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SAMPLE

Submitter: Green Mountain Utilities

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Project Site: XYZ Wastewater Treatment Plant

Parasite Levels in Effluent Samples - EPA Method 1623

Table 1. *Cryptosporidium* oocysts and *Giardia* cysts detected in secondary effluent and post filtration

Site	<i>Cryptosporidium</i> (oocysts/100L) (TOTAL #)	DAPI + (oocysts/100L)	Propidium Iodide - (oocysts/100L)	<i>Giardia</i> (cysts/100L) (TOTAL #)	DAPI + (cysts/100L)	Propidium Iodide - (cysts/100L)
ABC (SM 0011)	6	4	0	124	57	6
DEF (SM 0012)	104	18	0	1310	205	51
GHI (SM 0013)	6	6	0	105	84	4

Laboratory Comments

Pall Gelman Envirochek™ filters were received at the laboratory for processing on May 3, 2002. Filters arrived cold and were processed within 24 hours.

Elution

Captured organisms were eluted with a solution of phosphatebuffered saline containing 0.01% Tween 20. The eluant was concentrated by centrifugation, resuspended in the appropriate amount of reagent grade water, and was clarified using immunomagnetic separation (IMS) (see below).

Concentration and Visualization

The IMS procedure was performed as described in Method 1623 (USEPA, 1999). Briefly, each 10mL sample concentrate (eluate) was added to a Leighton tube containing appropriate buffers and anti - *Cryptosporidium* and anti-*Giardia* DynaBeads™. Samples were incubated and rotated for 1 hour in a circular rotator. After incubation, each sample was clarified by magnetic separation followed by two dissociation steps performed with 0.1N HCl. Recovered (oo)cysts (200?L) were spotted onto Dynal spot-on well slides, stained with immuno fluorescent antibody, DAPI, and Propidium iodide, and examined using epifluorescence microscopy as described in Method 1623 (USEPA, 1999). (See Below).

Vital Dye Staining

The inclusion or exclusion of the fluorogenic dyes 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) has been used as a marker of intact membranes in waterborne *Cryptosporidium* and *Giardia* and as an indicator of the presence of internal features such as nuclei (Shupp and Erlandsen, 1987; Campbell et al., 1992). PI is capable of passing through only damaged cell membranes, and intercalates with the nucleic acids of injured and dead cells to form a bright red fluorescent complex (Sauch et al., 1991). DAPI is an AT -selective DNA stain, which causes a 20 -fold enhancement in fluorescence when binding to DNA occurs (Campbell et al., 1992).

The fluorogenic vital dye assay or dye permeability assay tests the differential uptake of the fluorochromes 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) by the oocysts. Results from several investigations have demonstrated that the dye permeability assay correlates well with results from in vitro excystation assays and the standard mouse infectivity assay (Campbell et al., 1992; Jenkins et al., 1997). However, the interpretation of viability from fluorogenic dye inclusion or exclusion must be undertaken cautiously, since the dye tests are known to overestimate viability and the staining can be variable with a portion of the oocysts not staining with either dye (Campbell et al., 1992; Smith, 1996; Jenkins et al., 1997; Black et al., 1996; Neuman et al., 2000).

Aliquots of the purified water sample concentrate were fixed with methanol onto well slides. Stock solutions of 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide(PI) were prepared and stored at 4 °C in the dark.

Observations

DAPI/PI plus FITC stained (oo)cysts can give the following appearances: (i) (oo)cysts stained bright red with two or four visible internal nuclei when examined with the green filter block (PI+) and two or four visible internal nuclei when examined under the UV filter block and with an external apple green fluorescence along with a reddish color when examined with the blue filter block, (ii) empty cysts (DAPI-, PI-); (iii) very low numbers of cysts including PI into their nuclei only, (iv) a very reduced number of cysts showing a diffuse DAPI staining that are PI-. The results of the vital dye staining are shown in Table 1. For ease of interpretation, only DAPI+ and PI- (oo)cysts are presented in the Table. **DAPI+/PI- (oo)cysts can be preliminarily considered as viable. All PI+ (oo)cysts observed in this sample were also DAPI+.**

The results of the vital dye staining demonstrate that both fluorochromes DAPI and PI provide an additional confirmatory step to determine the presence of *Giardia* cysts and *Cryptosporidium* oocysts in wastewater samples and to distinguish the protozoan parasite from other nontarget organisms that can be recovered during sample collection and concentration/purification procedures. Some researchers have reported that the exclusion of the fluorogenic dye PI provides a general determination of living cells with intact cell membranes (Shupp and Erlandsen, 1987). Jenkins et al., (1997) consider that the dye permeability assay provides an economical method to determine viability and potential infectivity, although the estimate may be conservative.

Cryptosporidium oocysts isolated from wastewater samples and inoculated onto HCT-8 cells have shown to be viable/infectious after the whole process (filtration/IMS). For *Giardia* cysts more research is needed (i.e., cell culture infectivity assay).

References

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