inverted microscope with a DM580 filter and photomicrographs were captured using a Nikon N2000 35mm camera.

2.2.4. Feeding Experiments

For determining filtering rates of individual *Daphnia*, a technique was developed that did not require the use of immunofluorescent microscopy, which was found to be a time consuming and inaccurate method for *Giardia* cyst and *Cryptosporidium* oocyst enumeration. The new method used a SPECTRAmax GEMINI^{TM} microplate fluorometer

(Molecular Devices) to measure the intensity of fluorescent particles in suspension. Concentration was then interpolated using standard curves. A detailed account of the development and optimization of this protocol is outlined in Appendix II.

Feeding experiments were performed using adult (2mm) and juvenile (1mm) female *Daphnia pulex*, with no eggs or ephipia, chosen from cultured populations. Prior to each experiment the isolated *Daphnia* were starved in double filtered reservoir water for a period of one hour. For a single feeding experiment, a feeding solution of PI stained cysts or oocysts, or live *Chlamydomonas* was prepared in double filtered reservoir water at a known concentration. Four 15mL polypropylene tubes (Falcon™) were filled with this solution and used as feeding chambers for the experiment. Chambers containing PI stained cysts and oocysts were stored in the dark prior to the experiments while the live *Chlamydomonas* solutions were stored on the benchtop under normal light conditions.

To begin an experiment 5-7 isolated and starved female *Daphnia* were introduced into each of three feeding chambers. The fourth chamber was kept as a control for determining the initial food concentration. To maintain evenly distributed food suspensions during feeding experiments, feeding chambers were placed within a darkened outer tube attached to a tissue culture wheel turning at 1rpm. The animals were allowed to graze for 20 minutes, at which time they were removed.

The concentration in each of the four tubes after the 20 minute feeding period was determined using the microplate fluorometer. The machine was set for the food type's optimal excitation/emission wavelengths (see Appendix II, Table 6), and then the entire volume of each tube was aliquoted into the wells of two Greiner™ Microlon Black 96-well microplates. Standard curves were prepared for every experiment using the same PI

stained cysts/oocysts or live algae that the *Daphnia* were fed. Prepared microplates were placed into the fluorometer, shaken for 5 seconds and then read. Standard curve regressions and interpolated concentrations for each microplate were obtained using the Softmax Pro version 3.0 software.

Individual *Daphnia* filtering rates from each of the three tubes were determined using the following equation:

$$G = V (\ln C_0 - \ln C_t) / (tN) \qquad (7),$$

where V is 15mL (volume in each tube), C_0 is the concentration found in the fourth tube that contained no *Daphnia*, C_t is the concentration found in one of the three grazed tubes, t is the time of feeding in hours, and N is the number of Daphnia feeding in each tube. Individual ingestion rates were calculated by multiplying the filtering rate value by the C_0 value. A series of experiments were performed with *Giardia* cysts or *Chlamydomonas* using both 1mm and 2mm *Daphnia* feeding at food concentrations ranging from 2,000 to 20,000cells-cysts-oocysts/mL.

2.2.5. Statistical Analyses

Data were analyzed using both linear and non-linear techniques. For linear analyses, it was assumed that the functional response is linear with food concentration up to a point, after which the ingestion rate remains constant for further increases in food levels. Data below the ILL were regressed by size class and food type using an intercept-less linear model and slopes were tested for differences using t-tests on the regression coefficients. The maximum ingestion rates for each size class and food type were also compared by ignoring data below a food concentration of 10,000 particles/mL. For non-linear analyses was assumed that the ingestion rate response to food

concentration follows a type II functional response that can be described by a Michaelis-Menton function. This function can be described by two parameters- I_{max} and F_{half}. The I_{max} is the maximum rate of ingestion described by the function, while F_{half} is the food concentration at which the ingestion rate is half of I_{max}. Data from each size class and food type were fit with this function and 95% confidence intervals were calculated for I_{max} and F_{half}.

Due to the limited availability of *Cryptosporidium* oocysts, only three experiments were conducted using adult (2mm) *Daphnia* and three different food concentrations. The minimal data available for *Cryptosporidium* oocysts as a food source excluded the possibility of comparing *Cryptosporidium* to *Chlamydomonas* functions via the linear and non-linear methods described above. To test the two for differences, we simply compared 95% confidence intervals for the individual experimental ingestion rates at three different oocyst concentrations to the 95% confidence intervals of the non-linear functional response for *Chlamydomonas*.

2.3. Results

2.3.1. Ingestion

Photomicrographs of *Daphnia* that were fed high concentrations of IFA stained cysts or oocysts for a period of one hour show the presence of cysts and oocysts within the digestive tract and feces. *Giardia* cysts appeared as brightly fluorescing apple green oval objects that are 10-14μm long. *Cryptosporidium* oocysts also fluoresced apple green, but due to their smaller size (4-6μm) did not always appear as bright and obvious as *Giardia* cysts (see Figure 3). Both cysts and oocysts were observed in the filter screens, food

grooves, hindgut, and feces of exposed individual *Daphnia* (see Figures 4 and 5). No cysts or oocysts were detected when unexposed animals were observed.

Figure 3. Photomicrographs of fluorescently labeled *Giardia lamblia* cysts (A) and *Cryptosporidium parvum* oocysts (B). 200x magnification.

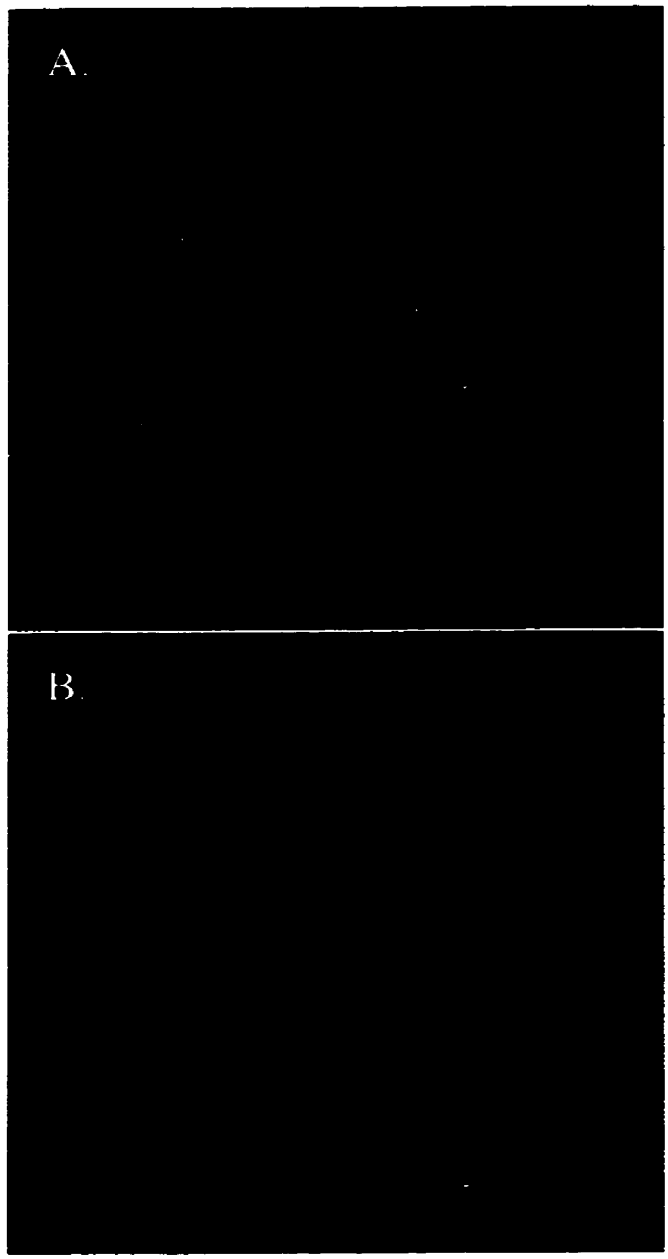

A.
B.

Figure 4. Photomicrographs of fluorescently labeled *Cryptosporidium parvum* oocysts in the food groove (A) and filter appendages (B) of female *Daphnia pulex*. Arrows indicate the oocysts. 200x and 400x magnification respectively.

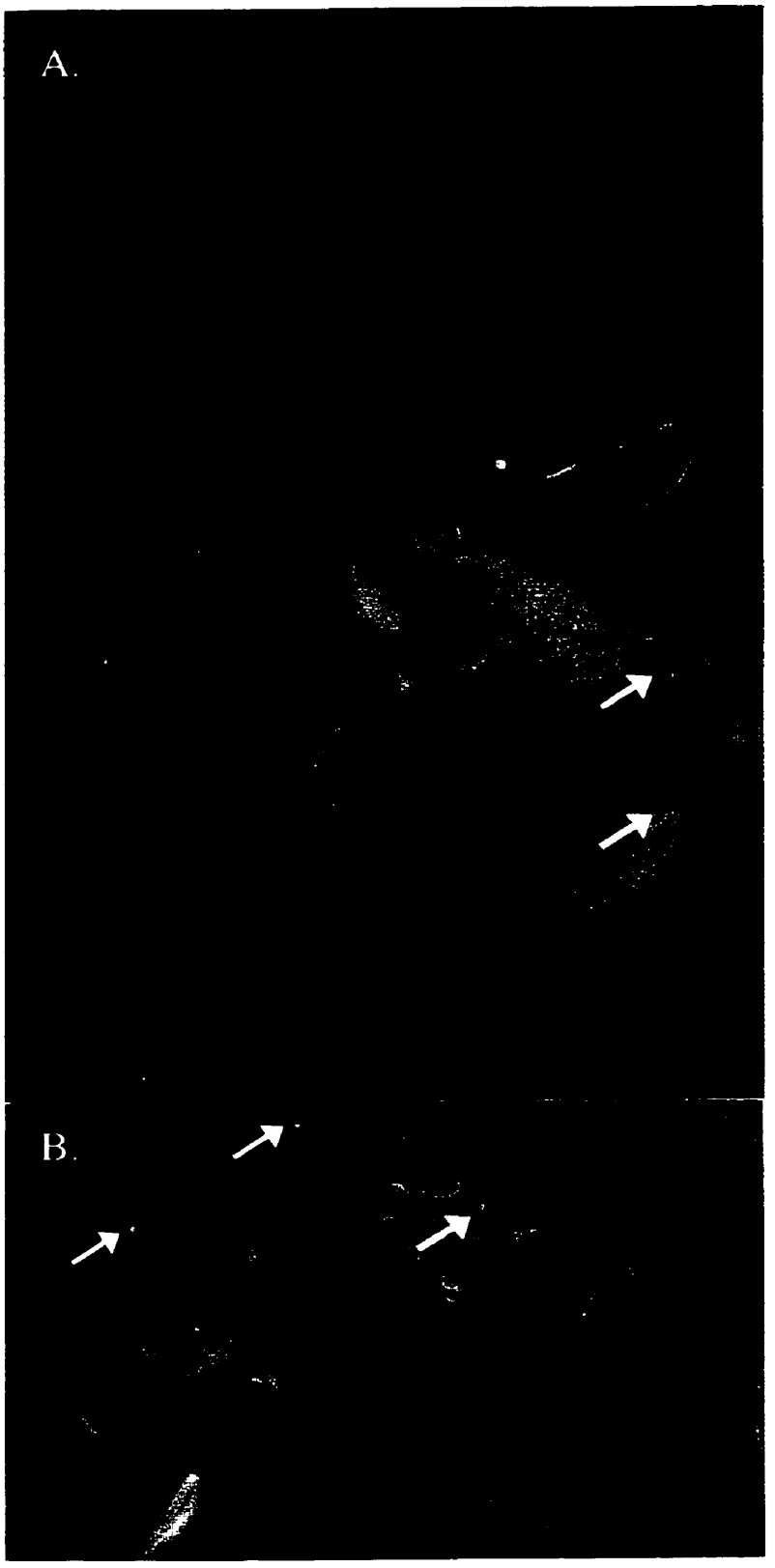
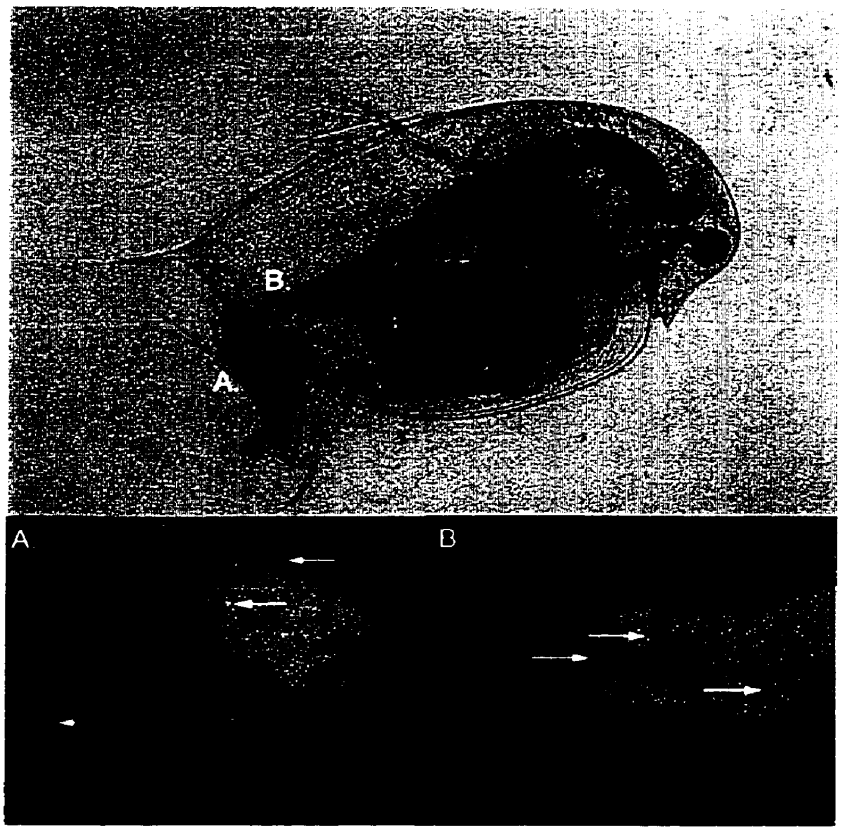

A.
B.

Figure 5. Upper panel- Photomicrograph of a female *Daphnia pulex*. The letters indicate the areas displayed in the two lower panels. 100x Magnification. Lower panels- Photomicrographs of fluorescently labeled *Giardia lamblia* cysts (B) in the mid-gut and *Cryptosporidium parvum* oocysts (A) in the hind-gut and feces. 200x magnification.


B
A
A
B

2.3.2. Feeding experiments

Individual juvenile (1mm) and adult (2mm) *Daphnia pulex* ingestion rates were determined at increasing concentrations of PI stained *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts, as well as live *Chalmydomonas*. Average adult and juvenile *Daphnia* filtering rates below food densities of 15,000 particles/mL are shown in Table 2. Adult *Daphnia* filtering rates for *Giardia* and *Chlamydomonas* were 2.26 and 2.77 times higher than juvenile filtering rates respectively. The responses of *Daphnia* ingestion rates to increasing food densities are shown in Figure 6. Ingestion rates for all food types increased with increasing food concentrations, and for *Giardia* and *Chlamydomonas*, ingestion rates appeared to level out above food densities of approximately 10,000 particles/mL.

Differences between ingestion rate functions for *Giardia* cysts and *Chlamydomonas* were tested for both adult and juvenile *Daphnia* using linear and non-linear techniques. Table 3 summarizes the results from linear analysis of juvenile and adult ingestion rate functions on *Giardia* and *Chlamydomonas*. Comparison of slopes from the linear regression of data below food concentrations of 10,000 particles/mL show significant differences ($P<0.05$) between ingestion rates of *Giardia* cysts and *Chlamydomonas* cells for adult daphnia but not for juveniles. Adult *Daphnia* ingest *Chlamydomonas* at significantly higher rates than *Giardia* cysts at a given density. Maximum ingestion rates were also compared with similar results. Both adult and juvenile *Daphnia* ingest *Chlamydomonas* at higher maximum ingestion rates than *Giardia* cysts. Comparison of upper and lower 95% confidence intervals indicate that this difference is significant for adult *Daphnia*, but not for juveniles. From the non-linear

analysis, 95% confidence intervals for I_{max} and F_{half} estimates show no significant difference between *Giardia* and *Chlamydomonas* ingestion by juvenile *Daphnia*. The same is true for adults but a substantial trend is indicated by the F_{half} estimates; while not significantly different, the F_{half} estimate for *Chlamydomonas* was much higher than the F_{half} estimate for *Giardia*.

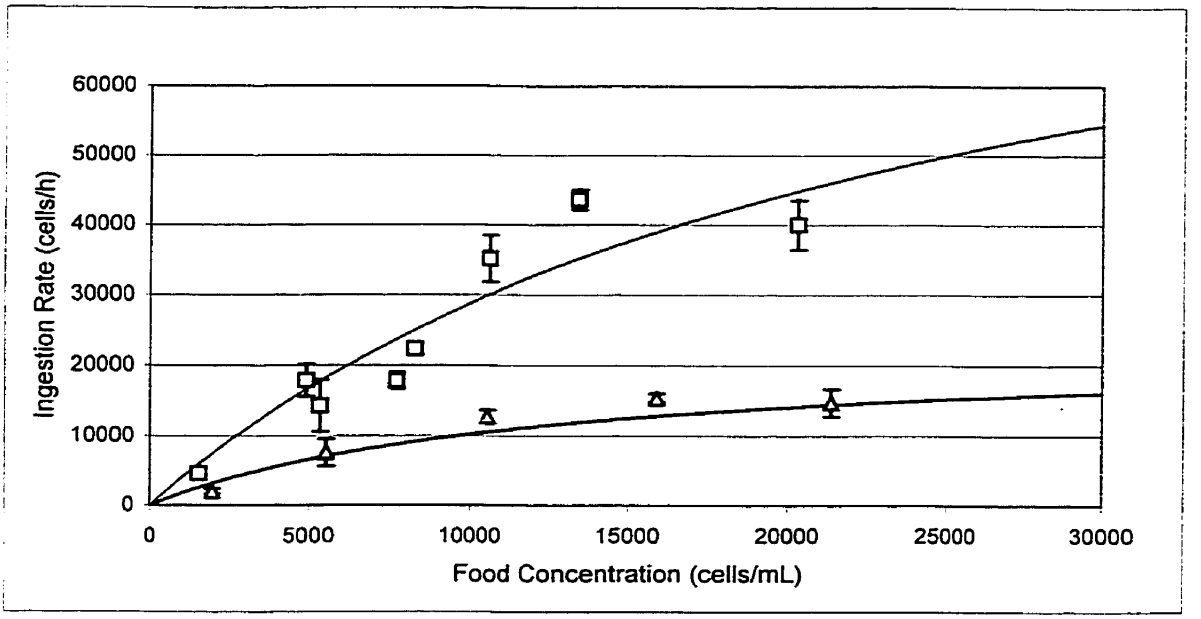
Due to the fewer data available for *Cryptosporidium* ingestion rates, linear and non-linear comparisons of functional responses could not be used. To test whether *Daphnia* ingestion rates of *Cryptosporidium* were different from *Chlamydomonas* at any given food density, the 95% confidence intervals for the parasite were compared to the confidence intervals of algal ingestion rate estimates at the same food density. This was done using the linear functional response determined for 2mm individuals feeding on *Chlamydomonas*. Table 4 shows these comparisons. It can be seen that while all of the algal ingestion rate estimates are substantially higher than the *Cryptosporidium* food source, only the middle food density shows any significant differences with respect to the 95% confidence intervals.

Table 2. Mean individual filtering rates for adult (2mm) and juvenile (1mm) *Daphnia* feeding on Propidium Iodide stained *Giardia* cysts and live *Chlamydomonas* at food densities below 15,000/mL.

Food type	*Daphnia* size	n	Average Individual Filtering Rate (mL/h)	Standard Deviation
Chlamydomonas	1mm	9	1.08	0.414
	2mm	17	3.00	0.730
Giardia	1mm	12	1.02	0.187
	2mm	11	2.31	0.498
Cryptosporidium	2mm	6	1.33	0.306

Figure 6. Ingestion Rates plotted as a function of food concentration for the food sources: A) *Chlamydomonas* and B) *Giardia* cysts and *Cryptosporidium* oocysts. On panel A. and B. the adult (2mm) *Daphnia* are represented by open squares while juvenile *Daphnia* are represented by open triangles; on panel B the open diamonds represent adult *Daphnia* feeding on *Cryptosporidium* oocysts. Each point represents one experiment (n=3) with error bars displaying the Standard Error. The lines are the non-linear fit for the data using a Michaelis-Menton function.

A.


60000
50000
40000
30000
20000
10000
0
Ingestion Rate (cells/h)
0
5000
10000
15000
20000
25000
30000
Food Concentration (cells/mL)

B.

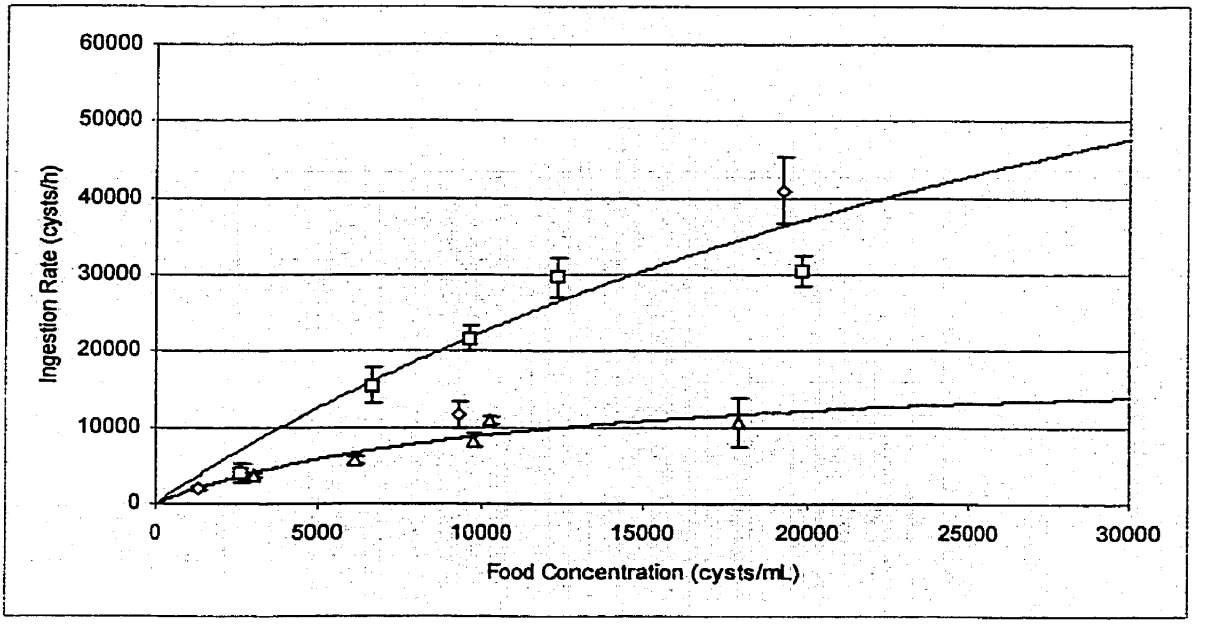

60000
50000
40000
30000
20000
10000
0
Ingestion Rate (cysts/h)
0
5000
10000
15000
20000
25000
30000
Food Concentration (cysts/mL)

Table 3. Linear analysis of ingestion rate functions for adult (2mm) and juvenile (1mm) *Daphnia* feeding on Propidium Iodide stained *Giardia* cysts or live *Chlamydomonas*.

	Slope		Maximum Ingestion Rate (#/h/individual)		
	Estimate	Standard Error	Mean	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Adults					
Giardia	2.33194	0.10216	30632.58	26201.61	35063.56
Chlamydomonas	3.06934	0.11662	39896.53	36296.26	43496.79
Juveniles					
Giardia	0.97516	0.04032	9872.41	7880.56	11864.26
Chlamydomonas	0.96214	0.09893	12278.57	9448.71	15108.42

Table 4. Comparison of adult *Daphnia pulex* ingestion rates of *Cryptosporidium parvum* oocysts and estimates of *Chlamydomonas* at three different food densities. Ingestion rate values and 95% confidence limits (CL) for *Cryptosporidium* were determined experimentally while the values for *Chlamydomonas* were estimated from the linear regression (see Table 2).

Food Density (mL^{-1})	*Cryptosporidium*			*Chlamydomonas*		
	Ingestion Rate Estimate (mL/h)	Lower 95% CL	Upper 95% CL	Ingestion Rate Estimate (mL/h)	Lower 95% CL	Upper 95% CL
1357.892	1901.21	793.41	3008.00	4167.83	219.73	8115.94
9235.529	11693.38	3969.77	19416.99	28346.98	26149.75	30544.20
19254.35	40965.99	22206.57	59725.42	59098.15	55264.58	62931.73

2.4. Discussion of Results

This study is the first of it's kind to describe the nature of an interaction between *Daphnia* and waterborne protozoan parasites. Both waterborne *Giardia* cysts and *Cryptosporidium* oocysts are filtered and ingested by juvenile and adult *Daphnia* as demonstrated by photomicrographs of fluorescent labeled cysts and oocysts in the filter-combs, food grooves, digestive tracts and feces of *Daphnia*. Furthermore, adult and juvenile *Daphnia* ingestion rate functions for *Giardia* cysts and *Cryptosporidium* oocysts and *Chlamydomonas* have similar shapes, following a type II response pattern to increasing food concentration. This type of ingestion rate functional response to increasing food concentration has been well documented for *Daphnia* [68; 57; 8] and suggests that *Daphnia* respond to changes in *Giardia* cyst and *Cryptosporidium* oocyst concentrations the same as they would respond to any other food source.

Research has shown *Daphnia* to be relatively non-selective filter feeders. For instance, feeding studies with flavored spheres have shown that while other freshwater zooplankton have chemosensory mechanisms of selecting polystyrene microspheres flavored with attractive algal factors over non-flavored ones, *Daphnia* do not [17]. Furthermore, studies with differently sized glass microbeads, algae, and bacteria have shown that there is no preference between particles based on size below a maximum diameter of approximately 20μm [57; 42; 26; 16]. The non-selective feeding pattern of *Daphnia* can be attributed to the passive sieving by the filter and the mechanism by which these particles are then collected. They feed by drawing currents of water through the gap in their carapace, trapping a volume of this water between their filtering appendages and then pressing it out through fine setae and setules that act as sieves.

Other appendages then act to "sweep" captured particles from the filter combs and into the ventral food groove [32]. The lower limit of ingested particle size is determined by the inter-setular distances of the filters [24] while the upper limit of particle size is established by the carapace gap size. Both the upper and lower limit will vary with body size, with smaller animals having smaller carapace gaps and smaller inter-setular gaps [43]. Using the linear relationship between maximum ingested particle diameter and *Daphnia* carapace length determined by Burns [7], the maximum particle size for ingestion by 1mm and 2mm *Daphnia* is estimated at 26.87 μm and 48.87μm respectively. This places cysts and oocysts well below the maximum consumable range for both juvenile (1mm) and adult (2mm) *Daphnia*.

Given the evidence for non-selective feeding for *Daphnia*, we expected that there would be very little difference between filtering and ingestion rate functions for parasites and algae. However, we found that the means of parasite filtering rates were lower than the means for algae below food densities of 15,000/mL (see Table 2). This was particularly evident for adult *Daphnia* filtering rates. Moreover, the linear and non-linear analysis of the ingestion rate functional responses for *Giardia* and *Chlamydomonas*, yielded statistically significant differences in feeding behavior for either the protozoa or the algae. Comparison of the ingestion rate slopes below food concentrations of 10,000 particles/mL showed significantly lower ingestion rates for the *Giardia* cysts. This is supported by a much higher F_{half} estimate for *Giardia* cysts, and while not significantly different from the *Chlamydomonas* F_{half} estimate, indicates lower ingestion rates for *Giardia* at a given food concentration. Furthermore, at food concentrations above

10.000/mL, we found that adult *Daphnia* had significantly lower maximum ingestion rates for *Giardia* cysts than for *Chlamydomonas*.

Comparison of *Cryptosporidium* ingestion rates and the corresponding ingestion rate estimates from the linear regression of *Chlamydomonas* show a similar trend (see Table 4). The ingestion rate estimates of *Chlamydomonas* were all much higher than those of *Cryptosporidium*. Results indicate that there was only significant differences in concentration near the mean of the food densities, and not at the upper and lower food densities. This is to be expected from this sort of analysis as the 95% confidence intervals around a linear regression are always smallest near the mean of the independent variable and greatest at the upper and lower extremes. The ingestion rate values for *Cryptosporidium* should also be interpreted carefully due to some inconsistencies we found with the standard curves (see Appendix II-II.C., Figure 14). The negative intercept value for all of the *Cryptosporidium* oocyst standard curves could not be corrected and could have been a result of interfering particles that were present in the oocyst samples. Based on our experience in developing these protocols this sort of deviation in standard curve interpolations could result in small over- or under-estimations of ingestion rates for experiments involving *Cryptosporidium*.

Overall, these findings suggest that while there is no active selection of *Chlamydomonas* over the two parasites, there is a difference in feeding efficiency between the parasite and algal food sources in adult *Daphnia*. This efficiency difference is likely related to the food particles and not active selection by *Daphnia*. A particle property that is known to cause feeding efficiency differences is the size of food particles. In the power relationship between body size and feeding rate:

$$R = aL^b \quad (8),$$

the exponent *b* is influenced by food size. As food particle size increases, so does *b*, which would result in higher rate (R) for a given body size (L) [43]. As previously mentioned, cysts and oocysts range in sizes between 6-10μm and 10-14μm respectively, while *Chlamydomonas reinhardtii* Dang. (UTEX 90) measures 5-7μm. By the very nature of the power function, small increases in *b* can significantly increase feeding rates for larger daphnia, but would have less effect on smaller ones. In other words, larger animals can feed more efficiently on larger particles than on small particles and vice versa. Table 3 clearly shows that parasite and algal filtering rate differences are more pronounced for adults than for juveniles. Also, the linear analysis of ingestion rate functions failed to show significant differences between *Giardia* and *Chlamydomonas* for juveniles, but not for adults. Estimates of *b* from the 1mm and 2mm grazing rate means from Table 3 show us that *b* is 1.700 for *Chlamydomonas* and 1.633 for *Giardia*. Based on weight as a measurement of *Daphnia* body size, *b* should be 2.5, and based on body length it should be 2 [5]. This however is not consistent with most experimental findings, due in part to the environmental influences that have been found to also influence *b*, such as temperature and diel effects [43]. Increases in temperature have been found to decrease the value of *b* according to the following relationship:

$$b=2.43-0.044T \text{ [43]} \quad (6).$$

Given that our experiments were conducted at a relatively high temperature (T=21°C) the value for *b* would be estimated as 1.506 which is more consistent with our findings.

The differences we observed between *Giardia* and *Cryptosporidium* ingestion rates can be explained by their size differences, however, the fact that higher ingestion

rates were observed for *Chlamydomonas* than for *Giardia* is not explained by the differing food sizes. One possible reason for this could be a property of the parasites that makes *Daphnia* filter feed less efficiently on them than on algal cells. A study by Gerritsen and Porter [25] reported that the surface chemistry of food particles does have an influence on the capture efficiency by *Daphnia*, and that neutrally charged particles were captured more readily than particles with net negative charges. It was also shown that adding a surfactant to the feeding suspension decreased capture efficiency. *Cryptosporidium* oocysts have been found to have global negative surface potentials in aqueous medium with low hydrophobicity [19]. *Giardia* also is known to adhere to glass and polystyrene which is why polypropylene tubes were used in these experiments. This may explain why *Chlamydomonas*, while being smaller in size was ingested at higher rates than *Giardia*; lower affinity of the parasites to *Daphnia* filtering appendages would decrease feeding efficiency.

Now that we can say with a high degree of certainty that *Daphnia* ingest waterborne cysts and oocysts, we must consider the likely consequences of this behavior. Whether either of the two parasites would be able to excyst and infect a daphnid, remains unknown. Even though we observed that fluorescently labeled cysts and oocysts passed through the digestive tract and into the feces intact, our parasites were not viable, being preserved in formalin. Even if *Daphnia* do not become infected by viable parasites it is conceivable that they may be intermediate vectors for infecting other animals such as fish and possibly predatory mammals. This is however, mere speculation.

From a water quality standpoint the impact *Daphnia* grazing would have on viable cysts and oocysts is of particular interest. *Daphnia* are known to possess digestive

enzymes such as proteases, lipases, and amylases [32] that could potentially damage ingested cysts and oocysts, making them less viable. After passing through the digestive tract and being exposed to these enzymes, cysts and oocysts may not be as viable when they are defecated back into the water column. Also, since *Daphnia* ingest small, suspended particles and then excrete them as heavier fecal pellets, it is likely that cysts and oocysts bound up in a mass of partly digested algae would settle out more quickly from suspension than they would if they were left to settle as individual particles. Therefore large resident populations of *Daphnia* in a drinking water system may act to improve water quality by reducing the number of parasites in suspension and possibly decreasing the infectivity of any that may make their way into drinking water.

This study may also present the water industry with a cost effective and potentially more sensitive method for detecting cysts and oocysts: using *Daphnia* as bio-indicators for the presence of waterborne protozoan parasites. We have already mentioned how poor the current methods of parasite detection are in the introduction. A significant portion of the error associated with concentrating and detecting waterborne protozoa occurs in the concentration step. We know that *Daphnia* are highly efficient filter feeders; populations have even been known to filter an entire body of water in a single day [52; 54; 53; 55]. They are also easily sampled in huge numbers by towing nets through the water column. If you consider a 2mm *Daphnia* with a gut passage time of 20min. and a filtering rate of 3mL/h, then the digestive tract would contain the filtrate removed from approximately 1mL of water. This means that a large sample from a *Daphnia* population will contain the filtrate from a significant volume of water; the exact volume will depend on the size structure of the population and the relative feeding

behaviors for each size. The larger the *Daphnia* sample, the larger the volume sampled, and the lower the minimum parasite detection level. Furthermore, there is no difficulties associated with removing the filtrate from the filter, only collect the discarded feces or homogenize the collected *Daphnia* and process as you would using standard protocols such as Method 1622 or the Information Collection Rule method. *Daphnia* may even be useful in detecting other disease organisms, such as coliform bacteria.

In conclusion, this study has shown that *Daphnia* do ingest suspended *Giardia* cysts and *Cryptosporidium* oocysts. While feeding efficiencies may be lower for the parasites than for *Chlamydomonas*, there seems to be no active selection mechanism that would prevent cysts and oocysts from being ingested if encountered among suspended algae and bacteria in a natural water system. Further study needs to be conducted to determine whether this occurs when natural populations of *Daphnia* encounter the parasites while grazing on phytoplankton. Also, the nature of the interaction between *Daphnia* and waterborne parasites needs to be further explored to determine what effect *Daphnia* have on parasite prevalence and viability.

3. Detection of *Giardia* cysts in natural water using populations of *Daphnia pulex*.

3.1. Introduction

Our previous research has shown that individual *Daphnia pulex* can filter and ingest waterborne *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts (see Section 2). These data on individual daphnia in isolation suggest that there is no active feeding selection against cysts or oocysts and that aside from small differences in feeding efficiency, adult and juvenile *Daphnia* consume the parasites at similar rates as they do a natural algal food source. This is supported by studies showing that *Daphnia* appear to be generalist filter feeders, choosing food based on a maximum particle dimension and not by taste or food quality [57; 42; 26; 16; 17; 3]. We cannot however, assume that *Daphnia* populations grazing on natural plankton communities will ingest the parasites when they are encountered.

Many man-made and natural water bodies contain resident populations of *Daphnia*. For instance, the Glenmore Reservoir in Calgary, Canada is a man made lake that supplies approximately half of the city with drinking water and contains significant populations of *Daphnia pulex* (150µg/L dry weight or approximately 5*Daphnia*/L) during the ice-free seasons [30]. If these animals are present in drinking water sources, why not try to use them as natural biological filters? We wished to test whether natural populations of *Daphnia pulex* ingest waterborne *Giardia* cysts and at the same time develop a method to address the detection problem the water industry is currently facing. To do this we exposed *Daphnia* populations of varied densities to waterborne *Giardia lamblia* cysts for a period of 48h, after which the populations were collected and processed to remove and enumerate any ingested cysts.

3.2. Materials and Methods

3.2.1. *Daphnia* populations

A preliminary study was done using *Daphnia pulex* cultured in a 700L tank. Due to the time consuming and intensive effort required to culture these high numbers of *Daphnia* it was decided that *Daphnia* should be collected directly from the Glenmore Reservoir. *Daphnia* were collected two days prior to the experiment by hauling zooplankton nets over the edge of the Screen-house catwalk at the Glenmore Reservoir Dam. While this method collects all zooplankton present, the Glenmore Reservoir primarily contained *Daphnia pulex* at the time of sampling. The samples were transferred into a plastic barrel containing 120L of non-filtered reservoir water located in a 15°C walk-in incubator.

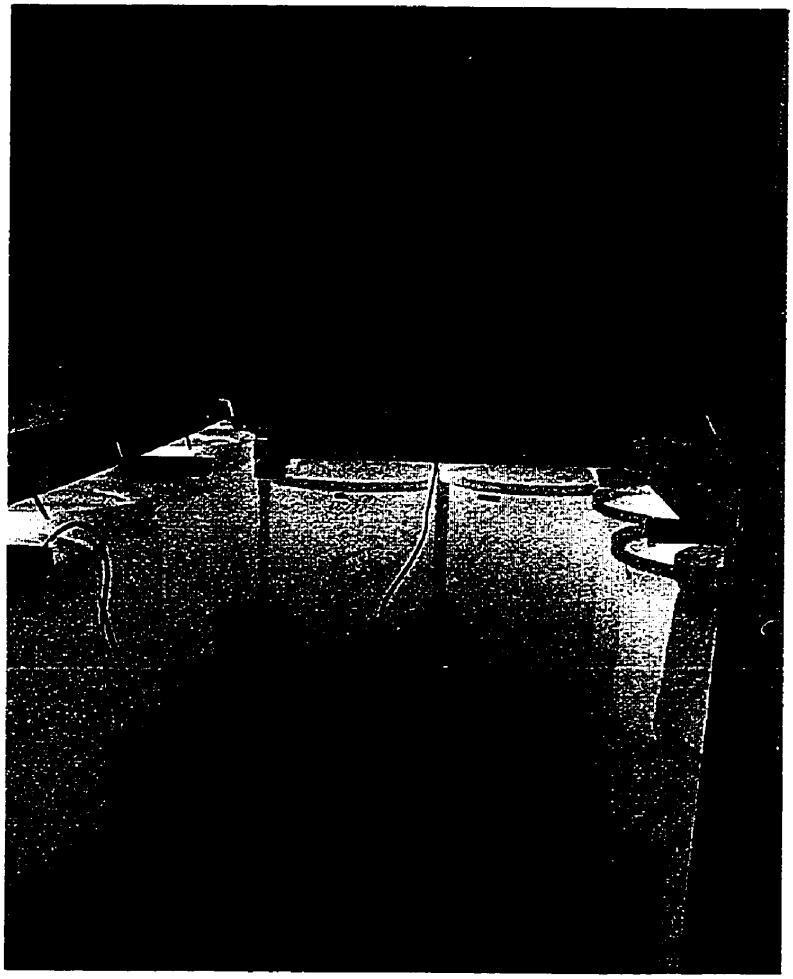
3.2.2. Mesocosm experiment

For the preliminary study, the experiment was set up in mid-July out-of-doors at ponds located at the University of Calgary. In order to more tightly control for environmental factors such as rain, light levels, and temperature the actual experiment was setup in a 15°C walk-in incubator (see Figure 7). A total of nine 200L plastic experimental barrels plus one inoculum barrel were placed in the incubator and ultraviolet lighting was supplied by five Sylvania Gro-Lux™ wide spectrum 40Watt lighting units. A photoperiod was chosen that was seasonal with sunrise and sunset times at Calgary for mid-September: on at 7am and off at 8pm. Each barrel was filled with 100L of unfiltered Glenmore Reservoir water, collected from the "Raw Water" tap at the Glenmore Reservoir Treatment Plant. The nine barrels were assigned to three experimental population density groups: 1/L, 5/L, and 10/L. To create these

experimental groups, the number of *Daphnia* required for each of the three treatment densities was determined by taking the product of the desired density and the volume of water in each of the treatments (100L). Next, the population density of the collected *Daphnia pulex* inoculum was estimated by averaging five 1L counts. This population average was then used to estimate the volume that needed to be sampled in order to collect enough *Daphnia* for inoculating each experimental treatment with the proper number. This was done by siphoning the calculated volume through a 200μm Nitex™ screen, and then transferring the captured *Daphnia* into the designated treatment barrel. The *Daphnia* were allowed to acclimate for a 24h period before beginning the feeding experiment with *Giardia* cysts.

To begin the experiment each treatment barrel received 10mL of formalin killed *Giardia lamblia* cysts suspended in filtered Glenmore Reservoir water at a concentration of 10,000cysts/mL. The final concentration was the same for each treatment barrel: 1000cysts/L. The contents of the barrels were gently stirred to mix the cysts.

After 48h post inoculum, the entire volume of each treatment barrel was pumped through a 30μm Nitex™ screen, collecting all of the *Daphnia* from each treatment and preserving them in 7% formaldehyde/sucrose solutions as for later processing. Phytoplankton samples were also taken from each treatment barrel and preserved with several drops of Lugol's iodine. All samples were stored in the dark at room temperature until later processing and counting.



3.2.3. *Giardia* Cyst Extraction

Before the *Daphnia* populations were processed each was classified by body length. This was done by enumerating the number of *Daphnia* in four different size classes: <1mm (neonatals), 1-1.5mm (Juveniles), 1.5-2mm (Young Adults), 2-2.5mm (Adults).

A. Homogenizing and Purifying

Each of the preserved samples were poured through a 30μm Nitex™ screen and the collected *Daphnia* were rinsed three times with deionized tap water. The samples were then rinsed off into a 50mL polypropylene tube with 10mL of double distilled water and then homogenized for 10min with an Ultra-Turax tissue homogenizer (Janke &Kunkel IKA-WERK, Type: TP 18/10 S1). The tip of the homogenizer was removed and rinsed with enough double distilled water to bring the volume up to 20mL. After homogenizing, the samples were sonicated for two 1min bursts, medium setting (550 Versonic Dismembrator, Fischer Scientific). The homogenized samples were then underlayed with 20mL of a cold 1M Sucrose solution using a polypropylene pipette and centrifuged at 1,050xg for 10min at 4°C (Beckman Centrifuge, Model J-6B). In order to ensure the interface was not disrupted, the centrifuge was accelerated slowly by manually turning up the velocity until the desired speed was reached. Very carefully, the top 30mL was removed from the centrifuged samples and placed into another 50mL polypropylene falcon tube, mixed well with a vortex, and then re-centrifuged at 1,200xg for 10min. The final step in purifying the samples involved carefully removing and discarding the supernatant, re-suspending in 100μL of PBS, and storing at 4°C until staining and microscopic enumeration.

A positive control was prepared by collecting 1L of *Daphnia pulex* culture population using a 30μm Nitex™ screen and preserving them in a 7%Formaldehyde/Sucrose solution several days prior to extraction procedure. This sample was processed as described above for the experimental samples with one exception: during homogenization, the positive control was spiked with approximately 1000 *Giardia* cysts suspended in 1mL of double distilled water.

B. Staining and Counting

Microscope slides were prepared by drawing approximated 20mm diameter circles with a silicon pen; only one circle was drawn per slide to minimize the possibility of cross contamination of samples. All of a single purified sample was pipetted into a silicon circle and allowed to air-dry. When the samples were completely dry they were each set by carefully overlaying 50μL of absolute methanol into the circle and air-drying. Samples were then stained with Giardi-a-Glo™ (1:20 dilution of stock in PBS; Waterborne™ Inc.) by carefully overlaying 50uL into the circle and incubating in a dark-box containing moist towels for 40min at 37°C. To aspirate off the stain, a Pasteur pipette was drawn out with a Bunsen burner to create a very narrow tip, and attached to a vacuum aspirator. While keeping the slide tilted, the tip was lightly touched to the bottom edge the sample until most of the stain was removed. Each slide was then rinsed three times by carefully overlaying 50μL of PBS over the sample and then aspirating off with the narrowed pipette. The samples were again allowed to air dry, this time at room temperature in a dark box. To finish the staining, 10μL of 2% 1,4-diazabicyclo [2,2,2]octane (DABCO; Sigma D-2522) /glycerol (60%glycerol, 40% PBS) was placed

over the stained samples, covered with a glass slip and sealed with clear nail polish. Slides were stored at 4°C in the dark until counting.

Each slide was analyzed using a Nikon inverted fluorescent microscope with a DM580 fluorescent filter at a magnification of 400X. The entire area within a circle was scanned and *Giardia* cysts were enumerated that were visible as bright fluorescent apple-green objects of the appropriate size and shape.

3.2.4. Phytoplankton Analysis

Phytoplankton samples were taken of each treatment tank. They were prepared for microscopic observation using the method described in Appendix II-IV. Settled samples were first observed under 200X magnification and the predominant species were noted. Phytoplankton were then classified into <10μm and 10<35μm size classes and then enumerated by counting random fields at 400X magnification and transects at 200X magnification respectively. Sizes were determined as the greatest measured dimension, including spikes or protrusions. Concentration was determined from the counts using the methods described in Appendix II-IV.

3.2.5. Statistical Analyses

Data were plotted as the number of cysts detected as a function of the treatment population's density. The linear regression of this function was calculated and the r^2 was determined. This was repeated again after the population density was converted into a biomass value (μg/L). Using the phytoplankton data collected from each treatment the grazing rates in each of the treatment populations was estimated using the non-linear regression equations determined in Section 2 for *Chlamydomonas*. The number of cysts

detected was then plotted as a function of filtering rate and the linear regression equation was calculated for this relationship as well.

3.3. Results

3.3.1. Population Structure

Figure 8 displays the *Daphnia* population size structures for each of the treatments. The most commonly sized *Daphnia* found in the treatments were 1-1.5mm in length (Juveniles), making up an average of 60% of the total populations. Adult *Daphnia* (>2mm) were only noted in treatments 5-1 and 5-3, totaling 1 and 3 individuals respectively.

The experiments were designed to create treatment populations with three specific densities: 1*Daphnia*/L, 5*Daphnia*/L, and 10*Daphnia*/L. However, the observed population densities for most of the treatments at the end of the experiment were not equal to their designated density; all of the treatments contained lower *Daphnia* concentrations than their designated density.

3.3.2. Phytoplankton Content

The treatments each contained a variety of edible phytoplankton, primarily *Scenedesmus spp.*, *Dinobryon spp.*, and *Ceratium spp.*. Phytoplankton from the treatments were characterized and enumerated by GALD. Figure 9 shows the amounts of edible phytoplankton, either <10μm or 10-35μm, in each treatments. It appears from this figure that samples with relatively low numbers of *Daphnia* such as treatments 1-1, 1-2, and 1-3, had higher numbers of 10-35μm and less <10μm phytoplankton than treatments with higher densities of *Daphnia* after the 48h feeding period.

3.3.3. Detection of *Giardia* Cysts

Giardia lamblia cysts were detected in all treatment populations except 1-2 and 1-3. Positive controls showed that extraction procedures recovered 33.1% of cysts from Experiment 2. Figure 10 displays the number of cysts detected in a treatment population plotted against the *Daphnia* density of that treatment. The highest number of detected cysts occurred in treatments 5-1 and 5-2 at *Daphnia* densities of $3.27L^{-1}$ and $2.26L^{-1}$ respectively, while no cysts were detected in treatments 1-1 and 1-3 with densities of $0.15L^{-1}$ and $0.22L^{-1}$ respectively.

In general, the number of cysts detected tended to be slightly higher in treatments with higher population densities. The linear regression indicated a positive relationship between the number of cysts detected and the population density as shown by the solid line in Figure 10. The probability that the slope of this relationship is greater than zero is 71.19%. When the *Daphnia* density was converted into total biomass (μg dry weight), the regression indicated a poorer relationship, with a probability of 60.87% that the slope is greater than zero (see Figure 11A). Interestingly, when the number of cysts detected was compared to the estimated total filtering rate for a population, the relationship was strongest at a 79.06% probability of slope greater than zero (see Figure 11B). The estimates of filtering rate were calculated using the linear regression equations determined for adult and juvenile individuals in the previous chapter and the edible algal densities shown in Figure 9.

Figure 8. Histograph depicting the *Daphnia pulex* population size structure of treatments. Populations were divided into four size classes: Adult (2-2.5mm), Young Adult (1.5-2mm), Juvenile (1-1.5mm) and Neonatals (<1mm). The size classes are combined to show the total number of the population; the different patterns designate the portion of the population that is composed of a particular size class.

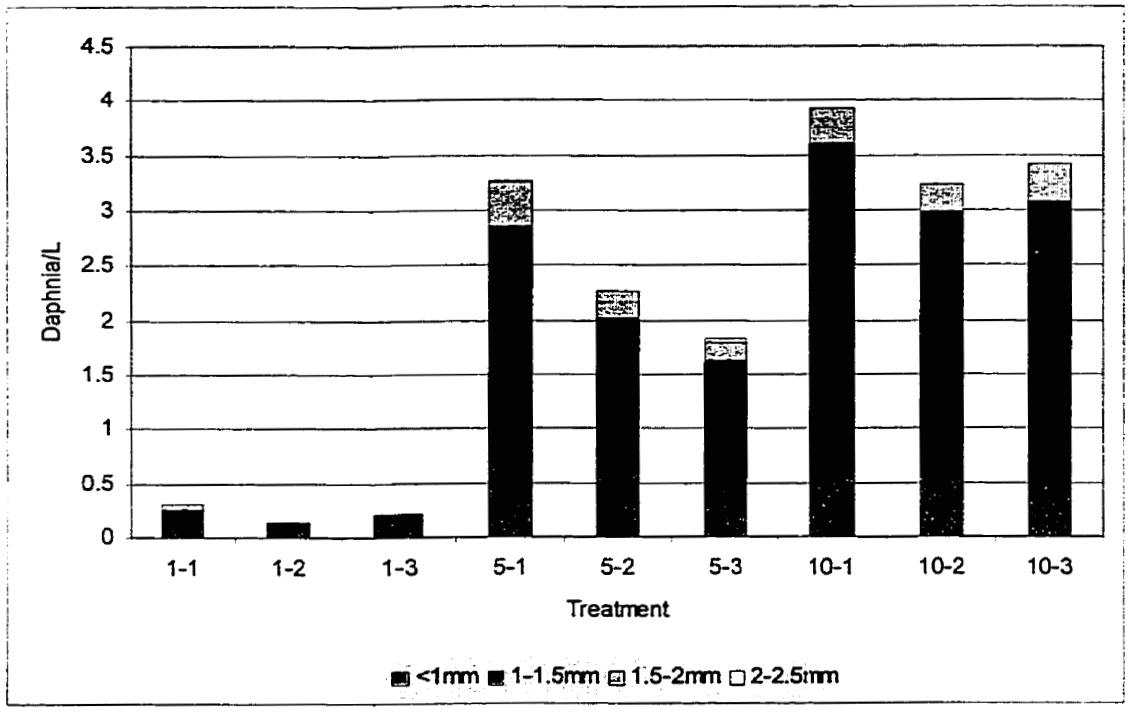

4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Daphnia/L
1-1
1-2
1-3
5-1
5-2
5-3
10-1
10-2
10-3
Treatment
<1mm
1-1.5mm
1.5-2mm
2-2.5mm

Figure 9. Histograph presenting sampled phytoplankton concentrations within treatments. Phytoplankton were classified by the greatest axial linear dimension (GALD) into two categories: 10μm<35μm and <10μm.

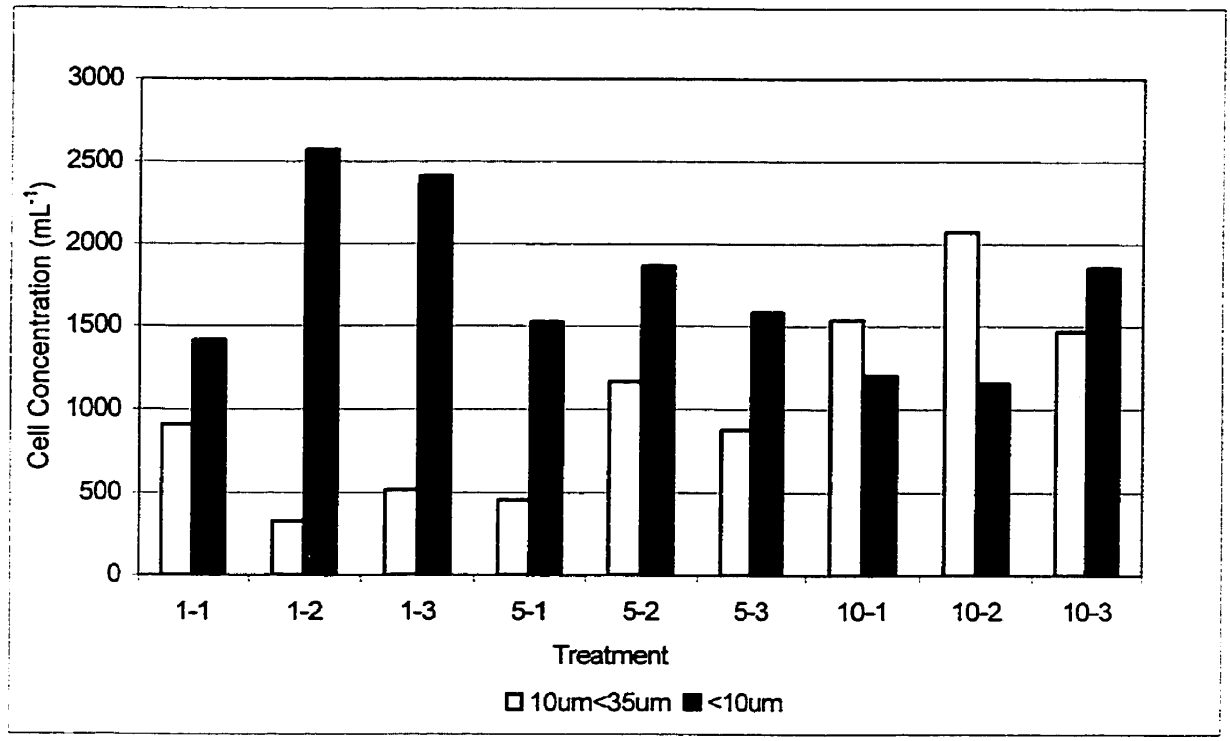

3000
2500
2000
1500
1000
500
0
Cell Concentration (mL-1)
1-1
1-2
1-3
5-1
5-2
5-3
10-1
10-2
10-3
Treatment
10um<35um
<10um

Figure 10. Relationship between number of *Giardia lamblia* cysts detected in the tissues of *Daphnia pulex* and the treatment population density. Solid line depicts the linear regression for the relationship (r^2=71.19).

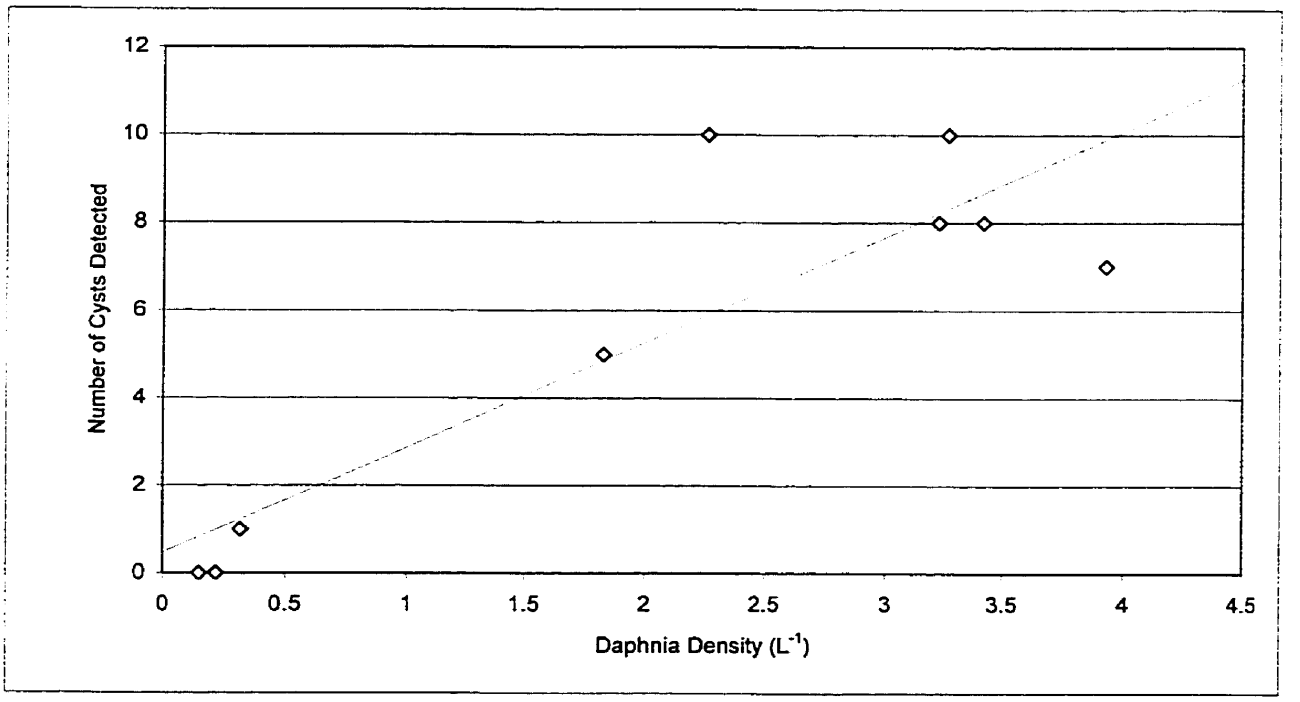
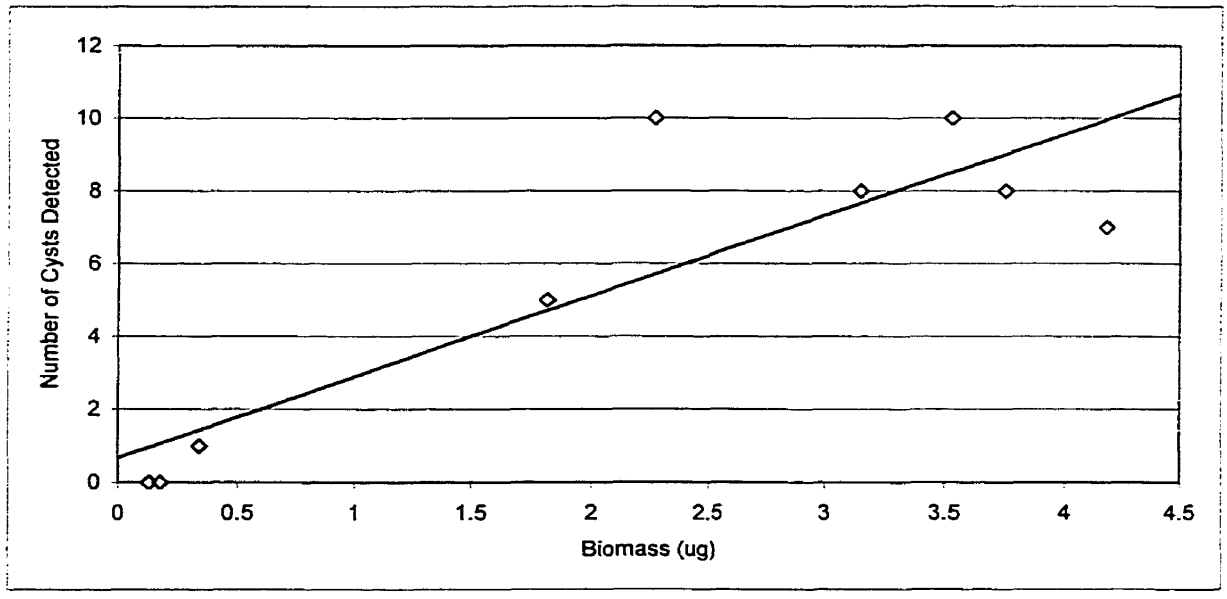
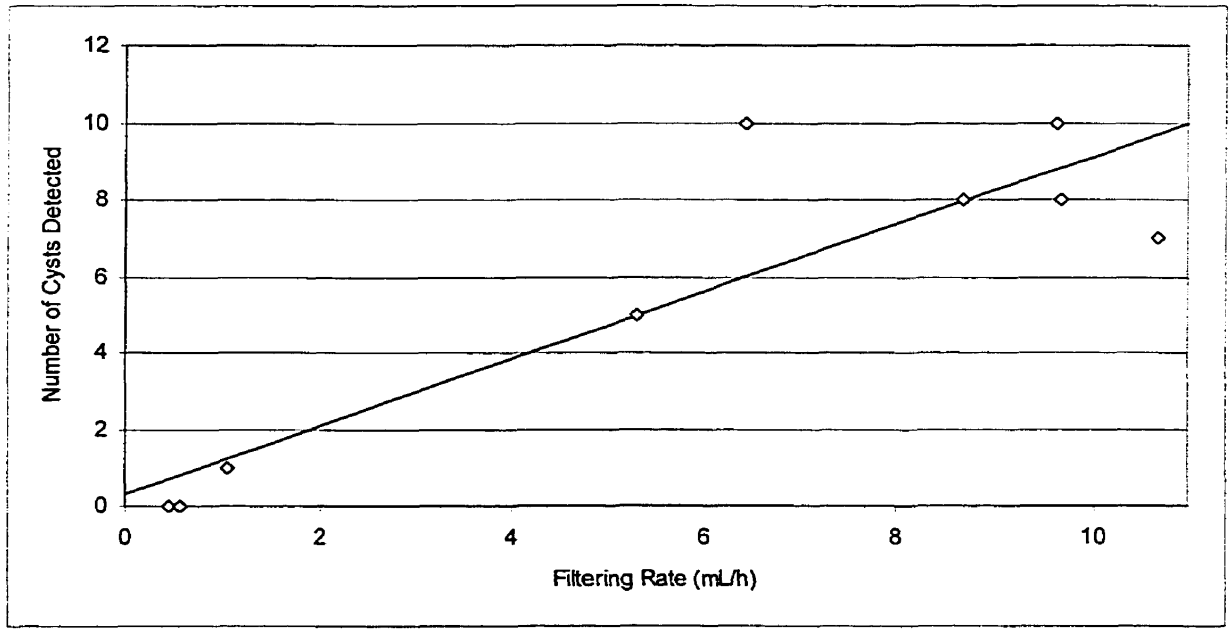

12
10
8
6
4
2
0
Number of Cysts Detected
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
Daphnia Density (L-1)

Figure 11. Relationship between number of *Giardia lamblia* cysts detected and: A) the estimated total biomass (μg) of *Daphnia* in each treatment, and B) the estimated total population filtering rate (mL/h) in each treatment. The solid line depicts the linear regression for each relationship (panel A. r^2=60.87, panel B. r^2=79.06).

A.


Number of Cysts Detected
12
10
8
6
4
2
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
Biomass (ug)

B.


Number of Cysts Detected
12
10
8
6
4
2
0
0
2
4
6
8
10
Filtering Rate (mL/h)

3.4. Discussion of Results

Data from Section 2 showed that individual *Daphnia* ingest waterborne *Giardia* cysts and *Cryptosporidium* oocysts as they do other natural algal food sources. This raised the question of whether populations of *Daphnia*, made up of variably sized individuals will ingest relatively small numbers of parasites when grazing collectively on natural phytoplankton populations and whether we can detect any parasites that may be ingested in community samples? Our experiments show that cysts from *Giardia lamblia* were found in the tissues of populations of individual *Daphnia*. This indicates two very important findings: 1) populations of *Daphnia* ingests cysts while grazing on natural phytoplankton under semi-natural conditions (i.e. in multi-species assemblages), and 2) that it is possible to detect waterborne *Giardia lamblia* cysts that have been ingested by populations of *Daphnia pulex* in multi-species communities.

There was a direct positive relationship between the population density and the number of cysts detected. Interestingly, the most significant relationship occurred when the total population filtering rate (mL/h) was calculated using the linear regression equations from Section 2 (see Figure 11B). This indicates a strong relationship between the volume filtered by a population and the number of cysts that can be detected. Our mesocosms represent very conservative tests for several reasons. First, our treatments contained on average 60% juvenile *Daphnia*, while natural populations such as those in the Glenmore Reservoir are primarily composed of adults [51]. It has been widely shown that there is a very clear and definable exponential relationship between *Daphnia* size and filtering rates; as size increases, so too does filtering rate (see Section 2.4). Secondly, with increased body size the gut passage times would also increase, meaning that each

Daphnia would contain more filtered volume. If the relationship between the number of cysts detected and *Daphnia* density is true for all populations, then higher densities of larger animals would mean an increase in the number of waterborne cysts that could be detected. Third, the largest *Daphnia* biomass/L in our treatments was only one tenth the biomass/L that is commonly present in the Glenmore Reservoir during the ice free seasons [30]. Given these considerations, we must assume that natural populations would enable more accurate detection than is indicated from our small study.

This has major implications for the detection of waterborne parasites in drinking water sources. Recall the problems outlined in Section 1 regarding the error associated with concentrating large volumes of water with nominal porosity filters. The fact that *Daphnia* populations do ingest waterborne parasites and that these can then be detected with some degree of certainty suggest a possible solution to this problem. Consider a drinking water reservoir containing a typical population of *Daphnia* with an adult (>2mm) density of $5L^{-1}$. It is reasonable to assume that each animal could filter 2mL/h and have a gut passage time given the environmental conditions of approximately 30min. This would mean a single *Daphnia* would contain 1mL of filtrate. Using standard conical zooplankton nets it would be very easy to sample as much as 10^5L of water, collecting a total of a $5x10^5$ *Daphnia*. That means that the filtrate from approximately 500L of water would be present in that sample of *Daphnia*!

Our experiments are very meager compared to these large numbers, but if we consider the same scenario for the largest treatment population in our experiments the *Daphnia* would contain the filtrate from a volume of 148.5mL. This value was obtained using a smaller gut passage time of 20min for the 95% juvenile content of the population

and a total population filtering rate of 445.55mL/h, as determined using the linear regression equations from Section 2. Given the cyst density in each treatments (1000cysts/L=1cyst/mL in each treatment) we would estimate that there would be approximately 148.5 cysts present in the digestive tracts of that population. Since we detected 7 cysts in that treatment this gives us a rough estimate of our percent recovery at 4.7%.

Coming back to a large natural reservoir environment with as few as 4 cysts/L, a zooplankton sample containing $5x10^5$ adults like the one described above would possibly contain 2,000 cysts and with only a 4.7% recovery, we would detect approximately 94 of them. While this seems like a tremendous loss of cysts, these numbers are significant if you consider that current parasite detection techniques commonly have false negative results [12]. The potential opportunity that this offers the water industry cannot be ignored. The recovery rates could easily be improved using more advanced techniques such as Immunomagnetic Separation and Flow Cytometry and it may even be possible to develop a method whereby the *Daphnia* are allowed to defecate out the parasites and they could be collected and analyzed using standard detection methods.

Using *Daphnia* populations to detect waterborne protozoa would have a further advantage of being a dynamic and adaptive method. Natural populations are continuously migrating up and down the water column during their daily feeding. A zooplankton sample would contain only the parasites present in the entire column of water at that exact spot in the body of water, making it possible to determine parasite distribution throughout a body of water quite easily. Also, because the populations are

continuously turning over, and that they have relatively short gut passage times, every new sample would contain a nearly instantaneous snapshot in time.

In conclusion this study has shown that it is possible to use populations of *Daphnia* to detect waterborne *Giardia lamblia* cysts. We found a clear positive relationship between the number of cysts that can be detected and the *Daphnia* population density. This means that natural populations may be used to detect very low numbers of parasites in any water source were *Daphnia* are present. The studies showing that clams, oysters and rotifers also ingest protozoan parasites may also offer alternatives in water systems where *Daphnia* are not present; the nature of these interactions should be more closely examined. Furthermore, mixed zooplankton samples may contain an entire host of herbivores such as copepods and other Cladocera that may ingest cysts or oocysts. This study lays the foundation for further research into using natural planktonic herbivores to detect waterborne *Giardia* and *Cryptosporidium*.

4. Summary

The objectives of this thesis as outlined in Section 1 were to test whether individual *Daphnia* would ingest waterborne *Giardia* cysts and *Cryptosporidium* oocysts and if so to determine whether the parasites were ingested at the same rates as natural algal food sources. The final objective was to test whether *Daphnia* populations could be used to detect the *Giardia* cysts in suspension in large volumes of water.

Section 2 clearly showed that both juvenile and adult *Daphnia* do respond to changes in *Cryptosporidium* and *Giardia* concentrations as they do a natural algal food source, indicating a generalist feeding behavior. While there were slight differences in feeding efficiencies between the parasites and algae. there appeared to be no active mechanism by which *Daphnia* can select against the parasites. In Section 3 we showed that individual *Daphnia*, foraging as part of a *Daphnia* population, ingest waterborne *Giardia* cysts suspended among natural phytoplankton communities. There was a strong direct positive relationship between the filtering rate of a population and the number of cysts that were detected. This has major implications for the water industry. Natural populations of *Daphnia* could provide a highly sensitive method for detecting protozoan parasites.

The questions raised by this study are many. Parasite prevalence and infectivity need to be examined to determine the impact from ingestion by herbivores. The fact that *Daphnia* defecate discrete fecal pellets that are far heavier than individual cysts or oocysts, the presence of these herbivores in a body of water may increase the rate at which these parasites reach the sediment and are removed from the water column. Also this study opens the door for examining other natural herbivores, planktonic or sedentary,

for use in detecting waterborne protozoa. Further work also needs to be done using populations of zooplankton from natural systems to determine just how sensitive this method can be.

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Appendix I. Algal Culture Techniques

I-I. Culture Media

Algal media was prepared in 1L volumetric flasks by combining the elements, metals, vitamins, and buffer solutions as detailed Table 5 into 1L of double distilled water. The pH was adjusted to 7.5 by adding 1N HCl or 1N NaHO as required. Approximately 50mL of this solution was then aliquoted into 100mL Erlenmeyer flasks, capped with foam and cloth, and autoclave sterilized for 20min in a liquid cycle. Media was stored in a dark cupboard at room temperature until inoculated.

I-II. Growth and Maintenance

Chlamydomonas reinhardtii Dang. (UTEX 90) were obtained from culture stocks maintained by Susan Watson. Flasks of media were inoculated aseptically with 5mL from an older culture of *Chlamydomonas*. The new flask was incubated at room temperature on benchtop under fluorescent lighting (Sylvania Gro-Lux^{TM} wide spectrum, 40Watt) with a 12h photoperiod. After approximately two weeks of growth, this was repeated with a fresh flask of media and the old culture discarded. Only cultures that were healthy in appearance were used for feeding experiments.

Table 5. Algal media; elements, metals, vitamins and buffers are combined in double distilled water at the noted concentrations and then adjusted to pH 7.5 before autoclave sterilizing.

Elements	**Media Concentration (mg/L)**
Ca	10.02
Mg	3.65
Na	3.45
Si	2.81
N	2.45
N	14.00
P	0.46
Metals	
EDTA	3.42
Fe	0.01
Zn	0.022
Co	0.003
Mn	0.009
Mo	0.0003
B	0.2
Cu	0.0022
Vitamins:	
Thiamin.HCl	0.0022
Biotin	0.0005
B12	0.0005
Buffer	
Hepes	25g/L

Appendix II. Protocol Development of Feeding Experiments

II-I. Optimization of Excitation/Emission Wavelengths

The problem with published excitation and emission wavelengths for a particular fluorophore is that these values are determined in standardized solutions and do not reflect the influence of different types of solutions and staining conditions on fluorescent behavior. The SPECTRAmax GEMINI™ microplate fluorometer is unique in that it allows optimization of a fluorescent solution for maximum excitation and emission intensities. Two aspects of fluorophore behavior are of particular concern during microplate fluorometry: the Stoke's shift, and the cutoff filter. The Stoke's shift is the distance between peak excitation and peak emission wavelengths for a fluorophore. It is important to maximize this difference by determining what wavelengths will provide the most resolution between the instruments excitation beam and the fluorophores emissions. The cutoff filters are important to reduce background fluorescence present in the sample or in the microplate, including solvents, water, and stray light [1]. The cutoff filter acts to increase the resolution between the excitation and emission wavelengths.

The Operator's Manual for the SPECTRAmax GEMINI details the method by which this optimization is achieved. Solutions of the fluorescent material were prepared. Cysts and oocyst were stained with PI as previously described and suspended at high concentrations (>100,000/mL) in double filtered reservoir water. *Chlamydomonas* cells were taken from cultures and suspended in double filtered reservoir water at similar concentrations as well. The exact concentration was not of importance, only that a significant amount of fluorescent material was present for analysis. 300µL of each

solution was plated into a single well of a Griener™ Microlon 96-well plate to be analyzed.

All three food sources were optimized using the same protocol, referred to as a "Spectral Scan." Using the published values for excitation and emission for the fluorophore the instrument was set to excite the sample over a range of wavelengths ranging from 50nm below literature value to 50nm above. Emissions were read at the tentative literature value for this step. The results were observed as a plot of fluorescence versus excitation wavelength and used to determine the optimal excitation wavelength at which emission intensity was highest. The manual suggests that if this value is less than 80nm from the emission wavelength that the optimal excitation wavelength should be chosen as the shortest wavelength that gives 90% maximal emission. This was not the case for any of the three food sources.

The next step involves performing the same type of Spectral Scan but with the excitation wavelength set at the optimum determined above and the emission wavelengths scanned over a 50nm range around the tentative literature value. The results were viewed as a plot of fluorescence versus emission wavelength and used to determine the optimal emission wavelength at which the intensity was highest. Finally, a cutoff filter was chosen that was near the optimized emission wavelength but at least 35nm greater than the optimal excitation wavelength. Values for optimized excitation and emission wavelengths and cutoff filters used are shown in Table 6.

Table 6. Optimal excitation and emission wavelengths (λ) for Propidium Iodide stained *Giardia* cysts and *Cryptosporidium* oocysts as well as live *Chlamydomonas* cells suspended in filtered water from the Glenmore Reservoir.

Optimized variable	PI stained *Giardia* cysts	Live *Chlamydomonas*	PI stained *Cryptosporidium* oocysts
Excitation wavelength (nm)	535	434	489
Emission wavelength (nm)	617	684	626
Cutoff filter wavelength (nm)	550	630	590

II-II. Development of Accurate Standard Curves

Before the microplate fluorometer could be of use in determining filtering rates of individual *Daphnia*, it was necessary to confirm that accurate concentrations of the various food types could be determined. A precise, and repeatable standard curve had to be developed that could be prepared for *Giardia*, *Chlamydomonas*, and *Cryptosporidium*. A protocol was tested first with *Giardia* and then applied and adapted for *Cryptosporidium* and *Chlamydomonas*.

A. *Giardia* – Standard curve suspensions were prepared using serial half dilutions in filtered reservoir water. Three 15mL polypropylene falcon tubes were filled with 5mL, and one tube with 10mL. With an Eppendorf, PI stained *Giardia* cysts were added to the 10mL volume to create a known maximum concentration level. The suspension was mixed thoroughly by continuously inverting the closed tube for several minutes. Serial half dilutions were performed using 5mL polypropylene graduated pipettes. An example of the resulting concentrations are: 10,000cysts/mL; 5,000cysts/mL; 2,500cysts/mL; 1,250cysts/mL. Maximum concentration was chosen for each individual experiment to ensure all data fell within the boundaries of the standard points. Suspensions were stored in the dark until aliquoted onto black Griener™ Microlon 96-well plates. Each standard concentration suspension was aliquoted into 16 wells, providing a high number of replicates for each point on the curve. Plates were read at the predetermined optimal Ex/Em wavelengths for PI stained *Giardia* cysts, after a 5sec premixing shake in the microplate fluorometer. The microplate expressed raw data as Relative Fluorescent Units (RFU) and standard curve data from the microplate reading are averaged and regressed linearly by the software. Sample data were automatically interpolated from

RFUs into a concentration value by the software, which used the linear regression equation from the standard curve.

In order to increase the accuracy of fluorometric analysis, as large a volume per well as possible was analyzed. While each well on a 96-well plate can hold up to 370µL, the premixing shake in the fluorometer caused spilling and loss of volume when they were filled to maximum. The loss of volume resulted in an increase in variation between replicate wells. An optimum single well volume of 300µL was established for 96-well microplates that completely eliminated occurrences of spillage.

The resulting standard curves had regression coefficients that were significant (>0.95), but variation between replicates contributed to large standard errors around the means of each point. Variation created by pipetting error was investigated as a possible cause. It was found that very small differences in well volume had a large influence on the fluorescent intensity. The accuracy of two different pipette brands were compared. Each pipette was used to aliquot a suspension of PI stained *Giardia* cysts into a 96-well plate. The scan results are displayed in Table 7 and show that a Gilson™ P1000 pipette had a coefficient of variance of 10.3%, and the Eppendorf Series 2000™ brand pipetman had a coefficient of variance of 4.8%. As a result of these findings, a fixed volume 300µL Eppendorf 2000 Series™ was chosen for use in all feeding experiments. This pipette was checked every two months and calibrated as needed.

Improper mixing as a source of variation was also investigated. During microscopic enumeration, cysts could be observed in clumps ranging in size from 2 to 15. The impact of four different methods of mixing was also examined. A well-mixed suspension of PI stained *Giardia* cysts was divided into four equal parts. One portion

was sonicated for two 20 second bursts on low. Two other portions were vortexed for 30 seconds, one with rotary motion and the other with linear motion. The last portion was simply inverted continuously for 20 seconds. The results are shown in Table 7. Coefficient of variations show little differences between treatments and ANOVA tests show no significant differences (P=0.998, n=11) between means. These results suggest that there is no benefit to sonication, or vortexing of samples, and to reduce the handling time of photosensitive PI stained cysts the experimental tubes were simply mixed by continuous inverting for 20 seconds. Figure 12 shows a typical standard concentration curve for PI stained *Giardia* cysts, after sources of variation were eliminated.

B. ***Chlamydomonas*** – Standard curves for *Chlamydomonas* were prepared using the same protocol that was outlined above for *Giardia*. Algal cells were collected aseptically from healthy culture flasks (see Appendix I). Healthy algal cells helped to insured that chlorophyll content was relatively equal between cells (personal communication with Dr. Susan Watson, University of Calgary). Even with healthy cultures, when suspensions of live cells were plated and scanned, variation in intensity was observed between replicate wells from the same suspension. This was attributed to changes in chlorophyll content or organelle shadowing of chloroplasts as a response to fluctuating light levels. To control for this effect, standard curve suspensions and feeding suspensions were acclimated together at experimental light levels for several hours prior to experiments and treated the same during experiments. Figure 13 shows a typical standard curve for live *Chlamydomonas* after fluctuating light levels were controlled for.

C. ***Cryptosporidium*** - Standard curves for *Cryptosporidium* were prepared using the same protocol that was outlined above for *Giardia*. Standard curves of PI stained oocysts

had significant regression coefficients (r^2>0.95) but the y-intercepts tended to be negative (see Figure 14). This could have been a result of fluorescent contamination of plate blanks or improper mixing during standard curve preparation. The exact reason could not be isolated and corrected and negative y-intercepts were observed for all standard curves prepared using PI stained *Cryptosporidium* oocysts. As a result, findings from feeding experiments on *Cryptosporidium* oocysts must be interpreted cautiously, possibly resulting in under-estimations of actual ingestion rates.

Table 7. Sources of variation of fluorescent intensity (RFU) between replicate wells of PI stained *Giardia lamblia* cysts in suspension.

Source of Variation	**n**	**Mean RFU**	**SD**	**CV%**
Volume				
Gilson	11	2.254	0.233	10.3
Eppendorf	11	2.225	0.107	4.8
Mixing				
Sonication	11	2.240	0.113	5.0
Vortex (linear)	11	2.232	0.122	5.5
Vortex (rotary)	11	2.236	0.108	4.8
Inverting	11	2.232	0.115	5.2

Figure 12. Screen capture from Softmax Pro software showing a typical standard curve for PI Stained *Giardia* cysts in filtered Reservoir water. Y-axis is the mean value of replicates in Relative Fluorescent Units (RFU), and X-axis is concentration (mL^{-1}).

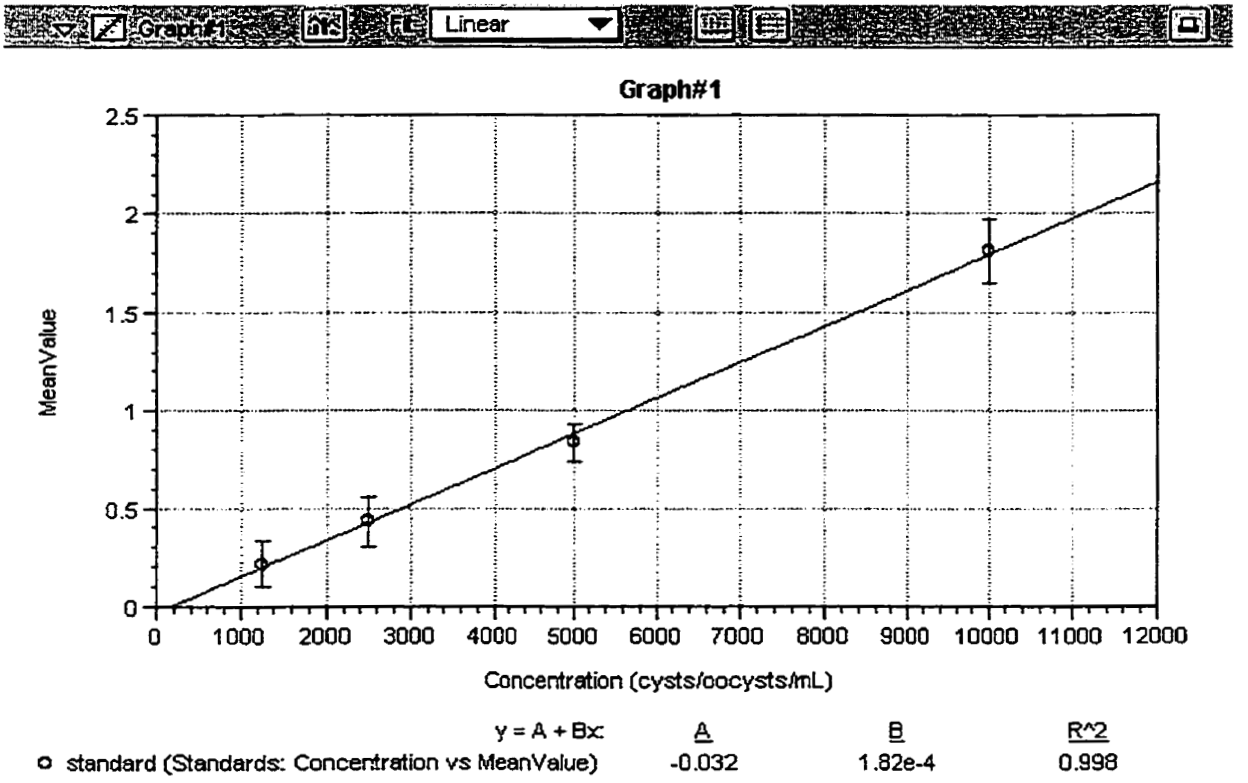

Graph#1
Linear
Graph#1
MeanValue
2.5
2
1.5
1
0.5
0
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
11000
12000
Concentration (cysts/oocysts/mL)
y = A + Bx:
A
B
R^2
standard (Standards: Concentration vs MeanValue)
-0.032
1.82e-4
0.998

Figure 13. Screen capture from Softmax Pro™ software showing a typical standard curve for live *Chlamydomonas* cells in filtered Reservoir water. Y-axis is the mean value of replicates in Relative Fluorescent Units (RFU), and X-axis is concentration (mL^{-1}).

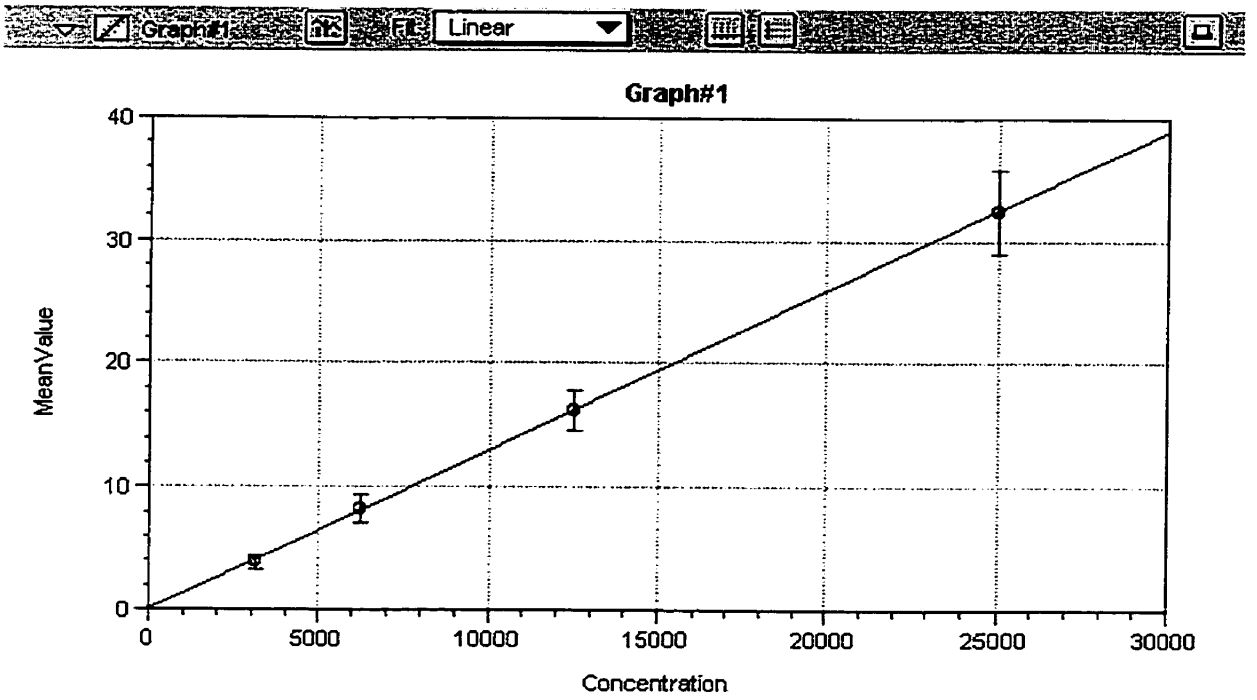
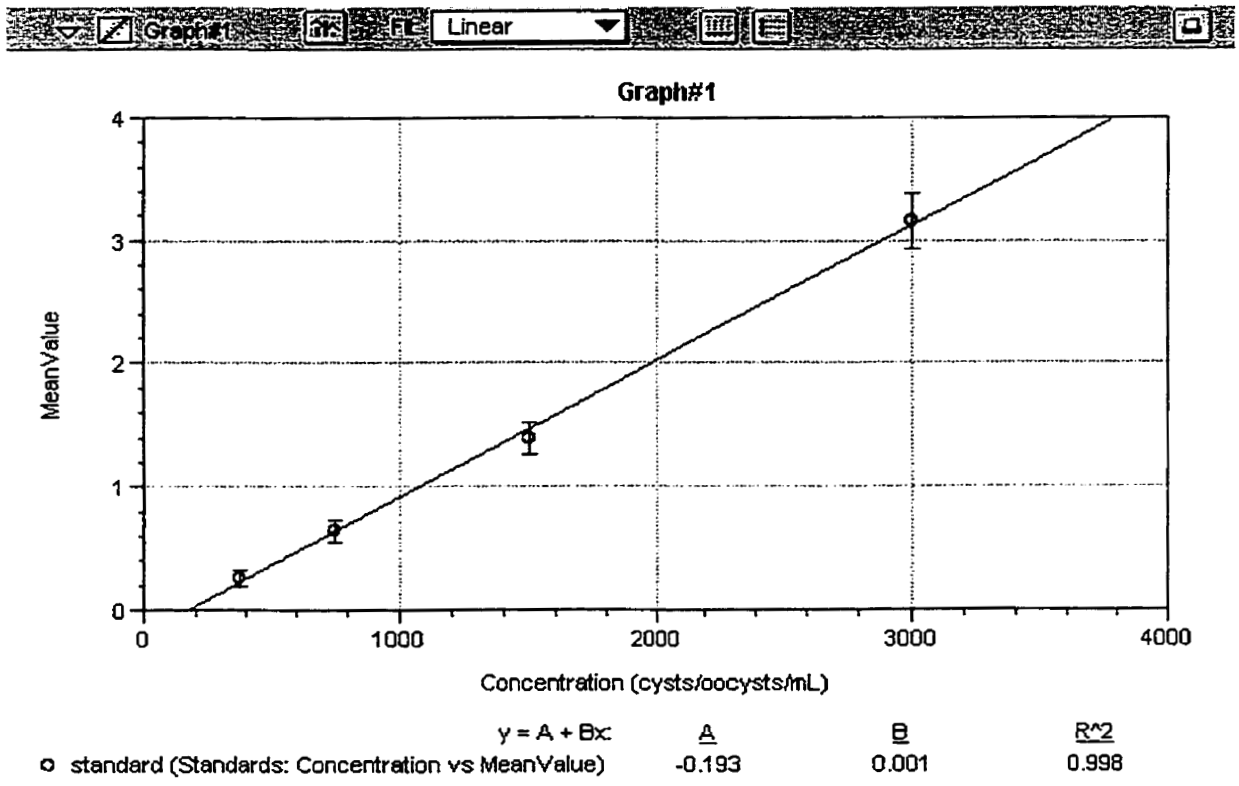

Graph#1
Fit:
Linear
Graph#1
MeanValue
Concentration
0
10
20
30
40
0
5000
10000
15000
20000
25000
30000
y = A + Bx:
A
B
R^2
Standards (Standards: Concentration vs MeanValue)
-0.025
0.001
1

Figure 14. Screen capture from Softmax Pro™ software showing a typical standard curve for PI stained *Cryptosporidium* oocysts in filtered Reservoir water. Y-axis is the mean value of replicates in Relative Fluorescent Units (RFU), and X-axis is concentration (mL^{-1}).


Graph#1
Linear
Graph#1
MeanValue
0
1
2
3
4
0
1000
2000
3000
4000
Concentration (cysts/oocysts/mL)
y = A + Bx
A
B
R^2
standard (Standards: Concentration vs MeanValue)
-0.193
0.001
0.998

II-III. Feeding Experiments

A. *Daphnia* Preparation – Great care was taken when isolating female *Daphnia* for feeding experiments. A portion of a population was captured in a small volume of water using a fine screen (30μm) and small petri dish. The screen was placed onto the petri dish and nearly all of the water was removed by aspirating from inside the petri dish, not inside the screen. It was essential to keep the *Daphnia* in enough water to keep them visibly filtering, while still being immobilized on the screen. The screen/petri dish was placed under a Nikon SMZ-10 dissecting microscope and visualized at 20X magnification.

To minimize the confounding influence of physiological factors on ingestion rates, selection of individual *Daphnia* had to be very consistent and accurate. Size classes were chosen at 1mm and 2mm lengths to examine both juvenile and adult feeding behavior. Size was determined at 20X magnification with an ocular micrometer to measure the distance from the head apex to the base of the tail spike. Animals of similar life stage were selected for by choosing only animals without eggs or ephipia. Only apparent healthy individuals with active thoracic appendages were chosen for feeding experiments.

Care was taken when collecting individual *Daphnia* from the screen's surface. Pasteur pipettes with shortened and widened tips were used to pick up selected individuals. The amount of pipette shortening depended on the size of individuals being isolated; small openings worked better for smaller *Daphnia*. To pick up a daphnid, a small volume of filtered reservoir was collected into the tip of the pipette and this was gently lowered until contact with the daphnid. Alternating gentle pressure was applied to

the bulb until the animal swam into the tip of the pipette. Isolated animals were then released into a 100mL beaker of filtered reservoir water with a low concentration of algae added as a food source. Swimming behavior was observed to ensure the animal was not injured during the isolation. Occasionally an individual would become trapped in the boundary layer of the collection beaker. The trapped animal was forced down into the water by gentle jets of water from a Pasteur pipette.

For a single feeding experiment 15 *Daphnia* would be used. To achieve this, at least 25 *Daphnia* were isolated to account for any accidental mortality or injury. Between the time of the isolation and the experiment itself the *Daphnia* were kept on the bench next to the experimental setup to ensure temperature and light fluctuations would not confound the results. The *Daphnia* were starved for a one-hour period prior to the experiment by carefully collecting and placing then into a 50mL polypropylene falcon tube with clean filtered reservoir water. During starvation, the tube was placed on the Tissue Culture Roller Drum (Model TC-7, NewBrunswick Scientific Co.) and turned at 1rpm to get the *Daphnia* used to the small currents created within the tubes.

B. Feeding Suspension Preparation – During the one-hour starvation period the Standard curve and the feeding suspensions were prepared. Feeding suspensions were prepared in a 100mL polypropylene Nalgene^{TM} bottle containing 65mL of filtered reservoir water. The concentration of the inoculum was determined with hemacytometer and the volume to be added to the feeding suspension to attain the desired concentration was determined; that volume was removed from the 65mL using an Eppendorf. Using the same setting on the Eppendorf the inoculum was added to the bottle, the lid placed on, and the suspension mixed by continuous inverting for 30 seconds. Four 15mL

polypropylene Falcon™ tubes were chosen for feeding containers and filled to the top with the feeding suspension. All suspensions, including the standard curve suspensions, were placed on the plankton wheel to maintain homogeneous distribution. Tubes containing PI stained cysts and oocysts were wrapped in aluminum foil to prevent fluorescent fading; suspensions of *Chlamydomonas* were not protected from light.

C. Experiment Setup – After the one-hour starvation period had elapsed, 15 *Daphnia* were isolated into three groups of five individuals. *Daphnia* that were actively swimming were chosen and placed into 1mL of filtered reservoir water. To begin the experiment five individuals were added to a feeding tube, a timer was started, and the tube placed in a darkened outer tube on the plankton wheel. This was repeated again at 5 and 10 minutes for the second and third groups of five *Daphnia* respectively. For a control, the fourth and final tube had 200μL of filtered reservoir water added to reflect the volume added to the other three tubes but without the *Daphnia*, and was placed on the plankton wheel with the others. Because of the sensitivity of PI to light, feeding experiments were conducted in darkened outer tubes. While this may not have been ideal for live *Chlamydomonas* cells, the confounding effect of diel feeding behavior would have been less favorable. For the live algal experiments, fluctuating light conditions were controlled for by placing the standard curve suspensions in darkened outer tubes during the experiment.

After twenty minutes of elapsed time the contents of the first feeding tube were passed through a wide screen to separate *Daphnia* from the suspension. The feeding suspension was collected in a clean 15mL polypropylene Falcon™ tube and the *Daphnia* were disposed of as biohazardous waste. This was repeated at 25, 30 and 35 minutes for

the second, third and control tubes respectively. A 20min feeding period was chosen to ensure that filtering rates were not underestimated by consumed food that was returned to suspension in feces.

D. Sampling – Feeding suspensions were mixed by continuous inverting for 20 seconds before being aliquoted onto the microplates. To increase accuracy, two plates were used for a total of 16 replicate wells per treatment, including standard suspensions. Blanks of filtered reservoir water were included on both plates. Microplates containing PI stained cysts and oocysts were covered to protect from bleaching and read within 5 minutes of aliquoting.

II-IV. Validation of Protocol

To validate the protocol, an experiment was performed with *Chlamydomonas* and 2mm *Daphnia*. After feeding suspensions were aliquoted onto microplates for fluorometric analysis the remaining (~10mL) were preserved with a drop of Lugol's Iodine and set aside for counting. Settling chambers with a 5mL capacity were filled with a preserved sample until the meniscus was slightly higher than the walls and then a coverslip placed over, forcing out excess fluid. These were settled in humid petri dishes for 24 hours and then observed at 400X magnification. Random fields were scanned and cells of *Chlamydomonas* were tallied. The cell concentration of each feeding suspension was determined using the Inverted Microscope method [48]. From these concentrations filtering rates were calculated as described in Appendix II-III.E. Results from both analyses are shown in Table 8. The means were tested with t-test and found to be not significantly different.

Table 8. Comparison of fluorometric analysis and cell count determination of filtering rates for 2mm individuals at 21,043 cells/mL.

Method:	N	Mean Filtering Rate (mL/h/individual)	Std. Dev.	SEM
Fluorometric	3	1.969	0.303	0.175
Cell counts	3	1.993	0.128	0.0741