THE FATE OF *GIARDIA* AND *CRYPTOSPORIDIUM* IN MARINE WATER

by

Dana Christine Johnson

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Dana Christine Johnson

entitled The Fate of Giardia and Cryptosporidium in Marine Water

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

Charles P. Gerba	April 30, 1996 Date
Norval Sinclair	4-30-96 Date
Rod Adam	4/30/96 Date
C.J. Mare'	5/1/96 Date
Ian L. Pepper	5/1/96 Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Charles P. Gerba — April 30, 1996
Dissertation Director — Date
Charles P. Gerba

STATEMENT BY AUTHOR

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SIGNED: Dana Christine Johnson

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3. ABSTRACT

The fate and occurrence of *Giardia* and *Cryptosporidium* were studied in the marine environment to assess their potential for transmission since their transmission to swimmers in fresh waters has been previously documented.

The first part of this study was designed to determine the occurrence of *Giardia* and *Cryptosporidium* at bathing beaches within the vicinity of an outfall discharging primary treated sewage into Mamala Bay, Hawaii. In addition, a bathing area not impacted by sewage discharge was studied as well as a canal discharging into Mamala Bay. Sites were monitored monthly and quarterly for parasites by passing 400 liters of marine water through a spun polyproplyene fiber filter. The filter eluent was concentrated, purified and labeled with antibodies and examined under UV fluorescent microscopy. *Giardia* and *Cryptosporidium* were detected in primary treated sewage from the Sand Island sewage treatment plant which discharges into Mamala Bay at concentrations of 2,560 cysts and 216 oocysts/liter, respectively. Ala Wai Canal as well as the bathing beaches within the vicinity of the outfall were also found to contain the parasites. However, only a single *Giardia* was found at the heavily populated

beach, Hanuama Bay, which is not influenced by the outfall. This suggests that the swimmers are not a major source of the parasites, but that the outfall and non-point sources are main sources.

The survival of *Giardia* in marine water was studied in direct sunlight and the dark in marine waters (33- to 35-ppt salinity), canal water (28-ppt salinity) and in phosphate-buffered saline (8-ppt) at 18 to 28 °C. *Giardia muris* was inactivated by 3-logs in the presence of sunlight in marine and canal waters. In comparison, *Giardia* survive up to 6 hours in PBS under the same conditions. Overall, it appears that salinity and sunlight quickly inactivate *Giardia* cysts. Thus, *Giardia* would pose a threat only if the cysts reach the bathing beaches within a few hours.

The last phase of this project was designed to develop a more efficient method of collection of cysts and oocysts from water. Methods for the concentration of enteric viruses, *Giardia* and *Cryptosporidium* from water requires the use of two different types of filters. An electropositive or electronegative microporous filter (0.2-0.45-μm porosity) is used for enteric viruses and a polypropylene spun fiber filter (1-μm porosity) for parasites. This study compared the efficiency of their concentration from tap water and tertiary

treated wastewater with a polypropylene fiber cartridge, and the Filterite electronegative and the 1MDS Virosorb electropositive microporous filters. Cysts, oocysts and poliovirus were added to the test water and then collected by passage through the filters. The filters were eluted with beef extract and a non-ionic detergent. The eluent was centrifuged and the pellet assayed for parasites and the supernatant for viruses. Results indicated that the overall efficiency was greatest for the Filterite filter for both *Giardia* and *Cryptosporidium* ($p=0.000762$ and $p=0.067069$, respectively); and in addition they are easier and faster to process than the polypropylene-wound parasite filter. Sampling for both viruses and parasites on the same filter reduces the time and cost of routine sampling and allows for direct comparison of viruses and parasites in the same sample.

4. INTRODUCTION

Problem definition

Off-shore sewage dumping into oceans is a common method of sewage disposal worldwide. Enteric microbial pathogens, such as *Giardia lamblia* and *Cryptosporidium parvum*, can be present in both treated and untreated sewage. A potential public health threat exists if these pathogens survive in marine waters and impact nearby bathing beaches. There have been several documented recreational outbreaks due to *Giardia* and *Cryptosporidium* (MMWR, 1990 and Krammer, 1996). Viability studies have demonstrated that *Giardia* and *Cryptosporidium* can survive several days to months in lake and river waters (DeRegnier *et al.*, 1989). To date, no studies have evaluated the occurrence and viability of *Giardia* and *Cryptosporidium* in marine waters.

To address these questions, a sewage outfall site at Mamala Bay, Oahu, Hawaii was studied for one year to determine the impact of *Giardia* and *Cryptosporidium* on nearby bathing beaches, as well as the survival of these pathogens in marine water. Other non-point sources, such as the Ala Wai Canal and Manoa Stream were also studied to determine if they could also be a source of these protozoan parasites.

Literature review

Waterborne protozoan diseases

Giardia lamblia

Giardia lamblia was first described in 1681 by Anton van Leeuwenhoek who found them in his own feces. He called the trophozoites "animalcules" (Dobell, 1960). In 1859 that Vilem Lambl rediscovered *Giardia* by finding the trophozoites in stools of young children with diarrhea (Farthing, 1995). It wasn't until the early 20th century that physicians began associating diarrhea with the presence of *Giardia* in the stools. Some doctors were convinced that *Giardia* caused diarrhea, such as Fantham and Porter (1916), who confirmed the infectivity of *Giardia* by oral administration of cysts to young animals. Later, in 1954 Robert Rendtorff confirmed infectivity in human volunteers with oral administration of *Giardia* cysts (Rendtorff, 1954). *Giardia* is the most frequently isolated intestinal parasite in the United States (Hill, 1990 and Adam, 1991). In the United States, the prevalence of *Giardia lamblia* infections has been estimated to be $<1\%$ in midwestern middle-class adults (McHenry, 1987) to as high as 10-13% in Oregon adults as well (Skeels *et al.*, 1986). In some groups, such as daycare, the incidence can be as high as 33% in children (Ginsberg *et al.*, 1994).

In the United States, it is the most frequently identified cause of waterborne disease (Craun, 1986).

Humans become infected with *Giardia lamblia* by ingesting the environmentally-resistant stage, the cyst (Figure 1). Once ingested, it passes through the stomach and into the upper intestine. The increase in acidity via passage through the stomach stimulates the cyst to excyst which releases two trophozoites into the upper intestine. The trophozoites attach to the epithelial cells of the duodenum, jejunum, and upper ileum of humans. It is believed that they use their sucking disks to adhere to the cells. The adherence to the cells flattens the villi causing malabsorption and diarrhea by not allowing adsorption of water and nutrients across the intestine (Despommier *et al.*, 1995). Once the trophozoites detach from the epithelial cell and travel down the intestine, cholesterol starvation stimulates the trophozoites to encyst and pass back into the environment as a cyst (Lujen, in press). In symptomatic patients, more trophozoites than cysts are excreted into the feces, which cannot withstand the harshness outside of the human body. In asymptomatic humans, mostly cysts are passed in stools, therefore *Giardia* carriers can serve as a source of cysts in the environment. Once the cysts are in the environment, they can remain

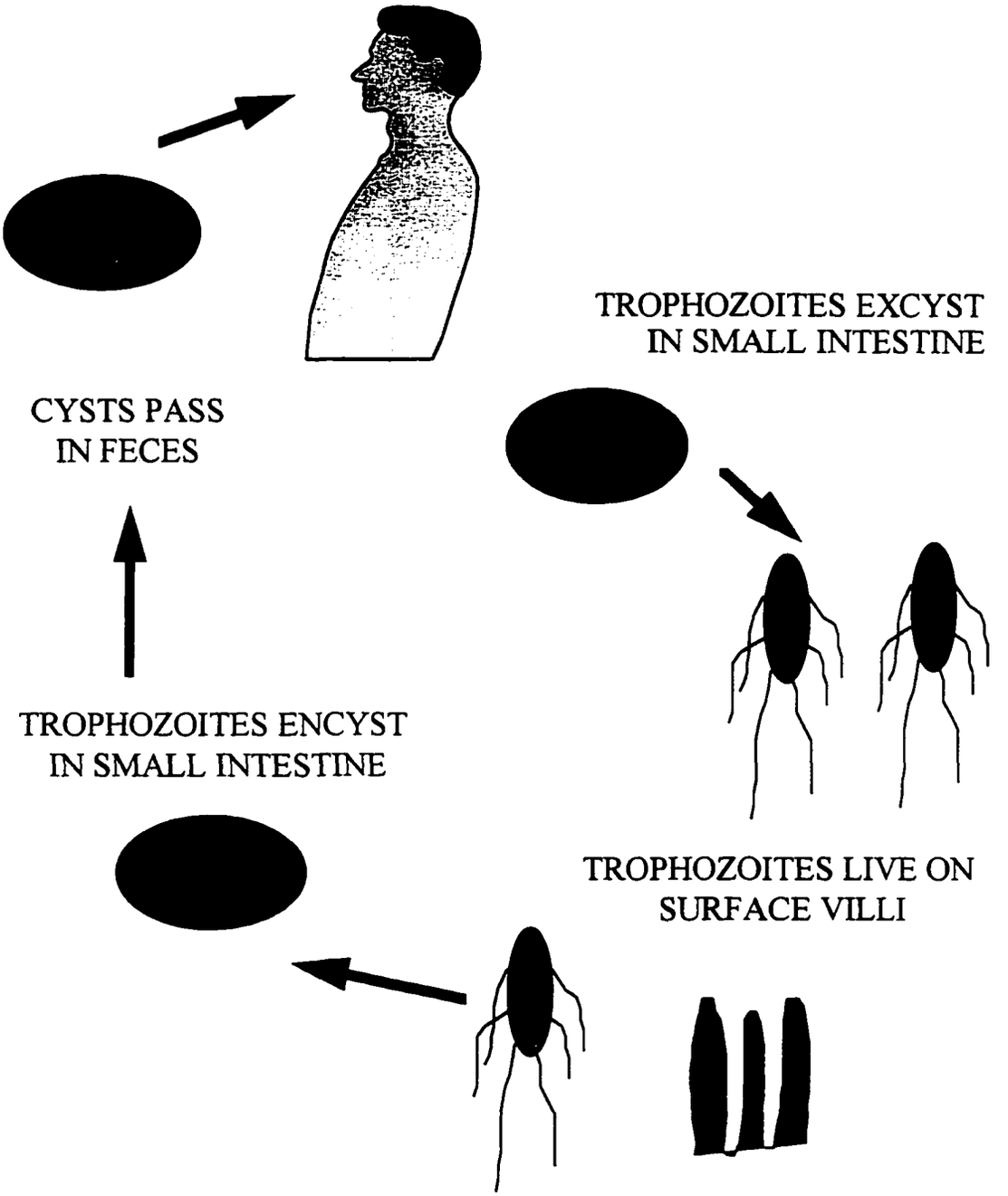
infectious for months under the right conditions.

The infectious dose of *Giardia* is quite low, which makes even low numbers in water a health threat. Rendtorff (1954) found human volunteers to become infected with as few as 10-25 cysts.

Giardia enters the environment from human and animal wastes, agricultural land runoff, broken sewer lines, and deficiency or error in water treatment. It is still controversial that animals such as beavers produce *Giardia lamblia* infectious for humans. Studies have shown they can be infected with *Giardia lamblia* from humans, but the reverse has not been demonstrated (Erlandsen *et al.*, 1988). In one study 40 to 45% of beavers in Colorado were found to be infected with *Giardia* and shedding up 1 x 10^8 cysts/animal/day, making them a major source of *Giardia* in the environment (Hibler and Hancock, 1990). Other animals which may contribute to *Giardia* in the environment are muskrats with prevalence found to be as high as 95%. Various other animals have been found to be infected with *Giardia lamblia*: cattle, goats, sheep, pigs, cats and dogs (Erlandsen, 1995). However, to date, no infections with *Giardia lamblia* in humans have been directly linked to an animal host, but there is evidence which suggests that animal-source *Giardia* could potentially

infect humans. Studies based upon isoenzyme analysis and PFGE (pulse-field gel electrophoresis) banding patterns did not find a difference between cysts from beaver hosts and human hosts (Isaac-Renton *et al.*, 1993).

Figure 1 ***Giardia lamblia*** **LIFE CYCLE**



Cryptosporidium parvum

Cryptosporidium was first described by Tyzzer in 1907 when he identified the organism in the intestinal epithelium of a mouse. It was not identified as a human pathogen until 1976 when Nime *et al.* and Meisel *et al.* described it in the diarrhea of immunocompromised hosts. Since this time, there have been several waterborne outbreaks, the most notable being the Milwaukee outbreak of April, 1993 which infected over 400,000 people, and killed more than 100 (Mac Kenzie *et al.*, 1994)

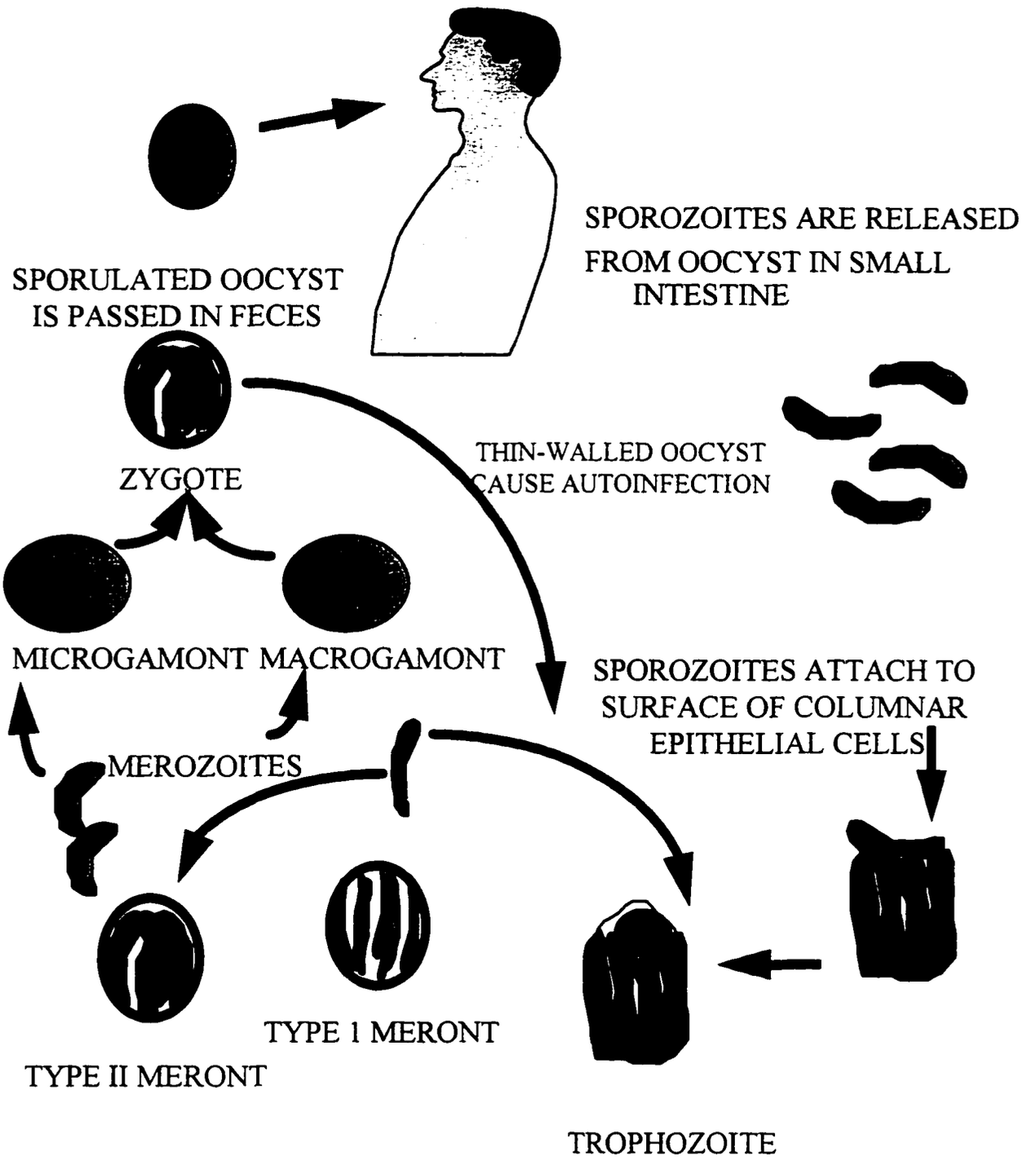
Cryptosporidium parvum has a complex life cycle involving both sexual and asexual stages (Figure 2). The host ingests sporulated oocysts. In the small intestine, the oocyst excysts, releasing four sporozoites which attach to the epithelial cells of the mucosa. The sporozoite becomes enveloped by the microvilli that fuse and elongate to cover the sporozoite. It then matures into a trophozoite and into a schizont. The schizont, an asexual reproductive form in which multiple mitosis occurs followed by cytokineses, results in eight first-generation merozoites. The cell ruptures, releasing the merozoite, which then infect neighboring epithelial cells and schizogony occurs again, but only forming four second-generation merozoites. When the cell ruptures, the merozoites

attach to uninfected epithelial cells and form either macrogametocytes or microgametocytes. The macrogametocyte and microgametocytes further divide and form macrogametes and microgametes, respectively. They join forming a zygote which differentiates to form the unsporulated oocyst which then is expelled from the cell surface, sporulates, and is shed in the host's feces (Despommier *et al.*, 1995)

The prevalence of cryptosporidiosis in the United States is 0.3 - 4.3% (Ungar, 1990). The infectious dose of *Cryptosporidium* has just recently been studied with human immunocompetent volunteers. DuPont *et al.* (1995) found the infectious $dose_{50}$ to be 132 oocysts, although 20% receiving just 30 oocysts also became infected.

Cryptosporidium can enter the environment in human and animal wastes. Cryptosporidiosis has been reported in many domestic animals, especially cattle. An infected calf can excrete 10^{10} oocysts per day. Agricultural land run-off can contaminate surface water. In the study by Kemp, farm drains were found to contain 0.06 to 19.4 oocysts per liter (Kemp *et al.*, 1995).

Figure 2 ***Cryptosporidium* LIFE CYCLE**



Outbreaks associated with water intended for drinking

Over 50% of gastrointestinal illnesses caused by waterborne outbreaks which occur in the United States do not have an identified agent (Herwaldt, 1992). The most common protozoan identified with waterborne outbreaks is *Giardia lamblia* (Hill, 1990 and Adam, 1991). The first suspected outbreak occurred in 1946 when sewage contaminated a water supply in a Tokyo apartment building (Davis and Ritchie, 1948). *Giardia*, as the causative agent, could not be confirmed because the organism could not be recovered from the water. However, *Giardia* was recovered from 77% of the occupants. The first documented waterborne outbreak occurred in Aspen, Colorado during the 1965-1966 ski season. The well water became contaminated by sewage leaking from defective pipes passing near wells (Moore *et al*, 1969). Over 11% of the 1,094 skiers became infected. Between 1965 and 1984 there were 90 documented *Giardia* outbreaks with over 23,776 cases of giardiasis (Akin, 1986). Between 1985 and 1988 there were 12 outbreaks and involving 1,910 cases (Levine and Craun, 1990). Between 1989 and 1990, there were seven outbreaks with 697 cases. *Giardia* was implicated in 27% of the total waterborne outbreaks for those two years, whereas viruses accounted for 12%, *E. coli* 4%, and CLB

(cyanobacteria-like bodies, now known as *Cyclospora*) for 4% (Herwaldt *et al.*, 1992). In 1991 to 1992, there were four outbreaks associated with 123 cases with water intended for drinking (Moore *et al.*, 1993).

Cryptosporidium is another well-documented waterborne protozoan. The first documented outbreak occurred in Texas in 1984 (D'Antonio *et al.*, 1985) when sewage contaminated a groundwater well. Oocysts could not be recovered from the well, but the rate of diarrhea was 12 times greater than in neighboring towns. Oocysts were detected in the majority of stools from patients with symptoms. An attack rate of 34% was determined with 2,006 people infected. The next documented outbreak occurred in Georgia in January 1987 (Hayes *et al.*, 1988). This outbreak involved 13,000 people when an operational irregularity occurred in the city's drinking water, although the treatment facilities revealed no physical or microbial violations of treatment standards. Since then there have been three more documented outbreaks, the most infamous occurring in Milwaukee in 1993 which involved over 400,000 people. In this case, *Cryptosporidium* penetrated the filtration system of the drinking water treatment plant when treatment plant facility operators switched from polyaluminum chloride coagulant back to alum and failed to adjust the coagulant

dosage with its streaming-current monitor (Lisle and Rose, 1995).

Water recreational exposure to *Giardia* and *Cryptosporidium*

Transmission of *Giardia* and *Cryptosporidium* through water recreational activities (swimming, snorkeling, surfing, etc.) has been documented. Haas *et al.*, (1995) has estimated that 30-ml of water is ingested during recreational activities. Protozoan parasites were responsible for 55% of the gastrointestinal recreational outbreaks in 1991-1992 (6/11) (Moore *et al.*, 1993).

During 1991-1992 there were five documented outbreaks: one *Giardia* outbreak occurred in June of 1991 involving 14 people in a swimming pool and in July 1991, a *Giardia* outbreak involved 16 persons who became infected via a wading pool in a daycare center. In July of 1991 there was yet another *Giardia* outbreak contracted from a lake at a campground. In Oregon, 1992 there was a *Cryptosporidium* outbreak in a wave pool infecting 500 persons. In August 1992 in Idaho there was a *Cryptosporidium* outbreak involving 26 persons who had used a water slide (Moore *et al.*, 1993). Contact with recreational water increases the risk of becoming infected two-fold with *Giardia* and *Cryptosporidium* (Isaac-Renton *et al.*, 1992).

Recreational activities in polluted marine waters have been correlated with increased risk of gastrointestinal illnesses. Stevenson (1953) found that swimmers who swam in polluted waters (defined by the presence of coliforms in water) had a higher incidence of gastrointestinal illness and upper respiratory infections. Cabelli *et. al.* (1982) found a linear relationship between swimming-associated gastrointestinal illness and bacteriological contamination of the water. He demonstrated that presence of enterococci correlated best to the risk of gastrointestinal illness among persons who bathed in marine waters. The causative agent of the documented gastrointestinal illnesses were not identified in these studies.

Transmission of *Giardia* and *Cryptosporidium* through recreational waters is well-documented, and it is possible that the previously observed gastrointestinal illness in marine waters could be associated with protozoan parasites.

Off-shore sewage disposal

Coastal cities have often relied upon direct discharge of sewage into the ocean. It is estimated that more than 3.5 billion gallons of wastewater is disposed

in oceans in the United States each day (Bishop, 1983). Pathogens- such as bacteria, viruses and protozoans- are released into the oceans via sewage, which may pose a public health risk to bathing and shell-fishing water. Although the presence and survival of enteric bacteria and viruses in oceans have been studied previously (Evison, 1988), no studies exist on *Giardia* or *Cryptosporidium*.

Sewage

The concentration of *Giardia lamblia* cysts in raw sewage has been observed to be as high as 3,375, 3,087 and 2,040 per liter from sewage treatment plants located in California, Florida and Vermont, respectively (Sykora *et al.*, 1991). The cyst concentration varied from 560 (Vermont) to 14,000 (Florida) per liter. There was a seasonal distribution of cysts in which the highest cyst concentrations were seen in late summer and winter to early spring. However, in Hawaii there was no significant difference throughout the year, as it is a tropical region.

Rose, *et al.* (1988) studied *Cryptosporidium* oocyst concentrations in raw sewage and found an average concentration of 5,291 oocysts per liter. In another study, raw sewage samples from Arizona had a range of 850 to 13,700 oocysts

per liter (Madore *et al.*, 1987). Generally, in temperate regions, more cysts are found in winter to early spring.

These various studies strongly support the proposal that sewage contains significant numbers of both *Giardia* and *Cryptosporidium.* The Sand Island sewage outfall in Hawaii disposes of more than 25 million gallons of raw sewage per day. This corresponds to releasing 2.4×10^{11} *Giardia* cysts and 2.0×10^{10} *Cryptosporidium* oocysts into Mamala Bay each day.

Removal of *Giardia* and *Cryptosporidium* by wastewater treatment

Today, there are more than 15,000 wastewater treatment facilities in the United States (Bitton, 1994). Collectively, these plants treat more than 37 billion gallons of wastewater a day. About 75% of the plants have the ability to treat to secondary treatment or better (Ouellette, 1991 and Bitton, 1994).

Preliminary and primary wastewater treatment involves the removal of debris and coarse material which are disposed of as sludge, which might interfere with plant equipment. Primary treatment is a further method of screening and sedimentation of debris. During primary settling, only 0 to 53% of *Giardia* cysts are removed (Casson, 1990). No data are available on the removal of

Cryptosporidium after primary treatment.

Secondary treatment is biological oxidation (e.g. trickling filter, oxidation ponds, or activated sludge). It reduces organic matter and reduces the biological oxygen demand (BOD) of raw sewage up to 95%. During activated sludge treatment, the sewage is aerated to encourage the growth of aerobic bacteria that can oxidize the dissolved organic matter to carbon dioxide and water (Tortora *et al.*, 1995). Secondary clarification removes anywhere from 98.6 to 99.7% of cysts (Casson *et al.*, 1990) and therefore secondary treatment of sewage would greatly reduce the number of protozoan parasites disposed of in marine waters.

Tertiary treatment or advanced treatment further removes the biologically-degradable organic matter, by using physical filtration and chemical precipitation to remove any remaining BOD, nitrogen and phosphorus. It can provide water that is suitable for drinking, whereas secondary treated effluent can only be used for water intended for irrigation.

Survival of *Giardia* in water

Giardia lamblia is able to survive for prolonged periods in various waters. *G. lamblia* cysts have been documented to survive for up to 77 days at 8 °C and

4 days at 37 °C in distilled water (Bingham *et al.*, 1979). In other studies, *Giardia muris* cysts were resuspended in lake, river and tap water. Plastic containers were filled with cysts with a mesh covering the container to allow the passage of water while retaining the cysts. Viability was determined by fluorogenic dye exclusion, animal models and Nomarski microscopy (DeRegnier *et al.*, 1989). Survival in lake water was dependent upon temperature and depth. Cysts were placed at a depth of 15 feet in the autumn (19.2 ±1.3 °C) and were viable for up to 28 days. At the 30-foot depth the temperature was 6.6 ±0.4 °C and the cysts remained viable for 56 days. In Mississippi river water in the autumn, the temperature ranged from 19 °C to 27 °C, and the cysts survived up to 28 days whereas in the winter the temperature was 0 °C to 2 °C and they survived more than 56 days (DeRegnier *et al.*, 1989).

Only one previous study has examined the survival of *Giardia muris* in hypertonic saline solution (artificial sea water) at 4 °C. The cysts survived less than 24 hours as determined by excystation (Erlandsen, 1984).

Detection of *Giardia* and *Cryptosporidium* in water

Due to the low infectious dose and low numbers of *Giardia lamblia* and

Cryptosporidium parvum in surface waters, large volumes of surface water must be collected and concentrated. There are three methods of collection and concentration from water that are currently in use today. The most common method in the United States is the use of the cartridge filter. Usually 400 liters of surface water or 1000 liters of groundwater are passed through a polypropylene cartridge filter (1-μm nominal porosity) (Rose *et al.*, 1986, Musial *et al.*, 1987, EPA, 1993 and APHA, 1994). The cysts and oocysts are collected and entrapped in the filter. They are eluted from the filter by cutting the filter longitudinally and shredding the fibers. The fibers are hand-washed in an eluting solution (phosphate-buffered saline, Tween 80 and sodium dodecyl sulfate). The eluent (containing the parasites) is concentrated by centrifugation. The pellet is preserved in 10% buffered formalin and stored at 4 °C .

Another method of collection is the membrane filtration method developed by Ongerth and Stibbs (1987). In this case, only small volumes of water can be concentrated. Usually 10 to 20 liters are transported to the laboratory and filtered through a 2-μm pore size polycarbonate filter. The surface of the membrane is scraped with a rubber blade and washed with detergent. The eluent is collected and concentrated via centrifugation. The

pellet is preserved in 10% buffered formalin and stored at 4 °C .

The calcium carbonate flocculation method is also used to concentrate small volumes (1 to 20 liters) of water. Sodium bicarbonate and calcium chloride are added to the water. The pH of the water is adjusted to 10 with sodium hydroxide. This causes calcium carbonate crystals to precipitate out of solution and transport particulate matter, such as cysts and oocysts, with the crystals. After a four-hour settling period, the supernatant is removed by vacuum and the calcium carbonate crystals are dissolved in 10% sulphamic acid. The eluent is concentrated via centrifugation.

There are advantages and disadvantages to each collection and concentration method. Only the cartridge filter allows for sampling of large volumes (100 to 1000 liters), however, recovery of organisms from the filter is variable. Watkins *et al.* (1995) reported 0.2 - 40% recovery for elution of the Cuno Filter (DPPPY, Merridian, CT). They also reported the recovery efficiencies to vary from 10 to 60% with membrane filtration and from 60 to 80% with calcium carbonate flocculation. Musial *et al.* (1987) found that the filter retained 80 to 90% of *Cryptosporidium* oocysts when testing 20-liter volumes. One study by Rose (personal communication) found the recovery of

Giardia and *Cryptosporidium* from the filter to be 82.5% and 62%, respectively. The results from the cartridge filter can be variable and depend upon the skill of the technician. The main disadvantages of the membrane filtration technique are the small assay size (10 to 20 liters) and the variability in recovery. The calcium carbonate does have a higher and more consistent efficiency, but again only small volumes can be assayed.

The pellet contains debris, such as sediment and algae, in addition to the parasites. The debris needs to be removed so that the parasites can be easily detected on a 25-mm diameter disk polycarbonate membrane (1.2-μm porosity) and so that the debris do not clog the membrane. There are three methods that are currently being used to separate the debris from the cysts and oocysts: flotation, flow cytometry and magnetic antibody selection. Flotation clarifies the sample in a percoll-sucrose density gradient (sp. grav. 1.10). The parasites are concentrated and clarified at the interface, while the debris is pelleted to the bottom. The interface is collected and washed and labeled with antibodies (EPA, 1993 and APHA, 1994). Flow cytometry is a cell sorter that is programmed to select cysts by size, shape and fluorescence from debris. The sample is labeled with fluorescein-labeled monoclonal antibody and then placed in the automated

flow cytometer (Vesey *et al.*, 1993). The purified sample is dropped onto a glass slide and ready for microscopy. Antibody-capture (Bifulco and Schaefer, 1993) concentrates *Giardia* from debris by mixing mouse IgG anti-*Giardia* antibody as a primary antibody and anti-mouse IgG antibody-coated magnetite particle as the secondary antibody. The sample and conjugated antibodies are mixed and then placed in a magnetic field. The debris can be easily aspirated, while the cysts are bound in the magnetic field. The recovery efficiencies vary for each procedure depending upon the skill of the technician and the turbidity of the water. The more turbid the water, the more likely the cysts will to adhere to debris. Fricker (1995) reported <5 to 81% recovery with the percoll-sucrose flotation. Rose reported 85.2 to 93.2% recovery of *Giardia* and *Cryptosporidium* respectively (personal communication). However, the percoll-sucrose density gradient does not remove all interfering substances, especially algae. Many algae the size of *Giardia* have the same specific gravity (1.05) and therefore become concentrated with the cysts and oocysts. Some algae contain pigments that fluoresce at the same wavelength as fluorescein isothiocyanate (Bifulco and Schaefer, 1993). The major drawback to the flow cytometer is the cost. A flow cytometer costs about $90,000 and requires a full-time technician capable of

operating it. C. Fricker claims the efficiency recoveries are in the magnitude of 90% (personal communication) and remove almost all interfering debris. Selective magnetic antibody capture is relatively new and still under development. A recovery rate of 82% has been reported by Bifulco and Schaefer, 1993. However, one problem with this method is that the antigenic sites must be preserved and available (not damaged or covered with material) to be available for attachment. The microscopy step is fairly universal. Usually a 25-mm round polycarbonate filter membrane and pre-filter are placed on a manifold. One percent bovine serum albumin (BSA) is filtered onto the membrane to prevent nonspecific antibody binding. Recently, Rodgers *et al.*, (1995) found the use of goat serum reduced nonspecific binding to algae and other nontarget organism. The sample is filtered onto the membrane and form a monolayer as to not cover any parasites. This step is not necessary for flow cytometry as the sample is labeled with antibodies for cell sorting and placed on a glass slide for microscopy.

Primary monoclonal antibody (EnSyn, Research Triangle Park, NC) is used for detection of *Giardia* and *Cryptosporidium*. Ensyn Inc. claims that the antibodies are pan-specific for all species of *Giardia*, however tests in our

laboratory and by Rose *et al.*, 1989 have shown that these antibodies will not react with *Giardia muris*. Ensyn Inc. also claim that the antibody is directed against *C. parvum*, but can cross react with *C. muris* and *C. meleagrids*, but not *C. baileyi*. The secondary antibody (goat anti-mouse IgA + IgG + IgM, KPL, Gaitherberg, MD) is fluorescently labelled with fluorescein isothiocyanate (FITC) which fluoresces green under UV light.

Viability testing

Giardia muris, the mouse pathogen, is used as a model instead of the human pathogen, *Giardia lamblia*, for viability studies since previous work with *G. lamblia* has demonstrated that it gives inconsistent results with excystation varying from 2 to 96% (Sauch, 1988). On the other hand, *G. muris* has been found to be a very good model for *G. lamblia* because excystation above 90% is consistent and reproducible. Animal studies with *Giardia muris* have also shown that it is a good model and that it is hardier than *G. lamblia*, making it a "worst-case" scenario (Erlandsen, 1984).

Viability of *Giardia* can currently be determined by three methods, inclusion/exclusion dyes, excystation, and *in vivo* mouse studies. Two common

dyes that are used to determine viability of *Giardia* cysts are inclusion dye fluorescein diacetate (FDA) and exclusion dye propidium iodine (PI). The dyes work on different principles to ascertain viability.

FDA is a polar vital dye and can cross the cell membrane. Once inside the cyst it is believed that lipase, acylase and/or proteinase enzymatically cleave the two acetate groups via their ester linkage producing nonpolar fluorescein which is then trapped in the cysts. It is detected by (450 - 490 wavelength) epifluorescent microscopy. However, if the lipid bilayer is damaged, the fluorescein leaks out and the cysts will not be detected under epifluorescent microscopy (Schupp and Erlandsen, 1987).

In contrast, PI does not cross intact membranes and therefore only cells with a disrupted membrane will take up the dye and fluoresce orange at 450 - 490 nm wavelength and red under 545 nm excitation wavelength (Schupp and Erlandsen, 1987). Frequently the FDA and PI are used in conjunction so that viable cysts stain apple-green and nonviable cysts stain red. Labatiuk *et al.* (1992) showed that FDA overestimates infectivity when compared to animal infectivity and excystation models because the FDA entered the cysts, but was not released and therefore in cases where the mode of inactivation does not involve cyst wall

or membrane damage, this dye should not be used. In other studies researchers have found that PI correlated with excystation with heat and inactivation due to the quaternary ammonium compound, but not with chlorine or monochlorine inactivation. It is because PI understained the cysts, the cysts were stripped, but the dye could not enter. (Sauch *et al.*, 1991) However, Schupp *et al.*, (1987) did find a correlation between FDA and PI dyes and infectivity studies. Over all, vital dyes have not proven to be reliable, especially in light of the fact that their inactivation in waters can occur via chemicals that alter cell wall permeability (Meyer and Schaefer, 1984). Excystation involves three steps and is an *in vitro* method which attempts to simulate the ingestion of cysts and the path it would take within the human body. First, a low pH induction step in which the cysts are pre-incubated with bile salts and sodium bicarbonate for 30 minutes. It is important that the redox potential is 120 mV and that CO_2 is produced as in the stomach (Meyer, 1984). Second, a washing step which is similar to how food passes from the stomach to the small intestine. Third, an incubation step in excystation media in which the cysts are incubated in a trypsin solution for 45 minutes (Rice and Schaefer, 1981 and EPA, 1991). The remaining cysts (considered nonviable because they did not excyst), trophozoites, shells and

partially excysted cysts are counted and calculated to determine viability. This method is frequently used in disinfection studies, and correlates well with mouse studies (Labatiuk *et al.*,1991 and Hoff *et al.*, 1985).

Viability of *Giardia muris* can be assessed with a variety of mice; C3H/HeN mouse (Labatiuk *et al.*, 1991), CF-1 Swiss mouse (Hoff *et al.*, 1985) and CF-1 non-Swiss mouse (Schupp *et al.*, 1987). The *Giardia*-free mouse is gavage fed the *Giardia* and five to seven days later the animal is sacrificed and the intestines are removed. The luminal contents of the intestine are rinsed and the mucosa are scraped. The contents are then microscopically observed for trophozoites. In addition, a section of the bowel (1 cm from the pylorus of the stomach) is also microscopically examined if the previous sample was negative. This type of test is very costly and time-consuming, but it is the most accurate and conservative measure of infectivity. However, it can only indicate infectivity for a group of cysts and not individual cysts (as dyes or excystation can) as the infectious dose (ID_{50}) for mice is 0.13 to 15.85 *G. muris* cysts (Hoff *et al.*, 1985). One advantage of using the animal model is the increased detection limit. Detection of cysts with dyes or excystation are limited to a maximum of 99.9% (3-log) reduction; which means that for a 3-log reduction, 1,000 cysts must

be enumerated. Haas *et al.* (1995) devised a volumetric method to detect inactivation in the 5- and 6-log range. The method is based upon counting only viable trophozoites and has the potential to replace the standard formula of enumerating cysts, shells, and partially excysted trophozoites. The current formula would be extremely tedious to use to achieve a 4-log (10,000 cysts) reduction. With animal studies, it is theoretically possible to identify up to a 6-log removal based on the inoculum fed to the mice.

Explanation of dissertation format:

The research reported in the appendices of this dissertation consists of three related experiments designed and undertaken by the candidate: 1) Occurrence of *Giardia* and *Cryptosporidium* in Marine Waters 2) Survival of *Giardia* in Marine Waters 3) Improved Method for Concentration of *Giardia*, *Cryptosporidium*, and viruses from water. There are two advantages to using this format. Each candidate for the advanced degree in the Department of Microbiology and Immunology is expected to submit original research papers to peer review scientific journals for publication. By using this format, these papers are ready for submission for publication.

Each of theses papers represents the candidate's research. Preparation and submission of the manuscripts were undertaken by the candidate. Dr. Charles P. Gerba, Peter Gerba and Kristina D. Crabtree served as co-editors of the manuscripts.

5. PRESENT STUDY

The methods, results, and conclusions of this study are presented in the papers appended to this dissertation. The three following papers describe the approach used to study the Sand Island Sewage Outfall in Oahu, Hawaii. These studies were part of a multi-collaborative project to study the impact of a sewage outfall on Mamala Bay. The project was commissioned by Drs. R. Colwell, F. Orlob and J. Schubel. Under their management several aspects were studied: pollution source identification (Dr. Stevenson), near-field modeling (Dr. Roberts), fate and transport of pathogens (Drs. Blumberg and Connolly), ocean current measurements, (Drs. Waddell and Hamilton), microbiology (Drs. Gerba, Pepper, Hill, Landry, Paul, Rose and Fujioka), ecosystem response to pollutional stresses (Drs. Bailey-Brock, Grigg, Mc Carthy, Kay, Ziemann, Smith and Parnell) environmental impacts on receptors and resources (Drs. Courtney, O'Connor, Oliveri and Cooper), environmental quality control and enhancement (Dr. Harleman), point source characterization and control options for Mamala Bay (Dr. Harleman), plume dynamics and dispersion (Drs. Jones and Dickey), Ala Wai Canal dye study (Dr.Connolly and Stevenson), and molecular detection of

Staphylococcus aureus in waters (Drs. Hill and Fujioka): The fate of *Giardia* and *Cryptosporidium* in marine water and the public health risk from a sewage outfall were the focus of this study.

The first paper describes the occurrence of *Giardia* and *Cryptosporidium* in marine waters. Bathing beaches were chosen according to their proximity to an outfall discharging primary sewage and monitored on a monthly or quarterly basis. Parasites were collected from marine water by entrapment onto a polypropylene filter. They were eluted from the filter and concentrated via centrifugation, labeled with fluorescent antibodies, and identified under UV microscopy.

The next study concerned the survival of *Giardia* in marine waters. Only *Giardia* was evaluated as this was a multi-collaborative study and another laboratory undertook the study of *Cryptosporidium* survival in marine water. The survival of *Giardia* was studied in three different marine waters collected from Mamala Bay: Waikiki beach, Hanauma Bay, Black Point beach, Ala Wai canal, in phosphate-buffered saline and tapwater. Survival was also evaluated in the presence of sunlight and in the dark. In addition, survival of *Giardia* was also studied *in situ* using McFeter chambers as a comparison to the results

obtained in the laboratory study. Survival in marine waters appears to be limited to a few hours in sunlight.

The final paper evaluated a more efficient method for collection of protozoan parasites from water. The current method of parasite collection has a highly variable efficiency with frequently low recovery rates. Two different filters, the Filterite filter and 1MDS filter, are both currently used for virus concentration from water and were examined to determine if they could replace the current polyproplyene filter and improve recovery. In addition, the method was simplified and would result in co-collection of enteric viruses and parasites. It was found that not only did the Filterite filter give greater recovery of cysts and oocysts but it also provided more consistent results. In addition, the concentrate from the Filterite filter was easier to process and gave less background fluorescence under UV microscopy than the current polypropylene filter.

6. Appendix A:

OCCURRENCE OF *GIARDIA* AND *CRYPTOSPORIDIUM* IN MARINE WATERS

Dana C. Johnson[1], Charles P. Gerba[1,2], Ian L. Pepper[2], Joan B. Rose[3] and Roger Fujioka[4]

Department of Microbiology and Immunology[1] and Department of Soil, Water and Environmental Science[2], University of Arizona, Tucson, AZ 85721. University of South Florida, Department of Marine Sciences, St. Peterburg, FL 33701[3], University of Hawaii at Manoa, Water Resources Research Center, Honolulu, HI 96822[4]

ABSTRACT

The purpose of this study was to determine the occurrence of *Giardia* and *Cryptosporidium* at bathing beaches within the vicinity of the Sand Island sewage outfall in Mamala Bay, Hawaii and, in addition, Hanauma Bay, a heavily visited bathing beach not in the vicinity of the outfall. The Ala Wai Canal and Manoa Stream were also studied to determine if non-point sources were also sources of the protozoan parasites. Sites were monitored monthly and/or quarterly for parasites, viruses etc.. The parasites were eluted from the filter and the eluent concentrated via centrifugation. Samples were clarified by the percoll-sucrose density gradient, and then stained by indirect immunofluorescent antibodies specific for *Giardia* and *Cryptosporidium*. *Giardia* and *Cryptosporidium* were detected at concentrations of 2.56×10^3 and 2.16×10^2 per liter, respectively, in the Sand Island primary treated sewage. They were also found at the Ala Wai canal (3.2 cysts and 1.9 oocysts), as well as at the bathing beaches within the vicinity of the outfall. However, only on one occasion was *Giardia* found at the heavily populated control beach (not influenced by the outfall), suggesting that the bathers were not a major contributor of microbial contamination, and that

the outfall and canal are likely sources. Both the outfall and canal were contributors of *Giardia* and *Cryptosporidium* at bathing beaches in Mamala Bay.

In addition, the presence of *Giardia* and *Cryptosporidium* correlated with each other, but not with viruses or *Salmonella*.

INTRODUCTION

The primary focus of this study was to determine if the Sand Island sewage outfall in Honolulu impacts the water quality of nearby bathing beaches. The occurrence of *Giardia* and *Cryptosporidium* at the sewage outfall, nearby bathing beaches, Ala Wai Canal, Moana Stream and Hanauma Bay was assessed in this study. *Giardia* is the most widely documented waterborne protozoan (Adam, 1991) and *Cryptosporidium* has also been implicated in waterborne outbreaks; the most well-known of which was the Milwaukee outbreak in 1993 (Mac Kenzie, 1994). In addition, both parasitic protozoans have caused outbreaks associated with recreational waters (Moore; 1993; Herwaldt *et al.*, 1992; and, Krammer, 1996). For these reasons, it is critical to assess the occurrence of the parasites at bathing beaches in the proximity of a sewage outfall. No previous studies exist on the occurrence of *Giardia* and *Cryptosporidium* at bathing beaches. The outfall which discharges primary treated effluent extends two miles offshore and is 75 feet below the water with diffusers located along the length of the pipeline (Figure 1). It is located approximately six miles west of Waikiki Beach, a heavily populated bathing beach. Ala Wai Canal and Manoa

Stream, which are in the vicinity of Waikiki Beach, are also potential sources of microbial contamination. The canal and stream are impacted by storm water and land runoff. In addition, Ala Wai Canal is also potentially contaminated by illegal dumping from boats and leakage from sewer pipes within the canal. The Hawaii Department of Health has determined that the canal is an unsuitable body of water for swimming and has posted "no swimming" signs along the canal because it exceeds the in-land recreational water quality standard of <200 fecal coliforms /100 ml (Fujioka, 1995). Hanauma Bay was chosen to determine if bathers are also a source of contamination since this beach is not considered to be influenced by either the outfall or the Ala Wai Canal. All sites were monitored quarterly and the bathing beaches and canal monitored monthly. Samples were collected from the surface and in the area of bather activity (within 5 to 15 feet of the shore).

MATERIALS AND METHODS

Giardia and *Cryptosporidium* were collected from the water by passing 400 liters of marine water through a polypropylene cartridge filter (1-μm nominal porosity, 25.4 cm) (Cuno, Meridian, CT). The parasites were eluted from the

filter by cutting the filter longitudinally and shredding the fibers (Figure 2). The fibers were hand washed in three liters of eluting solution (phosphate-buffered saline, 0.1% Tween 80 and 0.1% sodium dodecyl sulfate (Sigma, St. Louis, MO). The eluent containing the parasites was concentrated by centrifugation at 2800 x g for 10 minutes. The parasites were pelleted at the bottom of the container and the supernatant was aspirated and discarded. The pellet was preserved in 10% buffered formalin and stored at 4 °C (EPA, 1993 and APHA, 1994).

The sample was clarified of debris, which can interfere with visualization, by flotation of 1-ml or less of packed pellet. Flotation was performed by mixing the pellet with eluting solution to bring the final volume up to 20 ml. Frequently, the final pellet volume can be greater than 1 ml, and therefore, several simultaneous flotations must be completed to evaluate an equivalent volume of 200 liters per sample. The pellet and eluting solution were vortexed well and 25 ml of percoll-sucrose (Sigma, St. Louis, MO) (sp. grav. 1.10) was layered underneath the sample (Figure 2). The sample was centrifuged at 1050 x g for 10 minutes. The debris were pelleted to the bottom of the conical tube. The supernatant and the interface were collected and washed with water. The sample was further concentrated to 5 ml by centrifugation at 1700 x g for 10

minutes (APHA, 1994 and EPA, 1993).

The purified sample was filtered onto a 25-mm polycarbonate membrane (Costar, Pleasanton, CA). The membrane was pre-wetted with phosphate-buffered saline (PBS) and 1% bovine serum albumin (BSA) (Sigma, St. Louis, MO). BSA was used as a blocking agent to avoid non-specific antibody binding. An additional 2 ml of 1% BSA was filtered on top of the sample.

The primary antibodies (EnSyn, Research Triangle Park, NC) are monoclonal antibodies specific for *Giardia* cysts and *Cryptosporidium* oocyst wall antigens. The antibody was diluted to a 1:20 concentration and 0.5 ml was placed onto a membrane for a 30-minute contact time. The membrane was rinsed with 10 ml of PBS. Secondary antibody, goat anti-mouse IgA + IgG + IgM, (KPL, Gaitherburg, MD) fluorescently-labeled with fluorescein isothiocyanate (FITC), was diluted 1:20 and 0.5 ml was placed on the membrane. The samples were covered during the 30-minute contact time. The membranes were rinsed with 10 ml PBS and placed onto a glass slide. A drop of DABCO-glycerol (Mallinckrodt Chemical, Chesterfield, MO) was placed on top of the membrane and a glass coverslip was place on top of the membrane. Samples were stored at 4 °C and read within five days under 200 X - 400 X UV light

(BH-2, Olympus, Japan) (EPA, 1993).

Detection of *Giardia* and *Cryptosporidium* in sewage

Due to the high concentrations of *Giardia* and *Cryptosporidium* in sewage, it was not necessary to sample large volumes of sewage effluent. A one-liter grab sample was collected in a polyproplyene container. The sewage was centrifuged (2700 x g) which pelleted the cysts and oocysts. The supernatant was aspirated and discarded. The pellet was preserved in 10% buffered formalin and stored at 4 °C.

The sewage contained large amounts of debris that had a specific gravity similar to percoll-sucrose (sp. grav. 1.10). For this reason, percoll-sucrose did not clarify the sample. Sheather's flotation media (sucrose, sp. grav. 1.24) was used instead of percoll-sucrose to purify the sample. A fraction of the pellet was floated and the supernatant and interface collected, washed in water, and concentrated to 5 ml. The sample was stained and examined in the manner previously described. Frequently, only 0.5 ml of the 5-ml concentrated interface/supernatant was passed through the filter, because of the high number of cysts and oocysts in sewage. The entire membrane was scanned and cysts and

oocysts enumerated. Enteroviruses were detected by cell assay on Buffalo Green Monkey kidney cells (BGM) and adenoviruses were assayed on Primary Liver Carcinoma cells (PLC/PRF/5) (ATTC, Rockville, Maryland) (APHA, 1994).

Negative results were defined by the detection limit of the sample methodology (EPA, 1993). Factors that affect detection limit are pellet size, which is influenced by turbidity, sediments, and the volume of sample collected and assayed.

The colony and plaque forming units for bacteria and viruses, respectively and cysts and oocysts were transformed for analysis using $\log_{10}(y+1)$ for all data. Pearson's correlation coefficients were defined and two-by-five-way contingency tables were developed for determining relationships between presence of cysts or oocysts and adenoviruses, enteroviruses and *Salmonella* (Table 5).

RESULTS

During this study, *Giardia* or *Cryptosporidium* or both were detected at least once at all of the bathing beaches with the exception of Sand Island beach (Table 1). The second lowest occurrence of the protozoan parasites was at

Hanauma Bay (average concentration of 0.08 *Giardia* cysts and 0 *Cryptosporidium* oocysts per 200 l) (Table 5). Ala Moana Beach had the highest parasite density in which an average of 1.7 *Giardia* cysts and 0.7 *Cryptosporidium* oocysts were detected per 200 l. Waikiki and Queen's Beaches contained the next highest parasite densities with an average of 0.4 cysts and 0.5 oocysts and 0.2 cysts and oocysts, respectively.

Diamond Head was chosen as a control off-shore site because it was determined that it would be highly unlikely for the plume to travel eastward to Diamond Head as the currents are predominantly westward (Blumberg and Connolly, 1995). Pearl Harbor is located six miles west from the outfall. In addition to the possible impact by the sewage plume, the United States Navy also has a sewage outfall within Pearl Harbor that could potentially be a source of microbial contamination. Fifty percent (2/4) of the samples collected from Pearl Harbor contained either *Giardia* or *Cryptosporidium* with an average density of 0.8 cysts and 0.5 oocysts per 200 l (Tables 3 and 5).

Table 2 shows the sites that were considered possible sources of *Giardia* and *Cryptosporidium.* The outfall sampling site was directly above the pipeline in Mamala Bay at a depth of 75 feet, and contained an average density of 32

Giardia cysts and 17.3 *Cryptosporidium* oocysts per 200 liters (Table 5). Manoa stream emptied into Ala Wai Canal which discharged into Mamala Bay and both were possible sources of contamination for the bathing beaches. The stream was impacted by land run-off and storm drains (Fujioka and Loh, 1995). Parasites were detected in Manoa Stream in 50% (2/4) of the samples with an average density of 4.8 cysts and 1 oocyst per 200 liters. Ala Wai Canal is impacted by Manoa Stream, 65 storm drains, leaking sewer pipes and possible illegal boat dumping. The canal samples contained 3.2 cysts and 1.9 oocysts per 200 liters with 85% (11/13) of the samples collected containing parasites.

Table 1: Occurrence of *Giardia* and *Cryptosporidium* cysts or oocysts at bathing beaches (200 liters)

	Ala Moana		Waikiki		Queens		Sand Island		Hanauma Bay	
	Giardia	*Crypto*	*Giardia*	*Crypto*	*Giardia*	*Crypto*	*Giardia*	*Crypto*	*Giardia*	*Crypto*
Oct	<2	<2	<1	<1	NS	NS	NS	NS	<1	<1
Dec	<1	<1	<1	<1	<1	<1	NS	NS	<1	<1
Jan	<1	<1	**2**	**2**	**1**	<1	NS	NS	<1	<1
Feb	<1	<1	<1	<1	NS	NS	<1	<1	<1	<1
Mar	<1	<1	<1	<1	<1	<1	NS	NS	<1	<1
Apr	**16**	**1**	<1	**1**	<1	<1	NS	NS	**1**	<1
May	<1	<1	**1**	<1	**1**	**2**	NS	NS	<1	<1
Jun	**1**	**5**	<1	**2**	NS	NS	<1	<1	<1	<1
Jul	<1	<1	<1	<1	<1	<1	NS	NS	<1	<1
Aug	<1	<1	**1**	**1**	<1	<1	NS	NS	<1	<1
Sept	**2**	**3**	**1**	<1	<1	<1	NS	NS	<1	<1
Oct	<1	<1	<1	<1	<1	<1	NS	NS	<1	<1
Nov	**3**	<1	<1	<1	NS	NS	<1	<1	<1	<1

NS = No sample collected/assayed

Table 2: Concentrations of *Giardia* and *Cryptosporidium* (200 liters)

	Primary effluent x 10^3		Outfall		Ala Wai Canal		Canal/ocean interface		Manoa Stream	
	Giardia	*Crypto*	*Giardia*	*Crypto*	*Giardia*	*Crypto*	*Giardia*	*Crypto*	*Giardia*	*Crypto*
Oct	50	5	20	22	<2	<2	NS	NS	<2	<2
Dec	238	41	NS	NS	<9	<9	NS	NS	NS	NS
Jan	74	2	NS	NS	6	5	NS	NS	NS	NS
Feb	159	5	30	15	7	2	1	<1	<2	<2
Mar	60	15	NS	NS	1	3	NS	NS	NS	NS
Apr	300	18	NS	NS	2	2	NS	NS	NS	NS
May	1372	76	NS	NS	2	1	NS	NS	NS	NS
Jun	252	3	92	32	2	2	2	<1	9	11
Jul	160	64	NS	NS	4	4	NS	NS	NS	NS
Aug	416	160	NS	NS	<1	1	NS	NS	NS	NS
Sept	1100	100	NS	NS	2	2	NS	NS	NS	NS
Oct	736	32	NS	NS	14	2	NS	NS	NS	NS
Nov	1489	40	2	<1	2	1	2	<1	10	2

NS= no sample taken/assayed

Table 3: Occurrence of *Giardia* and *Cryptosporidium* at Diamond Head and Pearl Harbor (200 liters)

	Pearl Harbor		Diamond Head	
	Giardia	*Crypto*	*Giardia*	*Crypto*
Oct	<2	<2	<2	<2
Feb	2	<1	<1	<1
Jun	<1	2	<1	<1
Nov	1	<1	<1	<1

Table 4: Occurrence *of Giardia* or *Cryptosporidium* or both at Sampling Sites

Site	% positive for *Giardia*	% positive for *Crypto*	# samples
Ala Moana Beach	31	23	13
Waikiki Beach	31	31	13
Queens Beach	22	11	9
Sand Island Beach	0	0	3
Hanauma Bay	8	0	13
Diamond Head	0	0	4
Pearl Harbor	50	25	4
Manoa Stream	50	50	4
Ala Wai Canal	77	85	13
Ala Wai Canal/Ocean interface	100	0	3
Outfall	100	75	4
Primary effluent	100	100	13

Table 5 Average Density of Giardia and Cryptosporidium per 200 l at Various Sites

Site	Average *Giardia* Density	Average *Cryptosporidium* Density
Ala Moana Beach	1.7	0.7
Waikiki Beach	0.4	0.5
Queens Beach	0.2	0.2
Sand Island Beach	0	0
Hanauma Bay	0.08	0
Diamond Head	0	0
Pearl Harbor	0.8	0.5
Manoa Stream	4.8	1
Ala Wai Canal	3.2	1.9
Ala Wai Canal/Ocean interface	1.7	0
Outfall	36	17.3
Primary effluent	4.9×10^5	4.3×10^4

Table 6 Correlation Coefficients for *Giardia, Cryptosporidium,Salmonella*[1], enteroviruses[2] and adenoviruses[3] from all samples sites in Mamala Bay

	Giardia[a]	*Cryptosporidium*[b]	*Salmonella*[c]	enterovirus[d]	adenovirus[e]
Giardia	1.0	0.932	0.081	0.0223	0.030
Cryptosporidium	0.932	1.0	-0.029	0.192	0.017

[a&b&cd]N = 83 [c]N = 70 [e]N = 64

[1]Josephson, 1995
[2]Reynolds, 1995
[3]Enriquez, 1995

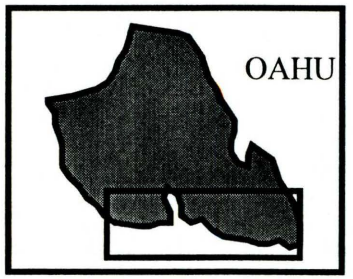

OAHU

Figure 1

Pearl Harbor
C
Monao
Stream
MS
SB
AM
AW
W
QB
D
Honouliulu
Outfall
Sand Island
Outfall
Ala Wai
Canal
HB
Diamond
Head
E
Hanauma
Bay
Mamala Bay
Key:
AM= Ala Moana Beach
AW= Ala Wai Canal
C= Pearl Harbor
D= Sand Island Outfall
E= Diamond Head
HB= Hanuama Bay
MS= Moana Stream
QB= Queen's Beach
W= Waikiki Beach

Figure 2 **Parasite Elution, Concentration and Evaluation Technique**

ELUTION

Cut Filter Apart

⬇

Divide Fibers into 3 containers containing 1 L eluting solution each

⬇

Handwash for 10 min

⬇

Concentrate via centrifugation at 1700 x g for 10 min

⬇

Store in 10% buffered formalin at 4 ^{O} C

FLOATATION

Take less than 1 mL of packed pellet and mix with 20 mL of eluting solution

⬇

25 mL of percoll-sucrose (sp. gr. 1.10) is layered under the sample

⬇

Centrifuge at 1050 xg for 10 min

⬇

Collect interface and concentrate to 5 mL via centrifugation

ANTIBODY STAINING

Filter sample onto a 1.2 micron polycarbonate filter

⬇

Stain with primary antibody for 30 min.

⬇

Rinse with PBS and Stain with Secondary antibody for 30 min

⬇

Rinse with PBS and place on glass slide with Dabco-glycerol and store at 4°C

DISCUSSION

The occurrence of *Giardia* and *Cryptosporidium* in marine waters has not been previously studied. Their occurrence in sewage has been well-documented and with the continuing discharge of sewage into the ocean, it is important to determine if *Giardia* and *Cryptosporidium* in sewage are potentially a risk for recreational transmission.

One possible source of contamination that was considered in this study was the bathers themselves. *Giardia* is the most commonly documented cause of gastrointestinal illness in the United States (Adam, 1991). Two negative control sites were chosen, Diamond Head and Hanauma Bay. Both sites were determined not to be impacted by the sewage plume (Blumberg and Connolly, 1995). No parasites were detected at the Diamond Head site. Hanauma Bay contained an average density of 0.08 *Giardia* cysts and 0 *Cryptosporidium* oocysts per 200 liters, the lowest from all the bathing beaches that were sampled monthly. This suggests that the bathers were not a major source of protozoan pathogens in marine waters. In comparison, the bathing beaches that contained the highest parasite density were Ala Moana > Waikiki > Queen's Surf. Both the outfall and Ala Wai Canal were important contributors to the contamination found within Mamala Bay. The canal appears to be a constant source of

pathogens, whereas the outfall can carry the plume to the shoreline during the Kona winds (low pressure cyclones which blow eastward accompanied by southerly winds during the winter) (Blumberg and Connolly, 1995). No parasites were detected at Sand Island Beach, the closest of all the beaches to the outfall, and in addition, it has a very small bathing population, however only a very limited number of samples (three) were collected from Sand Island Beach.

There were large numbers of *Giardia* and *Cryptosporidium* in the sewage (2.56×10^3 and 2.16×10^2 per liter, respectively) and at the outfall (average 36 cysts and 17.3 oocysts per liter). The amount of cysts and oocysts detected in the sewage compared to that reported in other studies. Sykora *et al.* (1991) reported an average of 3×10^3 *Giardia* cysts per liter of sewage from plants located in California and Florida. Less *Cryptosporidium* oocysts were found in Sand Island sewage than reported by Madore *et al.*, (1987). They reported a range of 850 to 13,700 oocysts per liter. Our lower numbers may reflect the lack of agricultural livestock farming on Oahu or lower infection rate among the population. The *Giardia* and *Cryptosporidium* were also present in 85% of the samples collected at Ala Wai Canal: 3.2 cysts and 1.9 oocysts.

Haas *et al.* (1995) calculated the risk of acquiring giardiasis in Mamala Bay using an exponential dose-response model. The assumptions he used were: all

Giardia cysts which detected were viable and 30-ml of water were ingested while swimming. He found that swimmers versus nonswimmers have a 6,200 times greater chance of acquiring giardiasis during certain periods of the year- when the Kona winds affect currents allowing the sewage plume to reach the bathing beaches (Haas *et al.*, 1995). For other periods of the year when currents carry the plume off-shore risks are significantly less.

This project was part of a multi-collaborative study in which sites were also monitored for *Salmonella*, enteroviruses and adenoviruses as well as *Giardia* and *Cryptosporidium*. There was a very strong correlation between the presence of *Giardia* and *Cryptosporidium* (r=0.932). No relationship could be defined between cysts or oocysts and presence of the other microorganisms. Strong correlation between the presence of *Giardia* and *Cryptosporidium* has been previously reported for freshwater rivers and lakes, but not other microorganisms (i.e. enteric viruses and coliforms) (Rose *et al.*, 1988; Rose *et al.*, 1991, Crabtree *et al.*, 1996).

Model of current patterns in Mamala Bay strongly suggest the influence of both the Sand Island sewage outfall and non-point sources such as Ala Wai Canal. Therefore, to reduce the risk of microbial illness and contamination, more than just treatment of sewage disposed in the ocean will be necessary. The non-

point sources must be further evaluated. In addition, the bathers seem to have the least impact as seen by the results at Hanauma Bay.

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7. Appendix B:

SURVIVAL OF *GIARDIA* IN MARINE WATERS

Dana C. Johnson[1], Carlos E. Enriquez[2], Ian L. Pepper[2], Charles P. Gerba[1,2] and Roger Fujioka[3]

Department of Microbiology and Immunology[1] and Department of Soil, Water and Environmental Science[2], University of Arizona, Tucson AZ 85721.

University of Hawaii at Manoa, Water Resources Research Center, Honolulu, HI 96822[3]

ABSTRACT

Discharge of sewage into the ocean is still a common method of disposal worldwide. Both treated and untreated sewage may contain significant concentrations of the waterborne protozoan parasite *Giardia*. However, information on the survival of protozoan parasites in marine waters is almost non-existent. This study examined the survival of *Giardia muris* cysts in marine waters. Viability was determined by excystation. *Giardia muris* was used as the model for this study because of its consistency and hardiness in excystation. *Giardia* cysts were placed in 500-ml plastic beakers containing 300-ml of marine water or in 100-ml McFeter chambers placed directly in the ocean. *Giardia muris* was found to survive in marine water from the coast of Hawaii up to 72 hours in the dark and less than three hours in the presence of sunlight. In the McFeter chambers, the cysts were reduced 66% (0.36 log) *in situ* compared to greater than 99.9% in the sunlight (roof) experiments within three hours. Under the conditions of this study, *Giardia* cysts are unlikely to survive for prolonged periods of time in marine water. In addition, *Giardia* does not survive as long as *Cryptosporidium* and poliovirus, but does persist longer than *E. coli* or *Salmonella* in marine water.

INTRODUCTION

Ocean outfalls are still a common method for disposal of domestic sewage worldwide. There is a concern that the microbial pathogens in the sewage might pose a health risk to nearby bathers and shellfish-harvesting areas. Infection with *Giardia* via recreational contact with contaminated water has been previously reported. During 1991-1992, there were four reported outbreaks of *Giardia* in the United States due to exposure to contaminated wading pools, swimming pools, and a lake (Moore *et al.*, 1993). Thus, the potential exists for the transmission of *Giardia* to persons who swim in marine waters. We have recently demonstrated the presence of *Giardia* in marine bathing waters (Johnson, *et al.*, 1995); however, information on its survival in these waters is not available. Previous studies on survival of *Giardia muris* cysts in lake, river and tap water demonstrate that it can survive for up to 28 and 56 days at 15 feet (19 °C, pH 7.7) and 30 feet (6.8 °C, pH 7.3), respectively, and up to 28 days (23 °C, pH 8.4) in a river (DeRegnier *et al.*, 1989). The focus of this study was to determine the survival of *Giardia* in marine waters. The only existing study on the survival of *Giardia muris* in artificial marine water (Jarroll *et al.*, 1984). Survival of *Giardia* was studied in marine water and in phosphate-buffered saline (PBS, pH 7.4). Samples were exposed to direct sunlight or placed in the dark and

assayed via excystation.

MATERIALS AND METHODS

The waters used in this study were collected from the shore at the surface from Hanauma Bay, Waikiki Beach, Black Point Beach and Ala Wai Canal located on Oahu, Hawaii. These sites were selected because previous studies have shown the occurrence of *Giardia* and *Cryptosporidium* (Johnson *et al.*, 1995) and they are heavily used for recreation (swimming and surfing). A 300 ml aliquot of test water was place in a 500-ml polyproplyene beaker. Samples not exposed to sunlight were covered with aluminum foil and placed in the dark. Samples exposed to sunlight were placed in polyproplyene beakers on the roof of a building (Hawaii and Arizona). The beakers exposed to light were placed in a styrofoam container filled with water and ice to regulate temperature (20 - 28 °C). The styrofoam container was placed on a stir plate so the samples could mix gently. A 40-ml aliquot was taken from each sample at the indicated intervals and viability was determined by excystation.

McFeter chambers were assembled as described by LaBelle and Gerba (1980) and tested in the water at Ala Moana Beach. This site was selected because previous studies found it to be contaminated with *Giardia* and

Cryptosporidium and the calm water would allow for the use of the chambers.

In addition, water was filter-sterilized via a 0.2-micron, 25-mm syringe filter (Nalgene, Rochester, NY) to compare sterile and nonsterile water and rates of inactivation. The experiments were later duplicated with autoclaved water.

Physical and chemical parameters of test waters were measured and recorded in Table 1. Salinity was measured with a refractometer and NTU was measured with a turbidity meter. Fujioka and Loh (1995) assayed for *Enterococci*, *Escherichia coli*, fecal coliforms and *Clostridium perfringens* as described in Standard Methods for the Examination of Water and Wastewater (APHA, 1992).

Giardia muris cysts (Shiwaji Ramaligam, Beaverton, OR) were produced in infected mice. Cysts were placed in 500-ml polyproplyene plastic beakers containing either marine water, canal water, or phosphate-buffered saline (PBS) pH 7.4. Cysts were between three and five days old at time of the experiment and gave greater than 90% excystation. Aliquots were periodically removed from the beakers or McFeter chambers and concentrated via centrifugation (1700 x g). The pellet was placed into a 2 ml polypropylene conical centrifuge tube with a screw cap (VWR Scientific, West Chester, PA). The sample was further concentrated to 0.2 ml. Next, 800 μl of reducing solution (1% glutathione, 1%

L-cysteine-HCl and Hank's balanced salt solution, Sigma, St. Louis, MO) and 800 μl of 0.1 M sodium bicarbonate (Fisher Scientific Co., Fairlawn NJ) were added to each sample and were incubated at 37 °C for 30 minutes. The cysts were then pelleted again and washed once in trypsin-tyrode's (0.5% trypsin and 1 X tyrode's solution) solution and then incubated at 37 °C for 45 minutes. Then 10-μl aliquots were removed from each tube and placed in a hemocytometer (Baxter Health Care Corp, McGraw Park, IL). Samples were examined under 400 X magnification with a phase-contrast microscope (BH-2, Olympus, Japan). All experiments were completed in duplicate or triplicate. A detailed protocol for excystation can be found in *Guidance Manual for Compliance with Filtration and Disinfection Requirements for Public Water Systems Using Surface Water Source* (U. S. EPA, 1991).

Empty, partially excysted, trophozoites, or intact cysts were enumerated. The percentage excystation was calculated using the following formula:

$$\% \text{ excystation} = \frac{ECW + PET}{ECW + PET + IC}$$

ECW = Empty cyst wall
PET = Partially excysted trophozoites
IC = Intact cysts

Table 1

Water Quality of Test Sites

Parameters	Black Pt. Beach	Waikiki Beach	Hanauma Bay	Ala Wai Canal	Ala Moana Beach	Phosphate buffered saline
Temp. °C	22-26	22-26	22-26	22-26	22-27	22-27
pH	8.0	8.3	8.3	8.2	8.1	7.4
Salinity (mg/l)	35	33-35	33-35	28	35	8
Turbidity (NTU)	0.4	2.5	2.6	4	2.6	<1
Enterococci*	no data available	3.9	6.1	116.6	3.4	
*E. coli**		2.8	3.3	206.3	2.9	
Fecal coliform*		2.8	5.9	403.3	3.5	
*C. perfringens**		0.3	0.6	15.9	0.3	

*Geometric average over one-year period- CFU/100 ml (Fujioka and Loh, 1995)

RESULTS

Cyst survival varied from water collected at the different sites Cysts placed in water from the Ala Wai Canal survived longer- up to 72 hours while those in water from Hanauma Bay or Waikiki Beach only survived 48 hours (Figure 3).

Viability of the cysts persisted longer in the dark than those held in direct sunlight. Figure 1 shows results of cysts in the dark and direct sunlight. Cysts in the Ala Wai Canal and Black Point Beach showed a three-log reduction in viability after three hours in direct sunlight, whereas cysts placed in the same water in the dark did not show a three-log reduction until 77 hours. The same effect of sunlight was observed when the cysts were held in phosphate-buffered saline (PBS) (Figures 1 and 2). The experiment shown in Figure 2 is a duplicate of the experiment in Figure 1. Because a three-log inactivation was observed in marine water in sunlight at three hours (the first sample point), the experiment was repeated to determine if the three-log inactivation occurred earlier.

The survival of cysts in water from Hanauma Bay, Waikiki Beach and Ala Wai Canal, and tap water before and after filtration through a 0.2-μm filter was evaluated. This was done to assess the effect of turbidity and/or bacteria on the survival of *Giardia*. There was no significant difference in the filtered and

unfiltered samples of marine water, suggesting that bacteria were not involved in the survival of the cysts during this period of time. However, cysts were inactivated more rapidly in the unfiltered Ala Wai Canal.

Figure 1 Effect of Sunlight on the Inactivation of *Giardia muris*

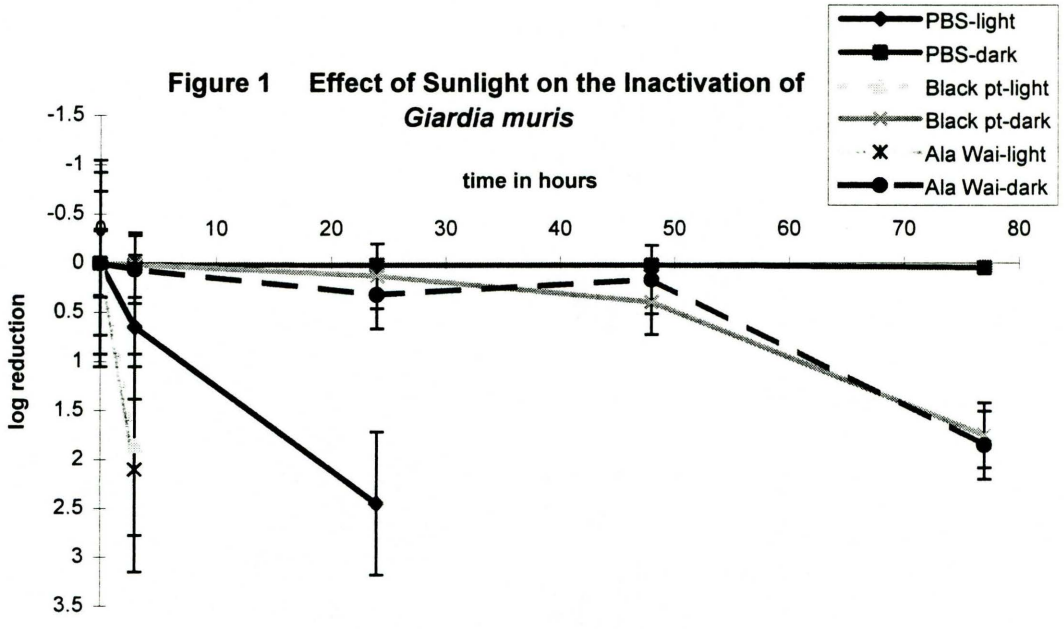


Figure 2 Effect of Sunlight on the Inactivation of *Giardia muris*

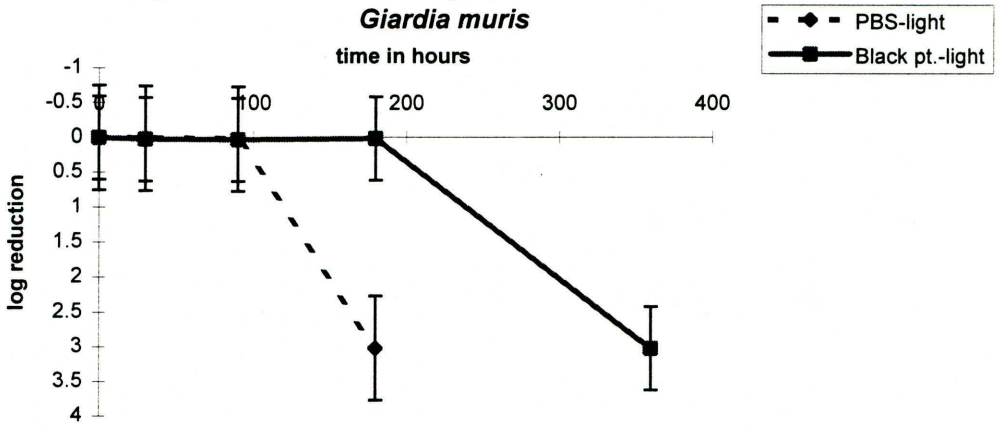


Figure 3 Survival of *Giardia muris* in the dark

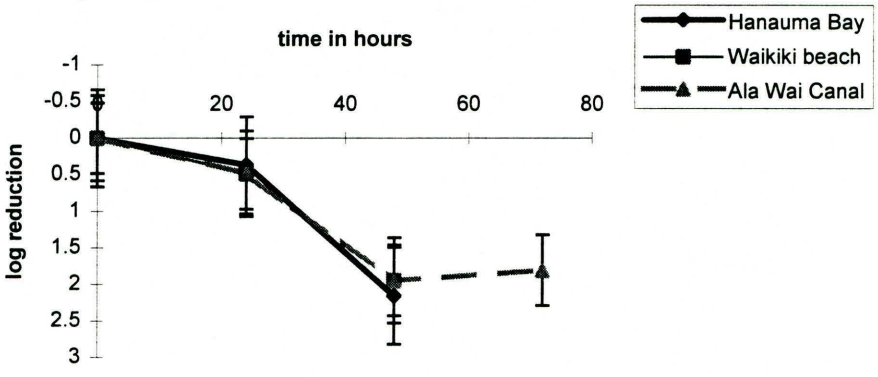
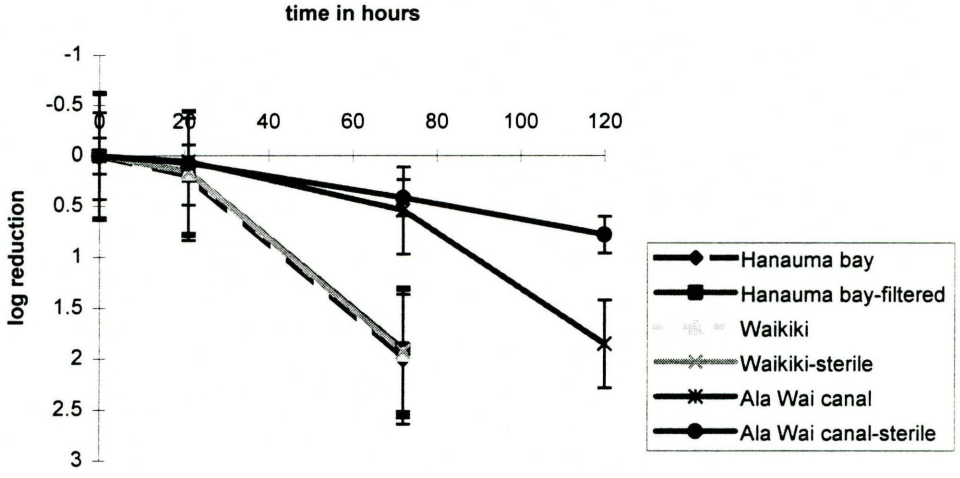


Figure 4 Inactivation of Giardia muris in filtered and unfiltered marine water



DISCUSSION

Salinity appears to play a significant role in the survival of *Giardia muris* (Figure 3). Cysts held at 35-ppt salinity were inactivated more rapidly than those in canal water (28-ppt), PBS (8-ppt) and tapwater (0-ppt) (Table 1). It was also observed that under phase-contrast microscopy the contents of the cysts hyperplasmolyzed, i.e., the contents of the cyst shrivel up inside the cyst as water moves out due to the hypotonic marine water outside of the cyst. In addition, there was no difference in the inactivation of cysts in sterile or nonsterile water (either by filtration or autoclaving), also supporting the hypothesis that salinity was responsible for inactivation and not a microbial antagonist (Figure 4).

The presence of light also appears to play a role in the inactivation of the cysts (Figure 1). In the absence of light, the cysts survived for as long as 77 hours in marine water and even longer in PBS (89% viability at 77 hours), whereas in the presence of light the cysts survived up to 3 hours in marine water and 6 hours in PBS (Figure 1). The lethality of sunlight (UV light) on microorganisms has been previously observed for enteric bacteria (Evison, 1988).

The experiment described in Figure 2 is a duplicate experiment of the experiment shown in Figure 1, designed to better define the rate of *Giardia*

inactivation. It was observed in the Black Point sample that at 30 and 90 minutes there was no inactivation, but after three hours there was a sudden three-log reduction. The same rapid inactivation was also observed in PBS. This sudden decrease in viability as opposed to gradual inactivation may be correlated with the permeability of the cyst wall due to the combination of sunlight and saline concentration.

McFeter chambers were used *in situ* at Ala Moana Beach to determine if inactivation was similar to that observed in experiments conducted in the laboratory. After exposure to direct sunlight in plastic beakers, there was a 99.9% reduction of cysts at three hours. However, *in situ* at three hours, there was only a 66.6% reduction. The difference between inactivation might be because the white Nylon membrane (MSI, Westboro, MA) used in the McFeter chamber blocked direct sunlight, which reduced the rate of inactivation. Together, both salinity and light inactivated the cysts within 3-6 hours.

The survival of *Cryptosporidium*, poliovirus-1, *Salmonella* and *E. coli* was concurrently conducted under the same set of conditions during the *Giardia* experiments. The T_{99} (time to achieve a 99% reduction) and T_{90} (90% reduction) are compared in Tables 2 and 3.

Sunlight had the greatest effect on survival of all microorganisms studied.

In all data sets, microorganisms in the dark and in phosphate-buffered saline survived longer than in the light. *Cryptosporidium* survived the longest of all the enteric organisms (Tables 1 and 2). The order of survival (T_{90}) (in the sunlight or dark, or canal water in the light) was: *Cryptosporidium* > poliovirus-1 > *Giardia* > *Salmonella.* The order of survival for T_{99} poliovirus-1 > *Giardia* > *Salmonella* > *E. coli.* In both experiments, poliovirus-1 survived longer than *Giardia* in marine water.

However, the order of survival (T_{90}) in canal water in the dark altered, in which *Giardia* survived slightly longer in canal water than in marine water. For poliovirus-1, however, one log was rapidly lost within 3.2 hours in marine water and two logs within 72 hours. The opposite trend is observed in canal water; poliovirus-1 required 30 hours to be reduced by one log, but then only needed 44 hours for a two log reduction. The fact that *Giardia* survives longer in canal water than in marine water suggests that salinity is primarily responsible for its inactivation. This does not appear to be the case for poliovirus-1, *Salmonella, E. coli* or *Cryptosporidium.*

Table 2 Extrapolated T_{90} of Microorganisms in Various Marine Waters and Phosphate-Buffered Saline (time in hours)

Water source	*Giardia muris*	*Cryptosporidium*[1]	Poliovirus-1[2]	*Salmonella*[3]
Black Pt Beach-dark	59	144*	3.2	60*
Black Pt Beach-light	1.9	72	3.1	0.15
Ala Wai Canal-dark	40 - 63	132*	30	30
Ala Wai Canal-light	1.9	72	6	0.15
PBS-dark	>77 [4]	720*	not done	104*
PBS-light	3.75	75	not done	1

[1]Rose and Davis, 1995
[2]Enriquez, 1995
[3]Pepper, 1995
[4]0.05 log reduction at 77 hours
* extrapolated

Table 3 Extrapolated T_{99} of Microorganisms in Various Marine Waters and Phosphate-Buffered Saline (time in hours)

Water source	*Giardia muris*	Poliovirus-1[1]	*Salmonella*[2]	*E. coli*[3]
Black Pt Beach-dark	78	72	120*	
Black Pt Beach-light	2.4	6	0.75	0.25
Ala Wai Canal-dark	83	42	44	
Ala Wai Canal-light	3	12	0.75	
PBS-dark	>77[4]	not done	208*	
PBS-light	4.6	not done	3	0.5

[1]Enriquez, 1995
[2]Pepper, 1995
[3]Fujioka and Loh, 1995
[4]0.05 log reduction at 77 hours
*extrapolated

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8. Appendix: C

IMPROVED METHOD FOR CONCENTRATION OF *GIARDIA*, *CRYPTOSPORIDIUM* AND ENTEROVIRUSES FROM WATER

Dana C. Johnson[1], Pamela M. Watt[2], and Charles P. Gerba[1,2]

Department of Microbiology and Immunology[1] and Department of Soil, Water and Environmental Science[2], University of Arizona, Tucson AZ 85721.

ABSTRACT

Methods for the concentration of enteric viruses, *Giardia* and *Cryptosporidium* from drinking water require the use of two different types of filters. Electropositive or electronegative microporous filters (0.2-0.45-μm nominal porosity) are used for collection of enteroviruses and a polyproplyene spun fiber filter (1-μm porosity) is used for collection of parasites from water. The filter for parasite concentration mechanically traps the organisms by size exclusion and therefore a microporous filter could conceivably be used for co-collection of parasites. This study compared the efficiency of their concentration from tap water with a polypropylene fiber cartridge (DPPPY) and two different microporous filters (Filterite and 1MDS). Cysts and oocysts were added to 400 liters of tapwater or tertiary-treated wastewater which was filtered through a polypropylene fiber cartridge filter at a flow rate of 8 liters per minute, and then eluted with Tween 80/SDS (sodium dodecyl sulfate) solution. For the Filterite filter, the pH of the dechlorinated water was adjusted to 3.5 and 0.005M aluminum chloride added to facilitate virus adsorption. No adjustment was necessary for the 1MDS filter as the pH of the water was below 8.5. Viruses and parasites were eluted from the filter by back-washing (elution in the opposite

direction of collection) under nitrogen pressure with two consecutive elutions of 900 ml of 1.5% beef extract/0.1% Tween 80. The eluent was collected and parasites concentrated via centrifugation and detected with indirect immunofluorescent antibodies. The supernatant was assayed for viruses on the BGM cell line. The overall efficiency was greater for the Filterite filter, 40.4% and 36.6%, when compared to the DPPPY filter, 10.1% and 16.0% for both *Giardia* and *Cryptosporidium*, respectively. The Filterite filters are easier and faster to process than the DPPPY filter. There was no significant difference in parasite recovery between the 1MDS and DPPPY filters. Sampling for both viruses and parasites on the same filter reduces both time and cost of routine sampling.

INTRODUCTION

Current methods for the concentration of viruses and parasites from large volumes of water requires the use of different filters. Viruses are concentrated by adsorption onto either a microporous electropositive or electronegative filter which function by adsorbing the virus by charge onto the filter surface (Gerba, 1983). Enteroviruses have an overall electronegative charge in neutral waters (pH 6-8) and therefore adsorb to electropositively charged filters (Sobsey and Jones, 1979). Adsorption to electronegative filters is enhanced by adjusting the pH of the water to 3.5 and by adding of cationic salts (trivalent aluminum chloride) which reduce the negative charge on the virus and filter and enhancing adsorption onto the negatively-charged filter (Gerba, 1983). Parasites are entrapped onto the filter by size exclusion. *Giardia lamblia* is 11 to 16 microns long and 5 to 9 microns wide (Meyer and Schaefer, 1985) and *Cryptosporidium parvum* is 4 to 6 microns in diameter (Schmidt and Roberts, 1989). They are trapped in the 1-μm nominal porosity polyproplyene fiber filter used for their collection from water. The microporous filters have a nominal porosity of 0.22- to 0.45-μm. Microporous filters would allow for entrapment of the parasites *Giardia* and *Cryptosporidium*. Payment *et al.* (1989) used 3- and 1-μm wound electronegative fiberglass Diamond Filter cartridges (Filterite, Timonium, MD)

in series to co-collect *Giardia lamblia*, *Legionella pneumophila*, *Clostridium perfringens*, human enteric viruses and *coliphages* from 1000 liters of tapwater. He found the recovery efficiency for *Giardia* to be 52%. Current methods for detection of *Giardia* and *Cryptosporidium* frequently have low and variable efficiency. Table 1 demonstrates the variability observed by different investigators.

Table 1 Reported Recovery Efficiencies For *Giardia* and *Cryptosporidium*

Filter type	Sample type	flow rate l/min	volume filtered (l)	% recovery *Giardia*	% recovery *Cryptosporidium*	Ref.
Micro Wynd II[1]	dechlorinated tapwater	3.78	378	ND	44	Musial *et al.*, 1987
Micro Wynd II[1]	Tapwater & activated sludge treated sewage effluent	not stated	378	13-22	29-58	Rose *et al*, 1991
Micro Wynd II[1] Filterite[2] Honeycomb Filter tube[3]	"raw" water[4]	NA	NA	0.8-22.3 ave. 9.15	1.3-5.5 ave. 2	Clancy *et al.*, 1994
No Filter-spiked sediment	tapwater sediment	NA	NA	66-93.7	61.7-89.9	LeChevallier *et al.*, 1990
No Filter-spiked sediment	raw water sediment	NA	NA	48	42	LeChevallier *et al.*, 1991
Filterite[2]	tapwater	10-40	1000	52	ND	Payment *et al.*,1989

[1]AMF/CUNO, Merridian, CT [2]Filterite Corp., Timonium, MD [3]Commercial Filters, Parker Hannafin Corp, Lebanon, IN
[4]0.25-g sediments mixed into 20-30 g water- 5 -ml inoculated into filter ND= not done NA=not applicable

MATERIALS AND METHODS

The electronegatively-charged, pleated, 25.4-cm Filterite cartridge filter (Filterite Corp., Timonium, MD) (0.45-μm nominal porosity) and Virosorb 1MDS (pleated, 25.4-cm) (Cuno, Merridian, CT) were chosen for this study because the nominal porosity allows for the mechanical entrapment of *Giardia* and *Cryptosporidium*, and they are commercially available for virus concentration from water.

The efficiency testing was conducted with both dechlorinated Tucson tapwater and unchlorinated tertiary treated effluent (activated sludge and coal-filtered) from a wastewater treatment plant (Table 2). A large plastic tank was filled with 400 liters of test water. Approximately 1 x 10^5 of *Giardia lamblia* cysts (Waterborne Inc., New Orleans, LA), *Cryptosporidium parvum* (Waterborne Inc., New Orleans, LA) and poliovirus (LSc-1 strain) (ATTC, Rockville, MD) were added to 200 ml of test water in a 500-ml polyproplyene beaker. The poliovirus was used only when evaluating the Filterite and 1MDS filters. A 200-μl aliquot of Tween 80 (Sigma, St. Louis, MO) was added to the test water to aid in the dispersion of the parasites. A 5-ml aliquot was removed from the seed stock for enumeration of the influent. The remaining 195 ml were added to the 400 liters test water and mixed with a submersible pump (Dayton Electric MFG., Chicago, IL) for ten minutes. The water was then filtered through the test filter

at a flow rate of 8 liters per minute using an electric pump (Homelite, Charlotte, NC).

The DPPPY filters were eluted by shredding and hand-washing the fibers in eluting solution (0.1% Tween 80/SDS) for 30 minutes (APHA, 1992). The eluent was concentrated via centrifugation and the pellet was collected for parasite assay (Figure 1).

The 1MDS and Filterite filters were immediately eluted by back-washing with 900 ml of 1.5% beef extract (pH 9.4) (Becton Dickinson, Cockeysville, MD) containing 0.01% Tween 80 (Sigma, St. Louis, MO). The process was repeated with an additional 900 ml of 1.5% beef extract/Tween 80. The eluents were combined and the pH adjusted to 7.2 - 7.3 with 1 N HCl and then centrifuged at 2700 x g. The supernatant was collected for virus assay and the pellet was collected for parasite assay (Figure 1).

Virus assay:

The supernatant was reconcentrated via organic flocculation by lowering the pH of the supernatant to 3.5 with 1 N HCl and stirring for 15 minutes. The precipitate was resuspended in sodium phosphate buffer (pH 7.2) and pelleted by centrifugation at 3000 x g for 30 minutes. The pellet was resuspended in 30 ml of sodium phosphate buffer and the pH was adjusted to 7.2 - 7.4 with 1 N NaOH. The reconcentrates were immediately assayed or stored at -70° C until

assayed.

Viruses were quantified on the Buffalo Green Monkey kidney cell line (BGM, ATTC, Rockville, MD) by the plaque assay technique (APHA, 1992). Dilutions of the supernatant were inoculated onto a cell monolayer, incubated for one hour, overlayed with Eagle's minimal essential media (Irvine Sci., Irvine, CA) containing 2% Fetal Bovine Serum (Sigma, St. Louis, MO) and 1% agar, and incubated for 3 - 5 days in a CO_2 incubator at 37 °C. Cells were stained with 1% crystal violet (Sigma, St. Louis, MO) to visualize the plaques.

Parasite assay:

The pellet was preserved in 10% buffered formalin and assayed for parasites by taking a portion of the pellet and purifying it in a percoll-sucrose (sp. grav. 1.10) (Sigma, St. Louis, MO) density gradient (APHA, 1994 and EPA, 1993). The cysts and oocysts were concentrated and clarified at the interface. The supernatant and the interface were collected, filtered through a polycarbonate membrane (Costar, Pleasanton, CA), and stained with antibodies specific for *Giardia* and *Cryptosporidium* (EnSyn, Research Triangle Park, NC). Samples were stored at 4 °C and read within five days (APHA, 1994 and EPA, 1993).

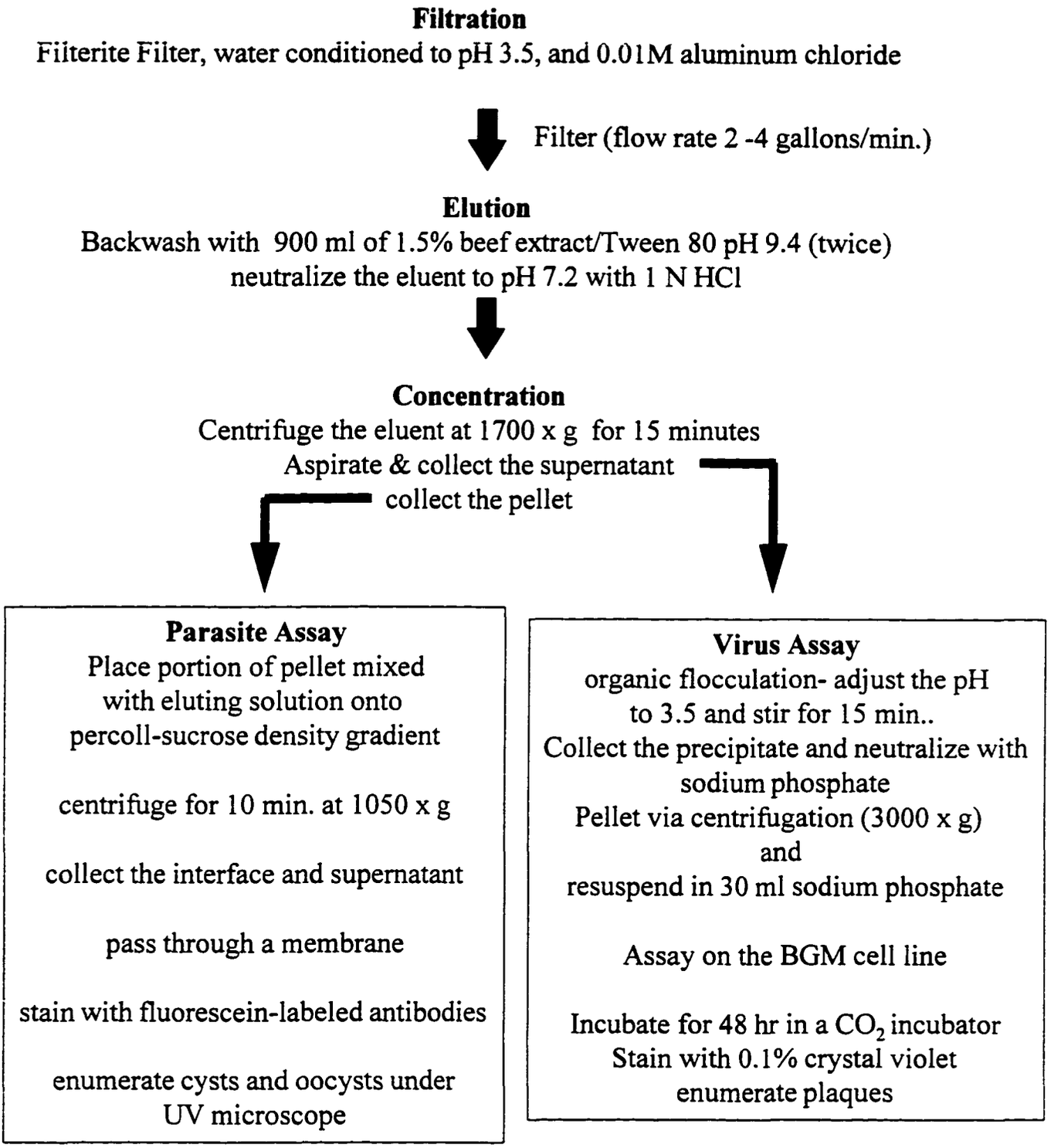
Membranes were read at 200 X magnification under a UV fluorescent microscope (BH-2 Olympus microscope). *Giardia* cysts and *Cryptosporidium*

oocysts were identified by size, shape and characteristic fluorescence at 400 X magnification (APHA, 1994 and EPA, 1993).

Table 2 Test water parameters

	Turbidity (NTU)	Total Dissolved solids (TDS) mg/L	pH
Tapwater	<1.0	200-300	7.1-7.9
Tertiary-treated wastewater	<1.0	500-600	6.2-7.0

Figure 1 **Co-collection of Parasites and Viruses** 104



RESULTS

Table 3 Percent Recovery of Cysts and Oocysts from 400 liters of Water with the DPPPY Filter

Water type	*Giardia*	*Cryptosporidium*
Tapwater	9.8	6.7
	37.8	17.1
	23.1	18.5
	13.7	27.8
	1.5	14.0
	4.0	58.2
Tertiary-treated Wastewater	10.3	7.2
	13.9	12.4

Table 4 Percent Recovery of Cysts, Oocysts and Poliovirus from 400 liters of Water with the Filterite Filter

Water type	*Giardia*	*Cryptosporidium*	Poliovirus-1
Tapwater	43.3	ND	ND
	43.5	29.0	ND
	32.4	20.6	ND
	31.3	24.0	55.9
	34.4	32.0	35.2
Tertiary-treated Wastewater	60.0	77.6	69.8
	44.3	47.9	ND

ND = not done

Table 5 Percent recovery of Cysts, Oocysts, and Poliovirus-1 from 400 liters of water with the 1MDS filter

Water type	*Giardia*	*Cryptosporidium*	Poliovirus-1
Tapwater	15.5	11.1	ND
	18.1	18.1	86.4
Tertiary-treated Wastewater	18.9	25.5	93.2
	32.9	20.7	ND

ND = not done

Table 6

Summary of Efficiency Results

		Giardia lamblia		*Cryptosporidium parvum*	
Filter type	Water type	Geometric mean	Standard deviation	Geometric mean	Standard deviation
DPPPY	Tapwater	9.4	±13.6	19.1	±18.2
	Tertiary-treated wastewater	12.0	±2.6	9.5	±3.7
	Tap & wastewater	10.1	±11.6	16.0	±16.8
Filterite	Tapwater	36.6	±6.0	28.4	±5.9
	Tertiary-treated wastewater	51.6	±11.1	61.0	±21.0
	Tap & wastewater	40.4	±10.0	36.6	±20.4
1MDS	Tapwater	16.8	±1.9	14.2	±5.0
	Tertiary-treated wastewater	24.9	±9.9	23.0	±3.4
	Tap & wastewater	20.4	±7.8	18.1	±6.0

Data Analysis:

Recovery efficiency results were obtained with the following formula:

$$\frac{\text{total seed influent}}{\text{total recovered in effluent}} \times 100 = \%\ \text{recovered}$$

The p value was calculated with Excel (Microsoft Corp, United States) ANOVA data analysis program and the geometric mean and standard deviation were also calculated with the Excel program. The geometric mean was employed because it utilizes the $\sqrt[n]{}$ which prevent atypical numbers from skewing the average.

DISCUSSION

The efficiency of current methods for the collection of parasites from water is frequently low and inconsistent. Previous results of other investigators have shown efficiencies to vary greatly (Table 1). The efficiency of recovery from the DPPPY filter (geometric mean and standard deviation of 10.1% $\pm$11.67 for *Giardia lamblia* and 16.0% $\pm$16.75 for *Cryptosporidium parvum*) was lower than previously reported (Tables 3 and 6). The recovery efficiency obtained with the Filterite filter is considerably higher ($p=0.00076$ and $p=0.067$ for *Giardia* and *Cryptosporidium*, respectively) with a geometric mean and standard deviation of 40.4% $\pm$10.0 for *Giardia* and 36.6% $\pm$20.4 for *Cryptosporidium* (Tables 4 and

6). The microporous filter may collect more efficiently because the nominal porosity is smaller (0.2-0.45μm) compared to 1.0-μm for the DPPPY polyproplyene filter. This smaller porosity may entrap the parasites onto the surface of the filter instead of becoming deeply embedded within the filter, the latter being more difficult to elute from the filter. In addition, the parasites are removed from the Filterite filter by backwashing under nitrogen pressure, whereas the DPPPY filter is shredded apart and handwashed. The hand-washing method may allow for more variability, depending how thoroughly the hand-washing step is completed. It was also observed that samples collected with the Filterite filters had less background on observation under UV fluorescence (autofluorescence and debris) than the DPPPY filters. The recovery efficiency for poliovirus-1 was within the range previously reported and was not evaluated further (Sobsey and Jones, 1979). The recovery efficiencies from the 1MDS filter (geometric mean and standard deviation of 20.4% $\pm$7.8 for *Giardia* and 18.1% $\pm$6.0 for *Cryptosporidium*) were not significantly different from the DPPPY filter, however, the standard deviation was less (Tables 3, 5 and 6). It is not understood why the Filterite filter may collect parasites better than the 1MDS filter. It may be because the positive charge on the filter or the filter matrix (glass and cellulose charge-modified electropositive medium) may interfere with

either adsorption or elution of parasites from the filter.

The recovery efficiency from tertiary-treated wastewater when compared to tapwater was usually better, especially with the Filterite filter (Table 6). This may be because the parasites (formed from the debris in the water) visually helps avoid aspirating away the pellet and parasites attached to the debris. However, too large a pellet may hinder recovery for it may be difficult to separate the cysts and oocysts from the debris during flotation.

An advantage of the Filterite filter is the elimination of the hand-washing step (a very tedious and variable step), which is replaced with the faster back-washing step. In addition, parasites and viruses can be collected on the same filter which saves both time and expense.

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