Improvement of the Microscopic Particulate Analysis Method

Peter Wallis and Matthew Henschke
Medicine Hat College, 299 College Dr. SE, Medicine Hat, AB T1A 3Y6

ABSTRACT

Method 1623 (United States Environmental Protection Agency), a protocol for the recovery of *Giardia* cysts and *Cryptosporidium* oocysts from water samples, was adapted for use in detecting surface water organisms from well water samples. After a number of initial trials the resulting procedure was tested for the recovery of *Giardia*, *Cryptosporidium* and two species of algae by spiking Filta-Max filters that had been pre-loaded by filtering 50 L of well water with cysts, oocysts, *Euglena gracilis* and *Sphaerocystis* sp. The observed recovery efficiencies (n=5) were; *Giardia* cysts 37% ± 26, *Cryptosporidium* oocysts 89% ± 17, *Euglena* cells 36% ± 30 and *Sphaerocystis* cells 2% ± 41. Comparable results from the established Microscopic Particulate Analysis were; *Giardia* cysts 7% ± 31, *Cryptosporidium* oocysts 1% ± 69, *Euglena* cells 4% ± 93 and *Sphaerocystis* cells 0%. The modified Method 1623 improved the recoveries of all the organisms tested but was still very inefficient for very small algae cells such as *Sphaerocystis* sp. Field sampling time was greatly reduced because of the much lower volume of water that needs to be filtered for Method 1623.

INTRODUCTION

Recent changes to legislation and regulations in Nova Scotia, Saskatchewan, and Alberta inspired by the Walkerton tragedy require that groundwater supplies be examined to determine if they are Groundwater Under the Direct Influence of Surface Water (GWUDI) and therefore subject to regulations that govern surface water drinking supplies. Similar action is proposed in British Columbia. Wells may be ruled non-GWUDI in BC if they meet criteria for installation, construction, hydrogeology and stable water quality as defined in a recent guidance document (Kohut 2007) drafted by Al Kohut of Hy-Geo Consulting. Wells that are judged to be potentially GWUDI by hydrogeological methods may be further investigated using the Microscopic Particulate Analysis (MPA) as specified by the United States Environmental Protection Agency (USEPA 1992) also known as the MPA Consensus Method. This test requires that approximately 3800 L of well water be filtered through a 1 μm wound cartridge and processed to recover particulate matter that is then examined microscopically. If the water contains significant numbers of aquatic organisms or if *Giardia* cysts or *Cryptosporidium* oocysts are detected, the well water is GWUDI and must be treated as surface water. A minimum of two samples must be collected to make a GWUDI judgment by MPA and it is recommended that one sample be collected in the spring after runoff and one in the fall after a prolonged dry period. Although this method has been in use since 1992 in the United States, it has only recently been employed to any great extent in Canada. The protocol was based on the methodology for detecting *Giardia* cysts and *Cryptosporidium* oocysts that dates back to 1979 and has never been updated. Its major drawbacks include:

- large sample volume and lengthy field time (~8h/sample),
- inefficient filter elution in the laboratory,
- poor separation by density gradient centrifugation of aquatic organisms from background material. Some samples are heavily contaminated with precipitated iron and/or manganese

hydroxide, exacerbated by the large sampling volume, which makes recovery of target cells very difficult because density gradient separation is required, and
- very low recovery efficiencies of algae, cysts and oocysts (10% or less).

In the past 10 years, the methodology for detection of *Giardia* and *Cryptosporidium* has been greatly improved by the utilization of better filters and eluting equipment and by the introduction of immunomagnetic separation of parasites from the background debris. The new Method 1623 uses a smaller filter which can be disassembled in the laboratory instead of just cut apart, and eluted much more efficiently than the old string-wound filters (USEPA 2001). The minimum sample volume required for a *Giardia/Cryptosporidium* analysis is 10 L but 100 L is more common for waters of low turbidity, a considerably smaller volume of water which reduces sampling time. The filter and filter housing are also designed to be used at higher operating pressures (up to 90 psi or 620 kPa) more typical of pumped well water which serves to further reduce field sampling time, potentially to about 30 minutes/sample. Any practical filtration/elution procedure could theoretically be used for GWUDI determination but it seems most sensible to employ an existing method that has a proven track record, at least for the recovery of *Giardia* cysts and *Cryptosporidium* oocysts. The laboratory charges for an analysis based on Method 1623 are higher but field expenses could be reduced, making the two methods comparable for cost.

METHODOLOGY

Algal Strain: Two isolates of algae were chosen for experiments based on their size and ease of culture. *Euglena* sp. were isolated from Kapo Cr. (SK) on April 14, 2008 and *Sphaerocystis* sp. was isolated from an Assiniboine River sample taken on October 23, 2007. *Euglena* is a photosynthetic protozoan and approximately the same size as *Giardia* cysts. *Sphaerocystis* is a green alga, about the same size as *Cryptosporidium* oocysts and grows in a mucilage sheath. Both were cultured in Tris-Acetate Phosphate (TAP) medium, supplemented with 10% peptone. Cultures were either diluted with deionized water or centrifuge concentrated (500 X g, 10 min) to produce stock cultures used for counting and spiking.

Cysts and Oocysts: *Giardia* cysts were prepared by infecting Mongolian gerbils (*Meriones unguiculatus*) with WB cysts, collecting faecal material 4-6 d after infection and recovering cysts by discontinuous gradient centrifugation over 1.0 M sucrose. Cysts were further purified by allowing the mixture to sediment through a continuous gradient of sucrose varying from s.g. 1.01 to s.g. 1.03 according to the method of Sauch (1984). *Cryptosporidium* oocysts were prepared by infecting C57/BL6 mice with the AZ1 strain of *C. parvum* (type II) and purifying them as described above. Cysts and oocysts were counted by haemocytometer (10 counts for each stock solution) and aliquoted into matrix material.

Matrix: Groundwater from Western Canada typically contains significant amounts of clay, precipitated iron and inert inorganic crystals, typically silica. Clay and silica are usually native materials but may also have been introduced during the drilling process from drilling mud and casing packing material. Iron is ubiquitous. The nature and quantity of the background material are crucial to the recovery of spiked organisms so a representative mixture was prepared by combining the left-over sediments from MPA samples received routinely by our laboratory that have proved to be negative for the test organisms after density gradient centrifugation and microscopic examination. The volume of sediment was adjusted to produce 1-2 mL of pellet when a 50 mL volume was centrifuge concentrated.

Test Filter Spiking: Matrix and target cells were prepared before each trial and stored in 50 mL quantities. Filters were mounted in the appropriate housing as shown in Figure 1 and filtered city water was used to flush the system. After an initial flush of 10 L, the spike was added to the loading chamber, flow was restored and the spike was pushed into the filter housing. For Filta-Max testing, the spike was diverted into the lower connection point. In both cases, an additional 40 L of filtered city water was passed through the test filter to ensure that all particulates were flushed through the system. The filter housing was then removed, washed, and a new filter put in place. Filters were removed, bagged and identified, and then refrigerated at 4 °C.

Filter Analysis: Filters were analyzed according to the MPA Consensus Method for string-wound filters and Method 1623 for Filta-Max cartridges. The Consensus Method was modified by arbitrarily splitting the pellet recovered from filter elution and density gradient centrifugation according to the ratio 75:25 with the smaller fraction used for immunofluorescent staining of cysts and oocysts. Method 1623 was modified by retaining the pellet from the immunomagnetic separation step and examining it directly by brightfield and phase contrast microscopy without any further treatment. If the pellet was too large to be examined completely, it was subsampled.

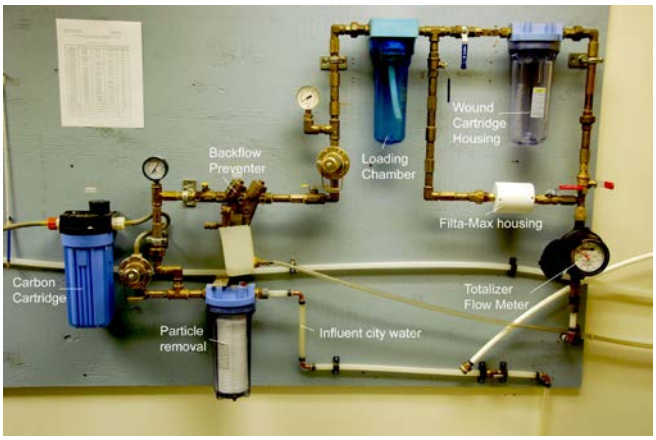

Figure 1. Apparatus for spiking filters in the laboratory.

Field Samples: A series of eight sequential samples was obtained from a production well on a First Nation site near Zaballos, BC, on Vancouver Island on July 17, 2008 by filtering 50-80 L of well water through a Filta-Max Filter using an FW12 sampler (Hyperion Research Ltd.). These samples were taken by Piteau Associates Engineering Ltd. in the course of regular MPA sampling and we are indebted to Chris Homes and Kathy Tixier for their generous assistance. Concurrent sampling on the same date gave negative results from this site for surface water organisms and parasites. The Filta-Max cartridges were mounted in the laboratory apparatus described above and spiked with known numbers of parasites and algal cells for recovery determination.

RESULTS

Initial Trials: Four initial laboratory trials yielded very low recoveries for both algae and parasites. The problems encountered were (*i*) difficulty in finding and counting very small algae cells (*Sphaerocystis*), (*ii*) high background iron concentration, (*iii*) quenching of the immunostaining reaction and (*iv*) low recovery caused by the deteriorating nature of the matrix spike preparation. These difficulties were largely overcome and a working protocol based on Method 1623 gradually evolved which is attached to this document as Appendix 1.

Field Samples: Three of the field sample spikes were expended on developing the working protocol. The recovery efficiencies from the remaining five samples are reported in Table 1.

	Giardia Cysts		*Cryptosporidium* oocysts		*Euglena* sp. cells		*Sphaerocystis* sp. cells	
	Spike	Recovery %	Spike	Recovery %	Spike	Recovery %	Spike	Recovery %
	1000	38.6	10000	86.9	1000	28.0	250000	1.5
	8000	47.0	8000	94.0	8000	48.1	200000	1.8
	7000	44.3	7000	94.9	6000	35.0	150000	1.4
	6000	33.0	6000	105.5	4000	23.3	100000	3.5
	5000	23.2	5000	64.6	2000	46.7	50000	2.3
Mean (%)		37.2		89.2		36.2		2.1
Std. Dev.		9.5		15.3		11.0		0.9
RSD (%)		25.6		17.1		30.4		40.9

Table 1. Recoveries of *Giardia* cysts, *Cryptosporidium* oocysts, *Euglena* sp. and *Sphaerocystis* cells from spiked Filta-Max cartridges pre-loaded with groundwater matrix. RSD = Residual Standard Deviation.

DISCUSSION

The development of the protocol in Appendix 1 was based upon trial and error and many details are omitted from this report. Many of the difficulties were resolved when the use of the artificial matrix prepared from MPA samples was abandoned. This mixture was a noxious combination of many different organic and inorganic compounds which began to ferment and was no longer representative of groundwater conditions. It was very useful for developing the protocol, however, because it presented so many difficulties. The groundwater matrix recovered from the field samples was easy to work with by comparison.

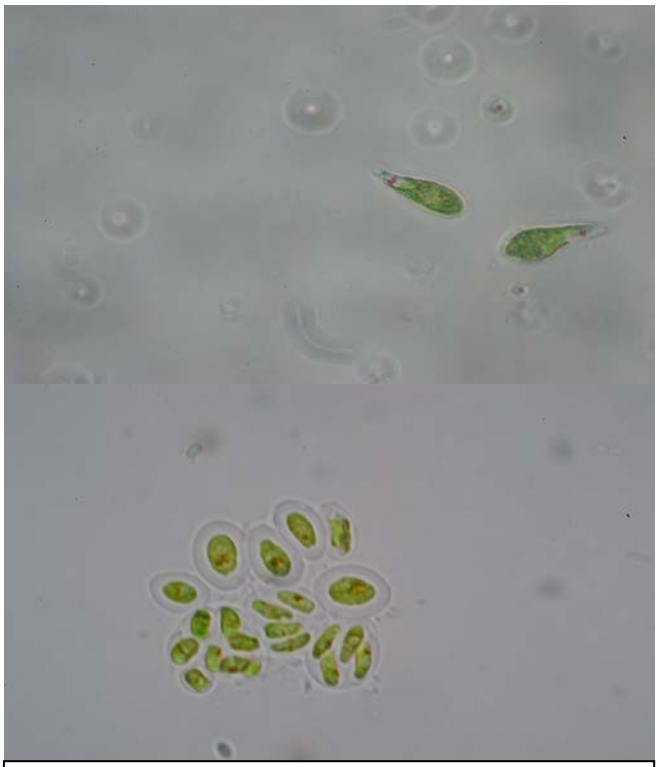

Figure 2. *Euglena* (top) and *Sphaerocystis* cells (not to scale).

The recovery of cells reported in Table 1 declined with the number spiked but was clearly best for *Cryptosporidium* oocysts with *Giardia* cysts and *Euglena* cells being roughly equal. In comparison, previous work in our laboratory demonstrated that the recovery efficiency for these organisms using the Consensus Method (USEPA,1992) was 6.5 ± 30.5% for *Giardia* cysts, 0.5 ± 69.1% for *Cryptosporidium* oocysts and 4.2 ± 92.8% for *Euglena* cells. The recovery efficiencies in Table 1 are a significant improvement for all but *Sphaerocystis* which is a very small and fragile cell which is difficult to recover and detect. No *Sphaerocystis* cells were recovered using the Consensus Method in early trials. The recovery efficiencies for *Giardia* cysts and *Cryptosporidium* oocysts are very comparable to those commonly reported for surface water samples using Method 1623 where the same pattern of better recovery for *Cryptosporidium* oocysts is frequently seen.

The advantage offered by this protocol in the field was clearly shown when Piteau Associates had no difficulty in taking eight Filta-Max samples with the new protocol in the same time that it took to collect one sample by the Consensus Method. This represents a considerable saving in field expenses.

One obvious drawback to these experiments is the relatively high numbers of cells that were used to spike filters. Algae cells are frequently abundant in surface waters and it would not be unusual to encounter concentrations in the thousands of cells per liter. *Giardia* cysts and *Cryptosporidium* oocysts are much rarer and so further work will be required to determine the lower limits of detection.

The next step in the development of this protocol is to test it with real field samples taken at a site that is known to be GWUDI positive (based on the Consensus Method). We hope to continue this work in 2009 so this project is not complete.

LITERATURE CITED

Kohut, A. Guidance document for determining ground water at risk of containing pathogens and ground water under direct influence of surface water. Draft December 14, 2007. BC Ministry of the Environment.

Sauch, J.A., 1984. Purification of *Giardia muris* cysts by velocity sedimentation. *Appl. Environ. Microbiol*. 48(2):484-5.

United States Environmental Protection Agency. 1992. Consensus method for determining groundwaters under the direct influence of surface water using microscopic particulate analysis. EPA 910/9-92-029.

United States Environmental Protection Agency. 2001. Method 1623: *Cryptosporidium* and *Giardia* in water by filtration/IMS/FA. EPA-821-R-01-025.

APPENDIX 1: Microscopic Particulate Analysis (MPA) for use with Method 1623

Prepared By	Date Adopted	Supersedes Procedure#
Peter Wallis	October 25, 2008	New Protocol

Review Date	Revision Date	Name

Distributed To	# of Copies	Distributed To	# of Copies
Main Lab	1		

This SOP has been authorized for use in the operation of Hyperion Research Ltd.

_____

Quynh Nguyen, B.Sc.
Lab Manager

Purpose: Combination test - Immunomagnetic separation of *Cryptosporidium* and *Giardia* from groundwater sample concentrates coupled with detection of surface water organisms

Equipment, Supplies & Media/Reagents:

Equipment	Supplies	Media/Reagents
Vortex mixer	Dynal L10 flat sided tubes	Dynal CG combo kit (see package insert for contents)
Micro pipettes (10 to 1000 µl)	Slides 3-well frosted, plain	0.05 N HCl
Rotating mixer	Pipette tips	PBST - Phosphate buffer saline with 0.01% Tween
Magnetic particle concentrators (Aureon and Dynal microcentrifuge)	Pipette aid	Reagent water – DI
Timer	Pasteur pipettes	100mM acid
Microcentrifuge	Pipette bulb or aid	Hyperion Trial buffer
	1.5 ml siliconized microcentrifuge tube	
	50 ml centrifuge tube	

Specimen: Water sample concentrates prepared by filtration and elution according to US EPA Method 1623 (See concentration elution SOP). Approximate 5 ml volume of PBST containing the pellet.

Procedure: Follow the steps in the table below

Step	Action
	Reagent preparation
1.	Dynal CG combo kit and sample concentrate allowed to equilibrate to room temperature (approximately 1 hour).
2.	Sample must be allowed to equilibrate to room temperature (approximately 1 hour).
3.	Prepare a 1X solution of PBST. For every 1ml of 1X PBST solution required, take 100 µl of 10X PBST concentrate and add 900 µl of DI.
4.	Retain 1X solution of PBST for use later in the procedure.
	Sample preparation
5.	Record the packed pellet volume of the sample. Refer to Volume Standards: Preparation and Use SOP. A maximum of 1 ml of packed pellet volume will be analyzed per sample. If sample contains more than 1 ml of pellet, refer to Dividing Pellets SOP.
6.	**Resuspend** sample concentrate pellet in 5 mL of DI in the original sample tube with Pasteur pipette and transfer to a Leighton tube.
7.	Rinse 50 ml tube out with 2X - 1.5 ml of DI, then transfer the DI to the Leighton tube. If iron colouration or floc is visible, place Leighton tube in Aureon magnet and rock through 180° for 1 minute. Withdraw suspension by pipette and transfer to a fresh Leighton tube. Discard magnetic particles removed from the sample unless needed for archive.

8.	Add 1 mL of 10X Hyperion Trial buffer concentrate. The colour should be orange-red. If it is orange, add 0.05N NaOH dropwise until the first traces of red show up. If too red, add 0.05N HCl dropwise until a red-orange colour is achieved. If in doubt, transfer mixture to a 15 mL disposable tube and check pH. The pH should be 7.0-7.2, adjust if necessary.
	Capture
9.	Vortex Dynal CG *Cryptosporidium* beads vial for 10 seconds. Add 95 µl of beads to Leighton 10 tube containing sample and buffers.
10	Vortex Dynal CG *Giardia* beads vial for 10 seconds. Add 95 µl of beads to Leighton tube containing sample, buffers and *Cryptosporidium* beads.
11	Rotate at 20 RPM for 120 minutes at room temperature.
12	Secure Leighton tube in Aureon magnet with flat side (capture window) against magnet, gently rock magnet with tube back and forth through 180°. Keeping beads covered with liquid at all times. Continue for 2 minutes with approximately 1 tilt per second.
13	Return to upright positions (cap at top) and let sit without disturbing for 10 min. Remove cap and withdraw all the supernatant from the tube by using a Pasteur pipette. Flat side of the tube facing upward so as not to wash beads off magnet while pouring supernatant. Transfer suspension to a 15 mL screw cap centrifuge tube and concentrate at 500 X g for 10 min. Record volume of pellet on MPA report sheet and store in frig for microscopic analysis. Add all saved wash solutions from previous steps to the pellet.
14	ONLY IF DIRTY! Remove tube from magnet and add 10 ml of PBST, rock and roll tube 3 times to suspend beads (DO NOT VORTEX).
15	Secure tube in Aureon magnet with flat side (capture window) against magnet, gently rock magnet with tube back and forth through 180°. Keeping beads covered with liquid at all times. Continue for 2 minutes with approximately 1 tilt per second.
16	Return to upright positions (cap at top). Remove cap and withdraw all the supernatant from the tube using the same Pasteur pipette employed in 13. Above and save. Flat side of the tube facing upward so as not to wash beads off magnet while withdrawing supernatant by pipette.
17	Hold magnet and Leighton tube at a 45° with the beads facing upward. Remove and withdraw all excess liquid and foam from the bottom of the tube with a Pasteur pipette. Save excess liquid to be added to residual after bead extraction. Be careful not to disturb beads attached to the magnet.
18	Remove Leighton tube from magnet and suspend beads by pipetting 500 µl of PBST solution down flat side of tube. Very gently suspend all material in tube within the flat area of the Leighton tube. DO NOT VORTEX. Transfer every drop to labeled microcentrifuge tube with glass Pasteur pipette.
19	Pipette an additional 500 µl of PBST down flat side of tube. Transfer every drop to microcentrifuge tube with glass Pasteur pipette
20	If processing multiple IMS samples, process all samples to this point (Repeat steps 10 through 17 for each sample).
21	With magnet removed from unit, place microcentrifuge tubes into Dynal unit. Insert magnet and gently rock/roll through 90° motion. Continue for 2 minutes with approximately 1 rock/roll per second.

22	Without removing the tube from the magnet, let sit for 1 minute, then gently aspirate the supernatant from the tube and cap with a Pasteur pipette and save. If more than 1 sample is being processed conduct 3 rock/roll actions before removing supernatant from each tube. Take extreme care not to disturb the material attached to the wall of the tube, but ensure that you remove particulate material and liquid off bottom tube and from inside the snap cap.
	Acid dissociation
23	Add 100 µl of 100mM acid to the microcentrifuge tube and vortex thoroughly for 20 seconds.
24	Allow samples to sit undisturbed for 10 minutes.
25	Vortex each tube thoroughly for 20 seconds.
26	With magnetic strip in Dynal unit, place microcentrifuge tubes in the unit. Rock carefully so the acid moves only to the top of the beads (this should help with bead carryover).
27	If the beads seems to be smeared on the side of the tube, slowly rotate microcentrifuge tube in the magnetic strip to collect all of the beads.
28	Allow to stand undisturbed for 20 seconds.
29	Transfer all liquid from microcentrifuge tube to the 1st well of the slide. Take care not to disturb beads at back-wall of the tube.
	2ND Acid dissociation
30	Repeat steps 21 through 28.
	Drying and fixing of slide preparations
31	Dry slides at 37°C for approximately 60-75 minutes in the incubator until completely dry or leave in incubator overnight for staining the next day. Label slide tray to indicate they are not stained.
32	Both a positive and a negative control must be prepared with each sample run. See Positive and Negative Control Protocol.
33	Fix slide immediately upon removal from the incubator with 50 µL of methanol (this results in better staining).
34	Slides should ideally be stained the next day (See SOP for Staining procedure for *Cryptosporidium* and *Giardia*)
	Reading and Interpretation of *Giardia/Crypto* on the Microscope
	Preparing a Slide to View
1.	Place 1 drop of immersion oil 518 C on each well to be viewed.
2.	Angle appropriate cover slip and place the cover slip onto the slide. Try not to cause bubbles to form. This is sometimes unavoidable.
3.	Place 1 drop of immersion oil 518 C on the slide.
4.	Place slide on the stage under the 40x/1,30 oil Plan-NEOFLUAR ocular lens.
5.	Adjust fine focus only. Scan under 40X and confirm cysts and/or oocysts under 100X.
6.	Course focus (stage) is NOT adjusted.

	Scanning Technique
1.	As you are looking into the scope, start in the middle of the well, move left to the side of the well, then move down to your start position.
2.	Read in an up and down pattern from upper left to lower right.
3.	Make sure the field of view is slightly overlapping the pervious one.
4.	Note that looking into the microscope is backwards from looking at the slide itself. i.e. left is right and up is down.
5.	Refer to Figure 1.0 for a visual representation of the field of view
	Examination of slide (Refer to Section 15.0 in US. EPA Method 1623 June 2005)
1.	Analyst must characterize a minimum of three Cryptosporidium oocysts and three Giardia cysts on the positive staining control slide before examining field sample slides.
2.	**Cy3 Examination of *Cryptosporidium* oocysts -** Use epifluorescence to scan the entire well for orange-red fluorescence of oocyst and cyst shapes. When brilliant fluorescing ovoid or spherical objects 4 to 6 µm in diameter are observed with brightly highlighted edges, increase magnification to 1000X and note size, shape, internal structures, etc.
3.	**DAPI Examination of *Cryptosporidium* oocysts** - Using the UV filter block for DAPI at 1000X, the object will exhibit one of the following characteristics: (a) Light blue internal staining (no distinct nuclei) with a orange-red rim. (b) Intense blue internal staining. (c) Up to four distinct, sky-blue nuclei. Look for atypical DAPI fluorescence, e.g., more than four stained nuclei, size of stained nuclei, and wall structure and color. Record oocysts in category (a) as DAPI-negative; record oocysts in categories (b) and (c) as DAPI-positive.
4.	**Hoffman Modulation Examination of *Cryptosporidium* oocysts** - Using the Hoffman 1000X objective, look for external or internal morphological characteristics atypical of Cryptosporidium oocysts (e.g., spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.) If atypical structures are not observed, then categorize each orange-red fluorescing object as: (a) An empty *Cryptosporidium* oocyst. (b) A *Cryptosporidium* oocyst with amorphous structure. (c) A *Cryptosporidium* oocyst with internal structure (one to four sporozoites/oocyst).
5.	**Cy3 Examination of *Giardia* cysts -** Use epifluorescence to scan the entire well for orange-red fluorescence of oocyst and cyst shapes. When brilliant fluorescing ovoid objects (8 - 18 µm long by 5 - 15 µm wide) are observed with brightly highlighted edges, increase magnification to 1000X and note size, shape, internal structures, etc.

6.	**DAPI Examination of *Giardia* cysts** - Using the UV filter block for DAPI at 1000X, the object will exhibit one of the following characteristics: (a) Light blue internal staining (no distinct nuclei) with a orange-red rim. (b) Intense blue internal staining. (c) Two to four sky-blue nuclei. Look for atypical DAPI fluorescence, e.g., more than four stained nuclei, size of stained nuclei, and wall structure and color. Record oocysts in category (a) as DAPI-negative; record oocysts in categories (b) and (c) as DAPI-positive.
7.	**Hoffman Modulation Examination of *Giardia* cysts** - Using the Hoffman 1000X objective, look for external or internal morphological characteristics atypical of *Giardia* cysts (e.g., spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.) If atypical structures are not observed, then categorize each orange-red fluorescing object as: (a) An empty *Giardia* cyst. (b) A *Giardia* cyst with amorphous structure (c) A Giardia cyst with one type of internal structure (nuclei, median body, or axonemes) or (d) A Giardia cyst with more than one type of internal structure.
8.	Record the date and time that sample examination was completed on the examination form.
9.	Report *Cryptosporidium* and *Giardia* concentrations as oocysts/L and cysts/L.
	Disposal of Sample Slides
1.	Positive slides are either kept for slide extraction for research purposes or placed in the disposal Glassware box which contains a biohazard bag and then once the box is full, the biohazard bag is autoclaved for 30 minutes, then placed into a stryofoam box for disposal in the back commercial garbage.
2.	Negative slides are placed in xylene for 24 hours to remove oil. Then washed according to the Slide Washing SOP.
	Quality Control
	Initial and Ongoing Precision and Recovery
1.	Principal Analysts will meet the initial and ongoing precision and recovery requirements specified in Method 1623. Refer to US. EPA Method 1623 June 2005 page 59 for predetermined limits.
2.	Method blanks will be performed after every 20 samples are analyzed as well as spikes with known numbers of organisms to ensure that there is no carryover between samples from laboratory equipment. Refer to Cryptosporidium oocyst and Giardia cyst spiking of Filta-max filters SOP.
	Verification of analyst performance:
1.	To ensure method quality control is maintained, monthly inter-technician comparison readings will be preformed. Refer to USEPA Method 1623 June 2005 Section 10.6.
	Proficiency Testing
1.	To ensure staff training is consistent and measurable amongst all analysts performing tests, proficiency testing using unknown spikes will be used.

2.	When available, unknown PT spikes from Wisconsin State Labs will be used.
3.	All staff members performing tests will participate in the proficiency testing up to and including the staining procedure.
4.	Reading and interpretation of samples on the microscope will be read by principal analysts only.
5.	Refer to *Cryptosporidium* oocyst and *Giardia* cyst spiking of Filta-max filters SOP for instructions on spiking procedures.
	Parallel Analysis
1.	To ensure method quality control is maintained, periodic parallel analysis will be done on a positive sample.
2.	May be combined with OPR, PT and monthly inter-technician microscope analyst verification.
	Microscopic Examination for Surface Water Organisms
1.	Resuspend pellet from 13 above in a minimal volume of DI. If pellet volume is very low, resuspend in 25 µL and mount entire volume on a plain microscope slide with a 18 X 18 mm coverslip. Seal the edges with nail polish.
2.	For larger pellets, resuspend in volumes up to 500 µL using DI. Mix thoroughly with a Pasteur pipette.
3.	Withdraw 25 µL aliquots and mount as in 1. Above.
4.	Scan entire coverslip at 20X using phase contrast.
5.	Photograph representative organisms of interest and record data according to the MPA SOP (HR Document #HR0165).

Method Limitations: Large amounts of particulate material in the concentrate used for IMS will reduce the recovery and make surface water organisms very difficult to detect. Control by adjusting dilution and aliquot volumes.

References: US. EPA Method 1623 June 2005.

Related Documents: SOP for Positive and Negative Control, SOP for Elution and concentration of Filta-Max filters for the detection and enumeration of *Cryptosporidium* and *Giardia* from water, SOP for Staining procedure for *Cryptosporidium* and *Giardia*, Volume Standards: Preparation and Use, Dividing Pellets SOP, Decontamination and Biohazardous Waste Disposal, Figure 1.0, Slide Washing SOP, *Cryptosporidium* oocyst and *Giardia* cyst spiking of Filta-max filters SOP.