Objectives

1. To determine the presence and levels of *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts in surface supplies of drinking water for six North Carolina piedmont cities.

2. To examine associations of *G. lamblia* cyst and/or *C. parvum* oocyst levels with levels of fecal coliforms, enterococci and turbidity in the above waters.

Introduction

Giardia lamblia and *Cryptosporidium parvum* are enteric pathogenic protozoans which follow the fecal-oral route of transmission. Their environmentally resistant forms (cysts and oocysts, respectively) can be carried by fecally contaminated water and may cause outbreaks of enteric disease (Craun, 1984; Craun 1985; D'Antonio et al., 1985; NPDWR, 1987; Veazie, 1969). These protozoans have the potential to be a serious public health threat. Since 1965, approximately 100 giardiasis and 2-3 cryptosporidiosis waterborne outbreaks, infecting more than 23,000 people, have been reported in the United States (Jakubowski, 1988).

The Environmental Protection Agency (EPA) considers all surface waters to be at risk of contamination by *G. lamblia* (NPDWR, 1987). The 1986 amendments to the Safe Drinking Water Act (SDWA) call for the promulgation of a National Primary Drinking Water Rule (NPDWR) by June 1989. This will regulate *G. lamblia* by way of treatment standards. The EPA has put *C. parvum*

on the first Drinking Water Priority List (DWPL), because it is at least as prevalent as G. lamblia in surface waters (Rose, 1988), and it may be as great a public health risk. This means that C. parvum will be considered for regulation in the near future (NPDWR, 1987). Projected costs to comply with these proposed regulations may reach $2.75 billion if plants now treating with disinfection-only are required to add filtration, the current best available treatment for removal/inactivation of cysts and oocysts (NPDWR, 1987). Thus, it is important for Water Treatment Plants to assess the G. lamblia and C. parvum contamination of their surface source waters.

Current methods used to assess microbiological quality of drinking water with respect to fecal contamination are now based on a bacterial indicator system, specifically the presence and concentrations of total coliform bacteria. Since G. lamblia and C. parvum are transmitted by the fecal-oral route, it is possible and would be fortuitous if G. lamblia and C. parvum presence and concentrations could be predicted using this system; especially since detection and quantitation of these protozoans is costly and technically difficult (NPDWR, 1987). However, no significant associations of G. lamblia or C. parvum with total coliforms have been found (Akin et al., 1986; Craun and Jakubowski, 1987; Rose et al., 1988).

It may be possible to predict or associate G. lamblia and/or C. parvum presence with the presence of other bacterial indicators of fecal contamination, such as fecal coliforms or

enterococci (NPDWR, 1987). A few studies have examined associations of fecal coliforms with these protozoans and found none (Craun and Jakubowski, 1987; Rose, 1988; Rose et al., 1988). However, no studies have been performed on surface waters in the southeastern U.S., and no studies have been performed examining associations of either G. lamblia or C. parvum with enterocci.

This study examines the untreated (raw) and fully treated (finished) surface drinking water supplies of six North Carolina Piedmont cities for the above protozoans and examines associations of either with fecal coliforms and enterococci.

Literature Review

Descriptions of the Agents

Giardia lamblia is a protozoan parasite with two stages in its life cycle: a flagellated trophozoite and an environmentally resistant cyst. The first description of *G. lamblia* and its first association with intestinal disease was reported in 1681 by Anton von Leeuwenhoek, in a letter to Robert Hooke, Secretary of the Royal Society of London, (Dobell, 1920).

Trophozoites are motile forms which normally inhabit the upper 1/3 of the small intestine. They are 12-15 μm by 6-8 μm, resemble a pear cut in half lengthwise, and are binucleate with 4 pairs of flagella arranged in bilateral symmetry. There is a ventral disc at the wide, anterior end, by which the trophozoites attach themselves to the microvillous border of the intestinal epithilial cells (Feely et al., 1984). As trophozoites are swept along the intestine they are induced to encyst (Schaeffer, 1988).

Cysts are the environmentally resistant forms which are responsible for the spread of *G. lamblia* to new hosts. They are ovoid, 6-12 μm long and enclosed in a cyst wall 0.3-0.5 μm thick (Feely et al., 1984).

C. parvum is a coccidian protozoan parasite which infects the mucosal epithelium of humans and various other animals, usually juveniles. (Angus, 1983; Jokipii et al., 1983; Navin and Juranek, 1984; Tzipori, 1983; Upton and Current, 1985). The first reported cases of human cryptosporidiosis occurred in 1976

in a 3 year old immunocompromised girl (Nime et al., 1976), and a 39 year old man undergoing chemotherapy (Meisel et al., 1976).

C. parvum goes through complete development in a single host (Soave and Armstrong, 1986). Its life cycle has 6 major developmental events: "...excystation, merogony (asexual reproduction), gametogony (gamete formation), fertilization, oocyst wall formation, and sporogony (sporozoite formation)." (Current, 1987). Infection occurs via oocysts which develop from zygotes. Oocysts are spherical to slightly ovoid, 4-6 μm in diameter, and have a suture line which spans 1/3-1/2 of their circumference (Jakubowski, 1988). Approximately 80% of these oocysts have thick walls (Soave and Armstrong, 1986; Current and Reese, 1986), sporulate within the host and most pass through the gut unaltered and into the environment (Jakubowski, 1988). The other 20% of oocysts develop with thin walls which rupture soon after being released by the host cell. Free sporozoites are released and reinitiate the endogenous developmental cycle; they are not excreted (Current and Reese, 1986). This is a developmental feature unique amongst coccidia infecting warm-blooded vertebrates and it gives them an enormous potential for host reinfection (Soave and Armstrong, 1986; Jakubowski, 1988).

Giardiasis and Cryptosporidiosis

The effects of ingesting G. lamblia cysts or C. parvum oocysts range from severe enteric illness, to asymptomatic

illness, to no illness (Rendtorff, 1954; Current et al., 1983; Jokipii et al., 1985; Baxby and Hart, 1986). The clinical symptoms of acute giardiasis and cryptosporidiosis are nearly indistinguishable and may include: profuse,watery diarrhea; abdominal cramping; nausea; vomiting; anorexia; flu-like headache or low-grade fever (Jokipii et al., 1983).

Infectious doses are dependent on the host. As few as 10 G. lamblia cysts have proven infectious to humans; however, the true infective dose may be smaller as these cysts were not checked for viability and the sample size was only 2 (Rendtorff, 1954). No tests have been performed on humans to determine an infective dose of C. parvum. 10 oocysts were infective for 2 infant non-human primates (Macacas nemestrina) (Miller et al., 1986), which suggests a similar low infective dose for humans.

Mean incubation and duration times for both diseases vary with the study. Incubation times determined for giardiasis are: 9 days (Rendtorff, 1954), 11.3 days (Kent et al., 1988), and 7 days (Jokipii et al., 1983). The incubation time for cryptosporidiosis is approximately 7 days (Jokipii et al., 1983; Jokipii and Jokipii, 1986). Acute cryptosporidiosis exhibits a median duration in immunocompetent patients of 10 days (Jokipii et al., 1983) with a range of 1-4 weeks (Jokipii and Jokipii, 1986; D'Antonio et al, 1985).

Giardiasis may last for periods ranging from days to years. The acute phase may last a few days, sometimes months, and resolves spontaneously. Often a chronic-subacute stage follows

with symptoms that are mild to moderate expressions of the acute stage and occur as brief episodes. These symptoms may continue for years but usually resolve sooner (Wolfe, 1984). Immunodeficient individuals are no more likely to become infected than immunocompetents, but they are more likely to exhibit (develop) symptoms (Meyer and Radulescu, 1979).

Three drugs available in the United States are used to treat giardiasis: Quinacrine (Atabrine), Metronidazole (Flagyl), and Furazolidone (Furoxone). All have side effects that may be troublesome and none have a 100% cure rate; the average is 90-95%. Furazolidone, though FDA approved, has caused mammary tumors in rats. Metronidazole is contra-indicated during pregnancy (Wolfe, 1984).

Cryptosporidiosis most commonly affects children (Soave and Armstrong, 1986; Navin and Juranek, 1984; Hunt et al., 1984; Wolfson et al., 1985), and it usually affects immunocompenent and immunocompromised individuals in different ways. Immunocompetents who experience symptoms usually develop an acute, self-limiting diarrheal illness similar to cholera (Current et al., 1983; Wolfson et al., 1985; Fletcher et al., 1982), with brown-green and offensive watery diarrhea usually lasting 3-12 days. Symptoms may persist for 2-8 weeks (Current, 1987; Tzipori, 1988). Often, oocysts are shed days to weeks after symptoms have resolved (D'Antonio et al., 1985; Jokipii and Jokipii, 1986).

In immunocompromised individuals, cryptosporidiosis may

develop into a chronic, life-threatening diarrheal illness (Current et al., 1983; Jokipii et al., 1983; Navin and Juranek, 1984). This can happen to individuals with congenital immunodeficiencies (Nime et al., 1976; Current et al., 1983), to individuals undergoing immunosuppressive chemotherapy (Meisel et al., 1976) or to patients with acquired immunodeficiency syndrome (AIDS) (Current et al, 1983; Laughon et al., 1988). Fluid loss amongst AIDS patients with cryptosporidiosis is often 2-6 L/day and has reached 17 L/day (Current et al, 1983).

This pattern of acute, self-limiting infection in immunocompetent individuals and chronic, life-threatening infection in immunocompromised individuals is not absolute. Some immunocompromised individuals have spontaneously recovered (Berkowitz and Sidel, 1985), and some immunocompetent individuals experience chronic symptoms (Isaacs et al., 1985).

Cryptosporidiosis is life threatening because so far no effective chemotheraputic agent (drug) has been found (MMWR, 1982a). Current (1987) has compiled a list of isolated reports in which at least 45 drugs as well as serum from 3 individuals with high titers of antibody to C. parvum have been tried. None of these attempts has proved successful. The best that can be done is to provide supportive therapy (Soave and Armstrong 1986; Current, 1987a).

Many individuals who have been exposed to G. lamblia or C. parvum are asymptomatic yet shed cysts or oocysts (Veazie, 1969; Meyer and Radulescu, 1979; Pickering et al., 1986; Wolfe, 1984)

or are never infected (Smith, 1984). And finally, there is good evidence for immune responses to both organisms (Akin, 1986; Istre et al., 1984; Navin and Juranek, 1984; Smith, 1984).

Transmission

Giardia lamblia and Cryptosporidium parvum are transmitted by the fecal-oral route. This may occur by direct contact with contaminated fecal material from humans (Koch, 1985), e.g. daycare centers (Casemore, 1983; Pickering et al., 1981; Soave and Armstrong, 1986; Taylor et al., 1985) or sexual activity (Kean et al., 1979; Quinn et al., 1983); or from animals, e.g. domestic animals (Current et al., 1983; Current, 1987; Ongerth, 1987), or pets (Current et al, 1983; Hahn et al., 1988).

Indirect transmission may occur by contact with contaminated fecal matter via food or water. Foodborne transmission may occur due to inadequate food handler hygiene or because uncooked food is washed in contaminated water.(Barnard and Jackson, 1984; Jephcott et al., 1986). Waterborne infection can be caused by drinking contaminated water (Veazie, 1969; D'Antonio et al., 1985; Jephcott et al., 1986; Craun and Jakubowski, 1987), or by inadvertent consumption while swimming (Porter et al., 1988; Gallaher et al., 1989). Good evidence exists for a dose-response to volume of water consumed (D'Antonio et al., 1985; Hopkins et al., 1985; Jephcott et al., 1986; Kent et al., 1988).

G. lamblia cysts and C. parvum oocysts are easily dessicated

and will not remain infective long in feces on land (Tzipori, 1988). However, fecal matter contaminating water directly or indirectly (i.e. washed into a water during a runoff event) (Current, 1987a; Gallaher et all., 1989) may remain infective for a long time. Cysts have been infective after 40 days in 4° C water (Schmidt and Roberts, 1977). No data exists on oocyst survival in the environment, though oocysts have proved 80% infective after 4.5 mo. at 4° C in 2.5% Potassium dichromate (Current, 1987).

The stool of an average infected human may contain from $3x10^3$ to $3x10^8$ cysts/gm (Schmidt and Roberts, 1977). Infected calves may shed 10^5-10^7 oocysts/gm (Ongerth and Stibbs, 1987). Since infectious doses are low for G. lamblia and probably low for C. parvum, any contaminated fecal matter entering a body of water may pose a risk of exposure and infection to humans ingesting the water.

Animal to human transmission of G. lamblia or C. parvum, directly or via water, is inadequately studied. There have been no experiments confirming that the Giardia spp. found in beaver, dogs, some ruminants and small rodents and resembling the G. lamblia found in humans will, in fact, infect humans (Jakubowski, 1988). Sources of G. lamblia in waterborne outbreaks could be solely of human origin (Kent et al., 1988; Jakubowski, 1988a). Recent studies by Capon et al. (1989) found no clear antigen differences between stocks of G. lamblia from humans, beaver or muskrats. These investigators noted that G. lamblia is also

known as G. intestinalis, that this "species" is considered a subgroup of G. duodenalis and that there may be no speciation within duodenalis. They suggest that the G. duodenalis group may be a species complex. Clearly more speciation and host range studies must be done (Jakubowski, 1988a).

Cryptosporidium speciation and host range also need clarification, despite many attempts to speciate. Previously, it was thought that these coccidia were host specific (Levine, 1984). However, recent work suggests little or no host specificity in cross-transmission studies between calves, lambs, mice, rats and humans (Anderson et al., 1982; MMWR 1982; Reese et al., 1982).

There had been some confusion between C. muris and C. parvum (Levine, 1984). They are now considered two separate species and C. parvum has been determined to be the species that causes severe diarrheal illness in many mammalian hosts (Upton and Current, 1985). Zoonotic transmission of C. parvum has been clearly shown to occur between calves and humans (Anderson et al., 1983; Current et al., 1983; MMWR, 1982). Rodents and pets such as dogs and cats may be reservoirs (Current et al., 1983; Koch et al., 1985). At least 46 mammals have been found to be infected with a Cryptosporidium spp. that can readily cross species barriers (Current, 1987), and at least 23 of these are common to the North Carolina Piedmont.

Occurrence of Giardia spp. and Cryptosporidium spp.

In Mammals

Many mammals may host *Giardia* spp. (table 1) and their cysts have been found in the feces of some North American mammals. Until some exact guidelines for species typing are developed, determining whether or not the cysts found in the environment are pathogenic to humans remains a problem (Jakubowski, 1988).

Beaver have long been implicated as *G. lamblia* reservoirs (Dykes et al., 1980; Kent et al., 1988). Many watershed management decisions are based on beaver or muskrat presence and control because some investigators believe that these rodents may act as reservoirs of *G. lamblia* (Dykes et al., 1980; Kent et al., 1988; Monzingo et al., 1986). These decisions may not be based on true cause and effect as there have been no beaver-human cross-infection studies or cross-infection studies with any other animals (Jakubowski, 1988). One very limited study found no conclusive association of increasing beaver populations with increased incidence of giardiasis, and that where giardiasis was most prevelant, beaver populations were often declining (Bemrick, 1984). The search continues for the organism(s) that carry *G. lamblia* to a watershed in the first place (Bermick, 1984).

C. parvum is prevalent in mammals; but cryptosporidiosisis is rare in mature mammals, probably due to immunity (Navin and Juranek, 1984). According to Current (1987a) " Any mammal examined can host *C. parvum*". It is especially prevalent in

TABLE 1

SOME MAMMALS WHICH HOST GIARDIA SPP

Dogs (*Canis familiaris*)[5,10]
Beaver (*Castor canadensis*)[1,2,4,5,6,8,9]
Muskrat (*Ondatra zibethica*)[2,4,7]
Elk (*Alces alces*)[5,8]
Deer (*Odocoileus* spp)[5]
Water voles (*Microtus richardsoni*)[3,8]
Long-tailed voles (*M. longicaudas*)[8,9]
Meadow voles (*M. pennsylvanicus*)[5,9]
Red-backed voles (*Clethrionomys gapperi*)[9]
Deer mice (*Peromyscus maniculatus*)[5,9]
Horses (*Equus caballus*)[5]
Sheep (*Ovis aries*)[5]

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1) Dykes et al., (1980); 2) Frost et al., (1980); 3) Grant te al., (1978); 4) Kent et al., (1988); 5) Metzmaker and Rosquist, (1986); 6) Monzingo et al., (1986); 7) Pacha et al., (1985); 8) Pacha et al., (1987); 9) Wallis et al., (1984); 10) Wallis et al., (1986).

calves (Bos taurus), 75% of which worldwide are infected with cryptosporidiosis by 3 weeks of age (Current, 1987a). This presents great potential for zoonotic transmission and considerable potential for waterborne transmission. Studies by Stibbs and Ongerth (1985) and Madore et al. (1987) have found over 2000 oocysts/L in waters downstream from a dairy operation.

Current (1987) has compiled a list of 46 mammals found to be infected with a Cryptosporidium spp "that readily crosses species barriers.". This list includes 23 mammals common to the North Carolina Piedmont (table 2).

Domestic animals and pets may easily pass C. parvum to humans (Anderson et al., 1982; Current et al., 1983; MMWR 1982; Reese et al., 1982), leading to the belief that zoonotic transmission is the most prevalent route by which humans are infected (Current, 1987). Calves have definitely passed cryptosporidiosis to humans (Current et al. 1983). Kittens and puppies are often asymptomatic, but still shed oocysts (Current et al., 1983).

In humans

G. lamblia is the most prevalent parasite in the United States and the United Kingdom (Meyer and Jarroll, 1980), with 1-24% infected in the U.S.(Healy, 1979). Giardiasis is common in day care centers, especially in children <3 yrs. old (Pickering et al., 1986).

C. parvum has a world-wide prevalence of 1-4% (Tzipori,

TABLE 2

SOME MAMMALS CARRYING NON-HOST SPECIFIC CRYPTOSPORIDIUM SPP

Human (*Homo sapiens*)
Calf (*Bos taurus*)
Lamb (*Ovis aries*)
Goat (*Capra hircus*)
Horse (*Equus caballus*)
Swine (*Sus scrofa*)
Red deer (*Cerous elaphus*)
White-tailed deer (*Odocolleus virginianus*)
Fox squirrel (*Sciurus niger*)
Gray squirrel (*S. carolinensis*)
Chipmunk (*Tamias striatus*)
Flying squirrel (*Glaucomys volans*)
Beaver (*Castor canadensis*)
Muskrat (*Onoatra zibethicus*)
Woodchuck (*Marmota monax*)
Cottontail rabbit (*Sylvilagus floridanus*)
Domestic dog (*Canis familiarus*)
Red fox (*Vulpes vulpes*)
Grey fox (*Orocyon cinereoargenteus*)
Domestic cat (*Felis catus*)
Striped skunk (*Mephitis mephitis*)
Raccoon (*Procyon lotor*)
Black bear (*Ursus americanus*).

1988) and a U.S. prevalence of 0.6-4.3% (Rose, 1988). This disease is usually most prevalent in children (Gallgher et al., 1989). Soave and Armstrong (1986) have found that up to 7% of children in developed countries and up to 16% of children in developing countries may be infected. Cryptosporidiosis prevalence in day care centers in the U.S. is 6-54% (Soave and Armstrong, 1986). Also, it may be the most common parasite in Finland, where 1% of diarrheal patients have been found positive for C. parvum (Jokipii et al., 1983).

In surface water

Cysts and oocysts in feces may enter surface water directly via contamination by feces in the water, indirectly by way of fecally contaminated runoff, or as effluent from wastewater treatment plants (WWTPs).

Some work has been done to determine G. lamblia cyst concentrations in surface waters. Cysts have been detected in many public surface water supplies around the United States and Canada (Akin and Jakubowski, 1986; Frost et al., 1980). The cyst concentrations found are: 0-0.6 cysts/L in the Colorado Rockies (Monzingo et al., 1986), 0.05-1.0 cysts/L in Washington (Ongerth et al., 1988), 0.03-15 cysts/L in Pennsylvania (Sykora et al, 1987), 0.005-0.05 cysts/L in Oregon (Glicker and Edwards, 1988), and 0-6.25 cysts/L in Arizona (Rose et al., 1988). G. lamblia has been found but not quantified across Washington (Frost et al., 1980) and in the Sierra Nevada mountains of California

(Sorenson et al., 1986). A nationwide survey of surface waters showed positive samples from 51% of the creeks, 51% of the rivers and 39% of the lakes tested (Hibler, 1987).

Human use has been examined for association with cyst presence. Some studies have found a positive association (Monzingo et al., 1986; Sorenson et al., 1986), and some have found none (Frost et al., 1980; Pacha et al., 1987; Rose, 1988), which suggests animal reservoirs. Pacha et al. (1987) found 82% of small rodent fecal samples positive for G. lamblia cysts in an area closed to humans for 80 years.

If a significant portion of the population was shedding cysts and/or oocysts, raw sewage could be a major source of contamination. Jakubowski (1984) calculated that if 1% of the population were shedding cysts,..".the concentration...in raw sewage would be 9.6×10^3 cysts/L.".

Determination of Cryptosporidium spp. oocyst concentrations in surface waters has only recently begun. Rose, et al (1988), have found 0-240 oocysts/L in Arizona, Ongerth and Stibbs (1987) have found 2-112 oocysts/L in 4 rivers in Washington and 2 in California. Sampling in western states shows positive samples in 77% of the rivers (0.02-0.08 oocysts/L) and 75% of the lakes (0.58-0.91 oocysts/L) tested (Rose, 1988). In Arizona the oocyst concentration was usually about one order of magnitude greater than cyst concentrations in the same waters (Rose er al., 1988).

Human use has also been examined for possible associations with oocyst concentrations in surface water and 83% of samples

from pristine waters with no human activity were positive for Cryptosporidium (Rose, 1988).

Conventional WWTPs may discharge significant numbers of cysts and/or oocysts into surface waters. Cyst concentration in treated sewage in one study was as high as 260 cysts/L, and 2 studies found oocyst concentrations in activated sludge to approach 1300 oocysts/L (Madore, et al., 1987). Ninety-one percent of WWTP effluents tested have been positive for C. parvum (Rose, 1988).

These cyst and oocyst concentrations suggest a serious potential for drinking water contamination, especially because of their ability to remain infective in waters for long periods of time.

Detection

Detection of G. lamblia cysts and C. parvum oocysts from environmental samples involves 6 basic steps: concentration, extraction, purification, detection, identification and quantitation (Jakubowski, 1988). Methods for G. lamblia have been available for 10 years and for C. parvum for 3 years. There is no "standard method" for either protozoan. The 15th edition of Standard Methods for the Examination of Water and Wastewater contained a tentative method for G. lamblia; the 16th edition contains a consensus method for G. lamblia and the 17th edition will have revisions of the consensus method for G. lamblia and will introduce a consensus method for C. parvum (Jakubowski,

1988).

Concentration from Water (and Wastewater)

Concentration is necessary because cysts and oocysts are usually present in low numbers. Neither protozoan reproduces in surface waters and concentrations decline over time or distance from the source of contamination (Craun and Jakubowski, 1987; Jakubowski, 1988). G. lamblia concentration methods have evolved from a sand-filled swimming pool filter used during the Rome, NY outbreak (Shaw et al., 1977), to an algal centrifuge (Holman et al., 1983), resin/fiberglass compound filters (Riggs et al., 1984; Sorenson et al., 1986a), membrane filters (Isaac-Renton et al., 1986; Ongerth et al., 1988; Ongerth and Stibbs, 1987; Wallis et al., 1985), and cartridge filters composed of orlon or polypropylene (Jakubowski, 1984). Filters for G. lamblia should be <5 μm pore size (Jakubowski, 1988), and 1.0 μm pore size for C. parvum (Ongerth and Stibbs, 1987).

Membrane filters are adequate in low turbidity waters (<2.5 NTU) (Ongerth and Stibbs, 1987; Ongerth et al., 1988),while cartridge filters allow sampling greater volumes of higher turbidity waters than membrane filters (Craun and Jakubowski, 1987). Other important filter criteria are: filter material that does not shed into the eluent, and filters that allow a practical flow rate (Jakubowski, 1988).

Extraction of concentrated cysts and oocysts from filters

Extraction is accomplished by washing (Ongerth and Stibbs, 1987; Ongerth et al., 1988) and/or backflushing the filters using solutions with detergent (Musial et al., 1987), or deionized (DI) water (Jakubowski, 1988; Ongerth and Stibbs, 1987). Particulates are then settled by overnight refrigerated storage or by centrifugation (Jakubowski, 1988; Musial et al., 1987; Ongerth and Stibbs, 1987).

Purification of cysts and oocysts from filter extracts

Purification of environmental samples is necessary because microscopic techniques are the only ones currently available and samples may contain an abundance of other material (algae, clays, leaf debris, invertebrates, yeasts and bacteria) which may mask the presence of the protozoans (Jakubowski, 1988). Most purification is done by flotation techniques involving over- or under-layering with sugar, salt or percoll density gradients, such as: percoll-sucrose (Kilani and Sekal, 1987; Sauch, 1985), potassium citrate (Ongerth and Stibbs, 1987; Ongerth et al., 1988), zinc sulfate (Jakubowski, 1984), or Sheather's solution (Rose et al., 1987).

All of these techniques clean up the samples, but none are completely effective; especially when there are materials present in the same size and density range as cysts and oocysts (Craun

and Jakubowski, 1987). "Algae can be a significant interference problem in some samples"...(Jakubowski, 1988), and Ongerth, et al. (1988) found that processing and microscopy became less efficient when sample turbidity was greater than 1.5 NTU.

Detection and Identification of Cysts and Oocysts from Environmental Samples

These two steps are often combined. For G. lamblia cyst detection, the purified concentrate may be stained with Lugol's iodine (Jakubowski, 1984), however, most assessments use various immunofluorescent antibody techniques to stain either G. lamblia cysts and/or C. parvum oocysts (Jakubowski, 1984; Jakubowski, 1988; Musial, 1987; Ongerth et al, 1988; Riggs et al., 1984; Rose et al, 1987; Sorenson et al.,1986a). The stained sample is either smeared on a slide and examined microscopically in a presence-absence test (Stetzenbach et al.,), or the purified concentrate is passed through a membrane filter, stained (labelled) and then viewed microscopically (Jakubowski, 1988). Size and shape are the criteria used for identifying cysts and oocysts, and many laboratories require the observation of two or more morphological characteristics to confirm identification (Jakubowski, 1988; Ongerth and Stibbs, 1987; Sauch, 1985).

False postives and false negatives in cyst and oocyst detection

Most assessments of cyst and oocyst presence and concentration are done using immunofluorescent assay (IFA) techniques. These have many advantages, but they also have some serious drawbacks. IFA techniques are much faster than other staining methods when it comes to detection and identification because it takes less time to locate cysts and oocysts amongst the debris (Sauch, 1985; Garcia et al., 1987). However, there may be problems with cross-reactivity of the antibodies with antigens in the walls of other cyst- and oocyst- like bodies (Darlington and Blagburn, 1988; Sauch, 1985). Observing internal morphology by phase contrast or bright field microscopy can contend with this problem. This is effective for G. lamblia but C. parvum has little visible internal morphology to distinguish it (Ongerth et al., 1988; Sauch, 1985). Observers are using very specific antibodies along with size, shape and the suture line as additional criteria to the FITC reaction (Ongerth and Stibbs, 1987).

A negative cyst or oocyst result does not mean that there are none present in the water. It may mean that they were not concentrated initially, or that they were lost in one or more of the processing steps.

Quantitation of cyst and oocyst concentrations

Quantitation, the final step, is a matter of careful, consistent laboratory technique and adequate record keeping of all sample, subsample and aliquot volumes handled. Seeded sample analyses with known cyst and oocyst concentrations in various raw and finished waters are important for accurate results (Ongerth and Stibbs, 1987; Ongerth et al., 1988).

Viability

Another problem with current analytical techniques is their inability to determine cyst and/or oocyst viability (infectivity). Cysts and oocysts may become inactivated or lose infectivity over time, distance or with disinfection; but cyst and oocyst bodies may remain intact. False positives may occur because most of the identification techniques use immunofluorescent antibodies targeted against these cyst and oocyst walls (Jakubowski, 1988).

Viability/infectivity assessments are thus important to correctly determine risk. To be effective an ideal viability assay would be reliable, easy to interpret, quick, easy to perform, cheap, amenable to automation and not affected by the inactivation method (Sauch, 1988).

Infectivity can be determined by using appropriate animal hosts, of proven cross-infectivity with humans, such as Mongolian

gerbils or neonatal mice. Sterling et al. (1989) found that a significant percentage of C. parvum oocysts disinfected with ozone or free chlorine tested positive for viability using an excystation assay, and that a significant percentage of oocysts disinfected with ozone tested positive for viability with metabolic dyes. However, these oocysts did not prove infective in neonatal mice. Thus, animal infectivity assessments have the advantage of truly testing infectivity, not just excystation or metabolic activity (Rose, 1988; Sauch, 1988; Schaeffer, 1987; Sterling et al., 1989). However, animal infectivity assays have important disadvantages. One is that infectivity studies are slow. Water treatment operators need to know the pathogen status of their water quickly (NPDWR, 1987). Infectivity determinations may take days: 4-12 day incubation for C. parvum and 2-28 day incubation for G. lamblia (Jokipii et al., 1983). Hosts may be asymptomatic, giving a false negative unless their feces are analyzed for cysts and/or oocysts (Hahn et al., 1988), which takes special equipment and expertise or the services of an independent laboratory. Also, animals are expensive to buy, house and feed (Sauch, 1988; Schaeffer, 1987)

Viability can be assessed by in vitro excystation, metabolic dyes or observing morphological differences (Sauch, 1988; Schaeffer, 1987). In vitro excystation is a frequently used method, but it takes a long time, requires large numbers of cysts or oocysts ($1\text{-}5 \times 10^5$) to get results and is currently unreliable for G. lamblia and C. parvum (Sauch, 1988; Schaeffer, 1987;

Sterling et al., 1989). Metabolic dyes may be taken up or excluded by metabolic processes and thus mark living cells. Advantages of dyes are that they are rapid, easy to apply, easy to read, inexpensive and capable of detecting small numbers of cysts or oocysts. However, eosin dye exclusion does not correlate well with excystation and fluorogenic dyes exhibit good correlations with G. muris excystation, but not with G. lamblia (Sauch, 1988; Schaeffer, 1987). Also, deactivation methods may affect dye uptake or exclusion (Sauch, 1988; Schaeffer, 1987; Sterling et al., 1989). Morphologic differences can be used to distinguish between living and dead cells. This is usually done by differential interference contrast microscopy. Advantages are that these techniques are quick, simple to perform, and applicable to small numbers of cysts or oocysts. However, their disadvantages include: subjectiveness, the need for very expensive optics, the lack of observable differences between viable and nonviable G. lamblia, and differences in morphology with inactivation method (Sauch, 1988; Schaeffer, 1987).

Indicator systems for Cysts and Oocysts

Indicator bacteria systems or turbidity may prove useful in predicting the possible presence of G. lamblia or C. parvum in surface waters. Little work has been done in this area. Rose, et al (1988), found a good correlation of cysts to oocysts but found no significant correlations of either protozoan with total or fecal coliforms. Other research has examined correlations of

cysts with total coliforms (Akin et al., 1986; Craun and Jakubowski, 1987; Hopkins et al., 1985; Rose et al. 1988) and with fecal coliforms (Craun and Jakubowski, 1987; Rose et al., 1988) and none were found.

Because cysts and oocysts are particulates, turbidity, measured as Nephelometric Turbidity Units (NTUs), has been suggested as a guideline indicator of their presence in finished water. (NPDWR, 1985, 1987). The current standard of 1.0 NTU is inadequate. Logsdon et al. (1981) suggest a maximum turbidity of 0.3 NTU to keep G. lamblia concentrations at an acceptably low level. This level may be too high as they found up to 200 cysts/L in finished water with a turbidity of 0.24 NTU, even with a small seed concentration. They also found up to 120 cysts/L in finished water with a turbidity of 0.08 NTU. Since C. parvum oocysts are smaller, and may have a similar infective dose to G. lamblia, one could infer that even 0.3 NTU may be too high to preclude their presence in finished drinking water. Cysts (Kirner et al., 1978; NPDWR, 1987; Rose, 1988) and oocysts (Rose, 1988) have been found in finished water meeting the 1.0 NTU turbidity limit.

Waterborne Outbreaks of Giardiasis and Cryptosporidiosis

Waterborne disease outbreaks have occurred throughout human history. In the United States there were 427 reported waterborne outbreaks between 1971-1983, infecting more than 106,000 individuals (NPDWR, 1985). 49% of these were of unknown etiology

(Craun and Jakubowski, 1987), and 77 were giardiasis outbreaks infecting approximately 23,000 individuals (NPDWR, 1985). Since 1965 there have been over 100 reported waterborne giardiasis and 2-3 reported waterborne cryptosporidiosis outbreaks in the United States (Jakubowski, 1988). Most giardiasis outbreaks have occurred in the Northeast, the Northwest and the Rocky Mountain states (Craun and Jakubowski, 1987). Waterborne cryptosporidiosis has occurred in Texas (D'Antonio et al, 1985) and Georgia (Rose, 1988).

Most waterborne outbreaks have occurred in small community systems using contaminated surface waters (Craun and Jakubowski, 1987). The greatest percentages of outbreaks and cases occur in systems using disinfection only (Craun and Jakubowski, 1987; NPDWR, 1985,1987)(table 3). Post-outbreak examination of these systems reveals various disinfection inadequacies, including insufficient disinfectant concentration, insufficient contact time, high turbidity, or no disinfectant residual in the distribution system (Craun and Jakubowski, 1987).

Waterborne outbreaks occurring in systems that use filtration have occurred because of: lack of chemical pretreatment (i.e. no chemical coagulation), various physical problems with the filters such as media loss or disruption (Kirner et al., 1978), filter breakthrough, or operational mistakes such as failing to filter-to-waste (Craun and Jakubowski, 1987)(table 3). Some outbreaks have been caused by the use of pressure filtration systems which are not suitable for

TABLE 3

WATERBORNE OUTBREAKS OF GIARDIASIS BY TREATMENT (1965-84)*

SOURCE	TREATMENT	OUTBREAKS	CASES
SURFACE WATER	UNTREATED	13%	1%
	DISINFECTION ONLY	43%	51%
	INEFFECTIVE FILTRATION	17%	31%
GROUND WATER	DISINFECTION ONLY	11%	ND
DISTRIBUTION SYSTEM CONTAMINATION	NA	6%	15%

* = Craun and Jakubowski, 1987, Status of Waterborne Giardiasis Outbreaks and Monitoring Methods, in: Proc. Int'l. Symp. on Water-related Health Issues

removing microbiological contaminants (Craun and Jakubowski, 1987). The Carrollton, Ga., cryptosporidiosis outbreak probably occurred because filters were used intermittently, they were not backwashed properly before putting them on line and they were heavily contaminated with oocysts (Rose, 1988).

Waterborne giardiasis outbreaks involving groundwater are usually caused by contamination of the ground water by human sewage (table 3) (Craun and Jakubowski, 1987). This was also true of the Texas outbreak of cryptosporidiosis (D'Antonio et al, 1985). A small percentage of outbreaks are caused by cross contamination of the drinking water distribution system from the wastewater system (table 3)(Craun and Jakubowski, 1987).

Treatment of Water to Remove and/or Inactivate Cysts and Oocysts

Current information shows that "no properly operated water treatment plant using conventional treatment, direct filtration, slow sand filtration, or diatomaceous earth filtration with disinfection has been implicated in a waterborne giardiasis outbreak." (NPDWR, 1987). The EPA proposed a MCLG of 0 G. lamblia cysts, realizing that for a MCL to be effective, there must be a method for determining cyst and oocyst concentrations before the tested water enters the distribution system. There is no such method available for either G. lamblia or C. parvum (Jakubowski, 1988) because there is no standard method. Current analytical

methods require expertise not typically available in WTPs, independent laboratory analyses are expensive, many large samples are needed to adequately test finished waters and it is not possible to guarantee that monitoring would detect cysts before adverse health effects occur due to less than 100% recovery efficiency and unknown viability (NPDWR, 1987).

As an alternative to a MCL the EPA has also proposed a treatment standard for G. lamblia under the 1986 SDWA amendments (NPDWR, 1987). C. parvum will probably soon be addressed in a similar fashion since it is on the first Drinking Water Priority List (DWPL) (EPA, 1988).

The proposed treatment standard requires that water treatment plants attain a 99.9% G. lamblia cyst removal/ inactivation and a 99.99% removal/inactivation of enteric viruses. WTPs may be excluded from filtration requiremants if they can meet specific source water criteria. Raw waters must meet total coliform, fecal coliform and turbidity standards, and the utility must implement an effective watershed control program including a yearly sanitary survey. There must have been no previous waterborne disease outbreaks, and, there must be redundancy in the disinfection system. If all the criteria are met then the WTP may use disinfection only, determined by CT (disinfection concentration times contact time) values, to achieve a 99.9% cyst inactivation and a 99.99% enteric virus inactivation (NPDWR, 1987).

States may grant temporary exemptions from requirements to

WTPs based on: economic hardship, no alternate source of raw water and no unreasonable health risk. If exemptions are granted states must also develop a compliance schedule (NPDWR, 1987).

Currently, approximately 1400 commercial and 1536 non-commercial WTPs serve unfiltered water to approximately 22 million people (NPDWR, 1985,1987). This includes parts of large urban areas such as New York City, Seattle and Boston. These utilities will require huge sums of money to comply with the proposed regulations. The EPA estimates costs of meeting these regulations to be approximately $1.95 billion assuming that all WTPs will not need to filter (NPDWR, 1987). This is an average increased cost of about $100/year per household by 1996 (Mainstream, 1989). If all disinfection-only WTPs have to put in filtration systems the total cost may be $2.75 billion (NPDWR, 1987). Because all studies show disinfection alone to be insufficient (Craun, 1988; Craun and Jakubowski, 1987; Kent et al., 1988; NPDWR, 1985,1987) and because watershed management may be ineffective, water may become a much more costly commodity.

However, disinfection to inactivate G. lamblia cysts may be effective if there is redundancy in the system and if all variables are controlled. For both G. lamblia and C. parvum, ozone is the most effective disinfectant, followed by chlorine dioxide, free chlorine, chloramines and UV light (Wickramanayake, 1986). CT values for inactivation of cysts have been computed (table 4) and are technically feasible. However, Sterling et.al. (1989) has found C. parvum to be 100-1000 times more resistant

TABLE 4

CT VALUES FOR ACHIEVING 99.9% INACTIVATION OF GIARDIA SPP.[1]

		Temperature			
Disinfectant	pH	0.5° C	5° C	10° C	15° C
Free chlorine[2]	6	170	120	90	60
	7	200	190	130	100
	8	380	270	190	140
	9	520	370	260	190
Ozone	6-9	4.5	3	2.5	2
Chlorine dioxide	6-9	81	54	40	27
Chloramines	6-9	3800	2200	1850	1500

1 = from the NPDWR, (1987)
2 = CT values vary with free chlorine concentration, here values are for 2.0 mg/L

than G. lamblia. For 99.9% inactivation 1.0 mg/L of ozone for 10 min (CT = 10 mg O_3-min / L) or 80 mg free chlorine for 2 hours, at T = 25° C, pH = 7.0 (CT ≈9600 mg Cl_2-min/L). This is compared to a CT of 100 mg Cl_2-min/L for 99.9% G. lamblia inactivation at 15° C and a pH of 7.0.

Cysts and oocysts have been found in finished water. In sampling from WTPs, plants that disinfect only were 17% positive and plants using conventional treatment 5.8% positive for G. lamblia in their finished water (Hibler, 1987).

The best approach for achieving the required treatment is by using multiple barriers (Wickramanayake, 1986; NPDWR, 1985,1987). Wickramanayake (1986) found that conventional treatment which includes all of the following will acheive 99.99% cyst removal: pretreatment with alum and/or polymer coagulant, sedimentation, filtration (rapid or slow, dual media or sand or diatomaceous earth (DE)), and disinfection. The EPA considers conventional treatment to be the best method for complying with the new regulations. This includes: coagulation, flocculation, sedimentation, rapid granular medium filtration and disinfection (NPDWR, 1985,1987). Conventional or slow sand filtration may adequately remove C. parvum oocysts (Logsdon et al., 1988).

Chemical pretreatment prior to filtration is very important. Filtration without chemical pretreatment to coagulate cysts may not adequately remove them (Craun, 1988; Craun and Jakubowski, 1987; NPDWR, 1985,1987; Wickramanayake, 1986), and although current disinfection practices may inactivate G. lamblia cysts

(NPDWR, 1987), these will not be effective for C. parvum oocysts. Pretreatment also reduces chlorine demand, making disinfection more effective (Craun, 1988).

A danger in using disinfection only is that increasing disinfectant concentrations high enough to deactivate cysts or oocysts increases disinfection by-product health risks. There are positive associations between drinking chlorinated water and bladder and colon cancer, especially for bladder cancer (Craun, 1988). This is another reason to use filtration with chemical pretreatment.

Potentially high treatment costs make it important for WTPs to assess G. lamblia and C. parvum contamination of their surface source waters to help them decide what treatment they must provide to protect public health. Part of the assessment effort could include a search for more effective indicator systems. If one is found it may obviate the need for a treatment standard.

Methods

Each sample analysis had three stages: sampling, sample processing and counting. Two samples of raw and finished water were taken at each of six water treatment plants (WTPs) in the North Carolina piedmont to determine concentrations of *G. lamblia* cysts, *C. parvum* oocysts, fecal coliforms and enterococci. Raw waters were sampled to determine cyst and oocyst presence. Finished waters were sampled to determine whether or not the treatment used by WTPs with cysts or oocysts in their raw waters was sufficient to reduce cyst or oocyst levels to below detection limits.

Samples of raw water were also analyzed for the above protozoans and fecal indicator bacteria to determine whether or not a statistically significant positive association existed between either protozoan and either bacterial indicator system. If a strong, consistent, positive association was found the bacterial indicator could be used as a predictor of the presence of that protozoan.

Samples of finished water were analyzed for the above bacterial indicators and protozoans to determine whether or not either survived treatment and, if so, would the fecal bacteria indicators be adequate predictors of the presence of cysts or oocysts.

Sample processing and counting were done in the laboratory (School of Public Health, University of North Carolina-Chapel

Hill).

Sampling

Samples were taken at the following water treatment plants: Raleigh (Johnson Plant), Durham Williams Plant), Orange Water and Sewer Authority (OWASA), Burlington, High Point and Winston-Salem (Neilsen Plant). Each plant was sampled twice to get enough data for studies of associations of cysts or oocysts with fecal coliforms or enterococci.

Source waters for the WTPs come from creeks and rivers in the piedmont. Winston-Salem obtains its raw water from a large river, the Yadkin. Raleigh obtains its raw water from a large impounded river, the Neuse. Both of these rivers are moderately impacted by industrial discharges and also flow through farmland and forests. Source waters for OWASA, Durham, Burlington and High Point are from impoundments which collect creek or small river waters. There is little to no impact by industries and all these waters flow mostly through farm and forested lands.

Water treatment plants were surveyed to determine what treatment the above waters received to fully treat them. If cysts, oocysts or fecal indicator bacteria are found in finished waters from any of the WTPs, treatment methods information may help point out which treatments should be further studied to assess their effectiveness.

All finished water samples were taken just prior to the passage into the distribution system from a tap in each plant.

Raw water samples were taken from taps in the plants at OWASA, Winston-Salem and High Point. Raw water samples at Raleigh, Durham and Burlington were pumped from the raw water storage reservoir using a negative displacement pump (Teel) powered by an electric motor.

Analyses for G. lamblia cysts and for C. parvum oocysts were performed simultaneously (because of time and budgetary constraints). Initially, a method from the University of Arizona was used (Musial et al., 1987). Recovery was low due to concentration and purification problems caused by high concentrations of particulates in the waters, especially diatoms and other algae. The selected method was developed by Sauch (1985) and Tom Trok, of the Western Pennsylvania Water Co.. This method worked well for the waters in N.C., as determined by recovery studies using spiked samples (see Methods to Evaluate Cyst and Oocyst Recovery).

Samples were concentrated by filtering a volume of water through a 10 inch, polypropylene cartridge filter, Microwynd II, nominal pore size 1.0 μm (AMF/CUNO Division, Meriden, CT). Approximately 100 gallons of raw water, as suggested by the EPA (Craun and Jakubowski, 1987), at 5-11 gpm, and 500 gallons of finished water, at 10-20 gpm, were filtered each time. Because of the risk of cyst and oocyst contamination, separate filter housings and hoses were used for raw and finished waters. After each sample was collected, the filter was removed from the housing, put in a one gallon "Zip-lock" style bag, labeled and

placed on ice in an insulated container for transport back to the laboratory. There the filters were stored at 4° C until extracted; which was done within 7 days. All sampling equipment, except the pump and hoses, was disinfected with a 10-15 mg/L solution of chlorine (NaOCl) for 30 min., throughly scrubbed and rinsed successively in tap and deionized (DI) water. The pump and hoses were flushed with at least 100 gal of tap water (Rose et al., 1986).

Samples for bacterial analyses were taken simultaneously with parasite samples. One liter volumes of raw and finished water were placed in sterile, wide mouth polyethylene sample bottles (Nalgene), labeled and transported back to the laboratory on ice. There they were processed within 6 hrs of the sampling time.

Sample processing

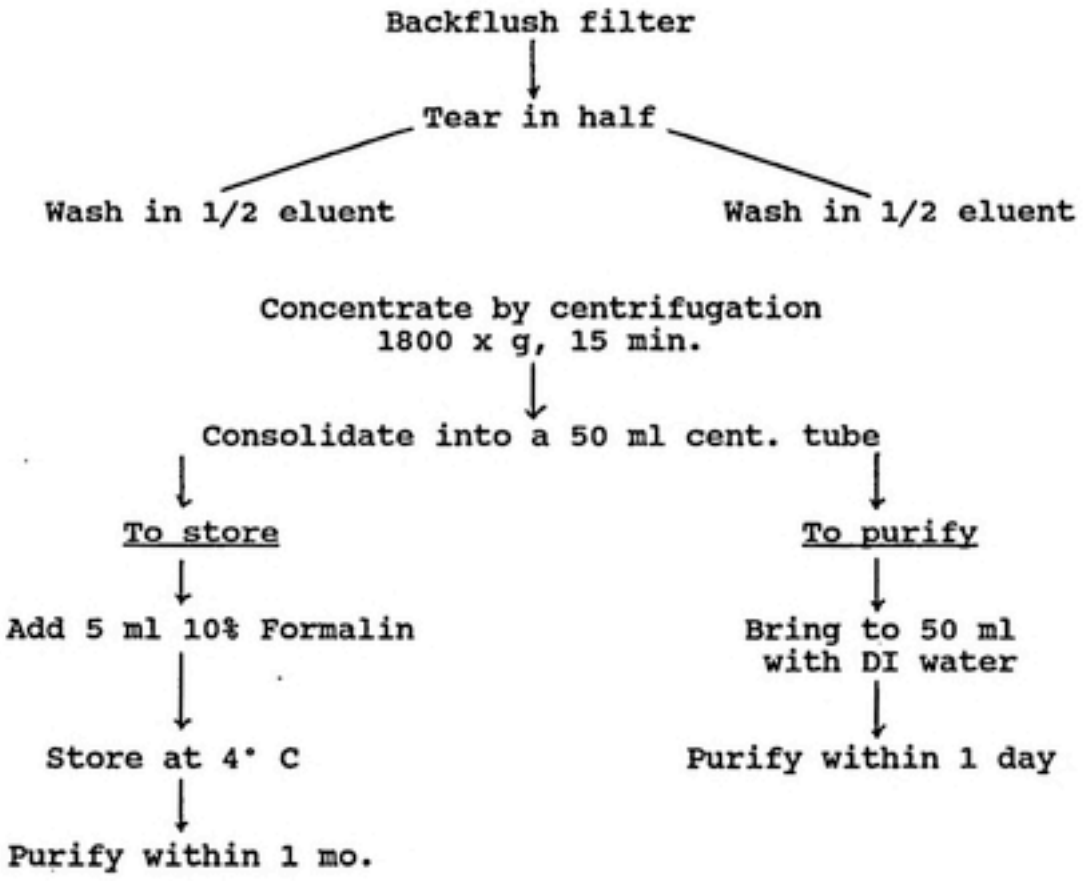
Protozoans

Processing for G. lamblia and C. parvum was accomplished by: extraction by backflushing and washing, concentration by sedimentation, and purification by flotation and a final sedimentation.

Particulates from each filter were extracted by back-flushing with 2700 ml 0.1% Tween 80 (J.T.Baker Chemical Co., Phillipsburg, NJ) (figure 1). The eluent was saved and used as a wash solution for the filter. The filter was cut off its tubular support, torn in half, thoroughly teased apart, and each half was

FIGURE 1

EXTRACTION



washed in 1/2 of the eluent in a 4 L flask on a shaker for 10 min. The eluent was wrung by hand from the filter material and poured into 4 one liter centrifuge bottles. (Rose, personal comm. 1987). The bottles were centrifuged at 1800 x g for 15 min (Rose, personal comm. 1988), the supernatant aspirated off and discarded, and the pellets plus a small amount of residual supernatant, were transferred to one 50 ml centrifuge tube. This was either brought to 50 ml with deionized (DI) water and processed further or 5 ml 10% Formalin was added and the sample stored at 4° C for later processing (Trok, 1987).

Purification

If the sample had been stored in Formalin it was washed in DI water by centrifugation as follows (figure 2): the sample was centrifuged (1800 x g for 15 min), the resulting supernatant containing the formalin was aspirated off to 6 ml and discarded, and the centrifuge tube was filled to 50 ml with DI water and vortexed. This was again centrifuged as above and the supernatant aspirated off to complete the wash. The pellet was brought to 50 ml with DI water and vortexed. 20% of the sample, a 10 ml subsample, was processed further. The rest of the sample was preserved by adding 5 ml of 10% Formalin, vortexing and storing at 4° C.

The subsample was purified by flotation and cleaned by washing with DI water (figure 3). The 10 ml subsample was placed in a 50 ml centrifuge tube and brought to 20 ml with DI water (to

FIGURE 2

WASHING BY CENTRIFUGATION

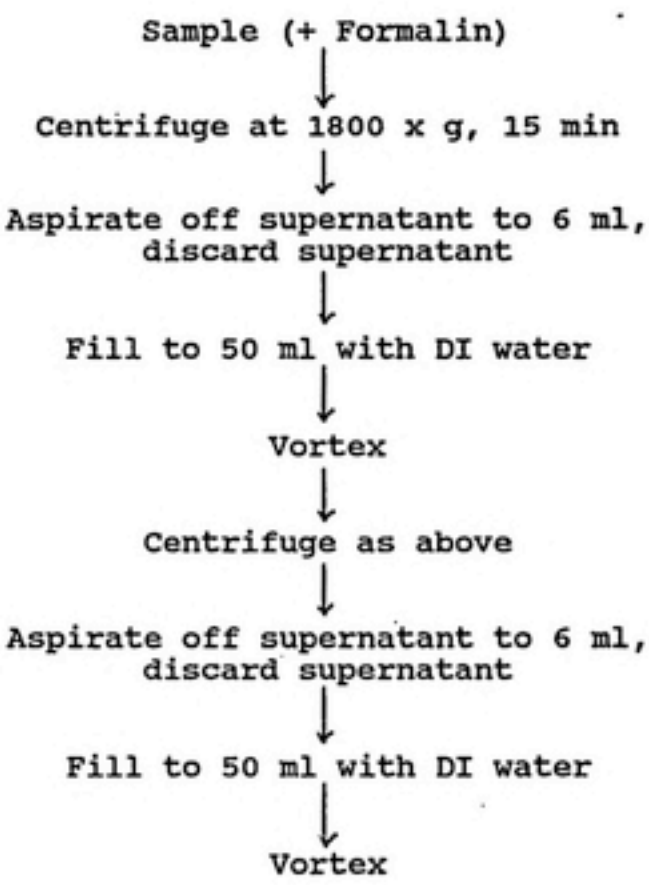
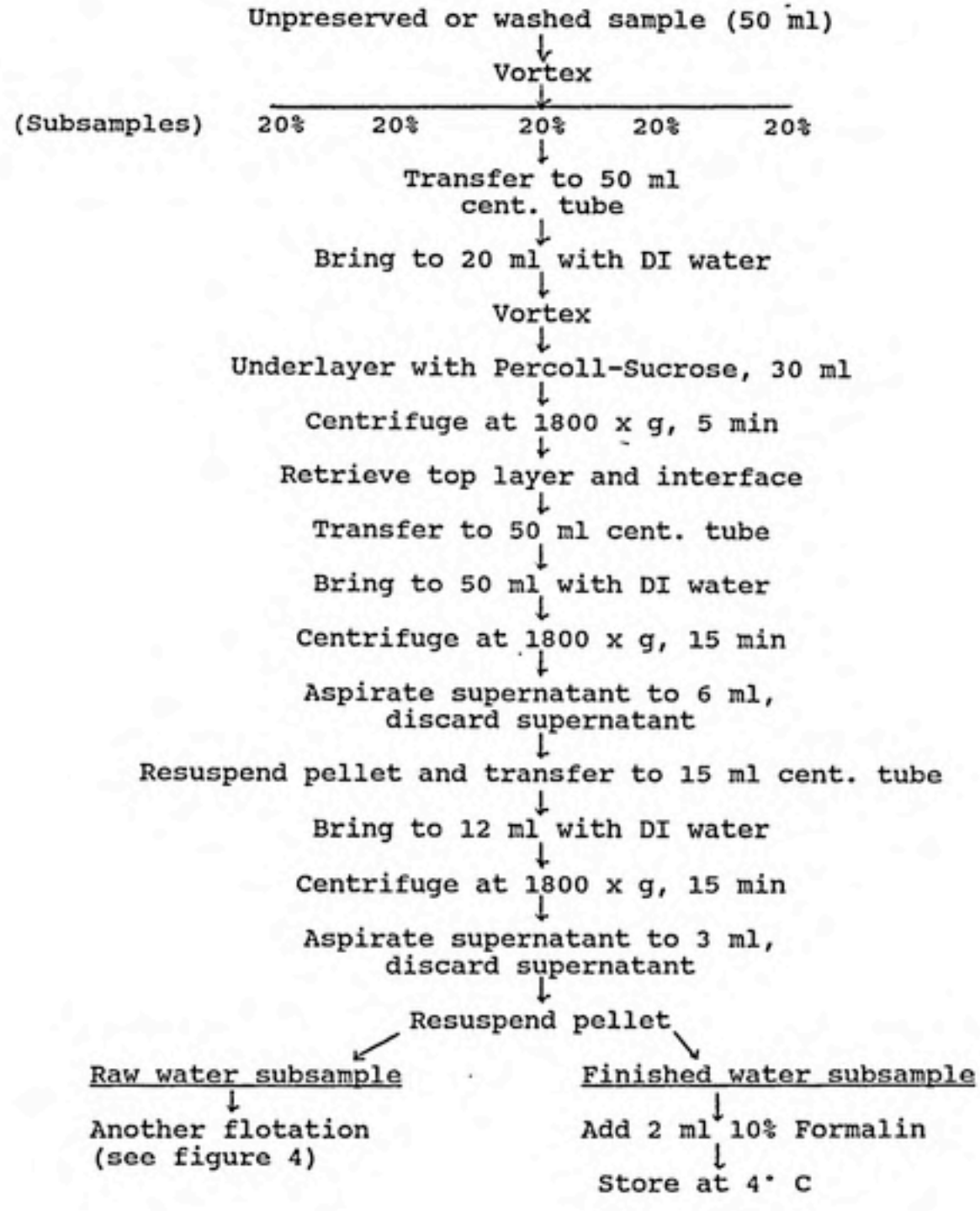


FIGURE 3

PURIFICATION



dilute the subsample and avoid collapsing the density gradient) and vortexed. Then it was under-layered with 30 ml of 1.08-1.09 g/ml Percoll-Sucrose (Sauch, 1985). This was centrifuged at 1800 x g for 5 min (Trok, 1987). The top layer and interface were retrieved and placed in a 50 ml centrifuge tube. This was brought to 50 ml with DI water and centrifuged at 1800 x g for 15 min. The supernatant was aspirated and discarded leaving 6 ml. This was resuspended, placed in a 15 ml centrifuge tube and spun as above. The supernatant was aspirated to 3 ml and the pellet resuspended. Finished water subsamples were preserved with 2 ml of 10% Formalin at 4° C. (Trok, 1987)

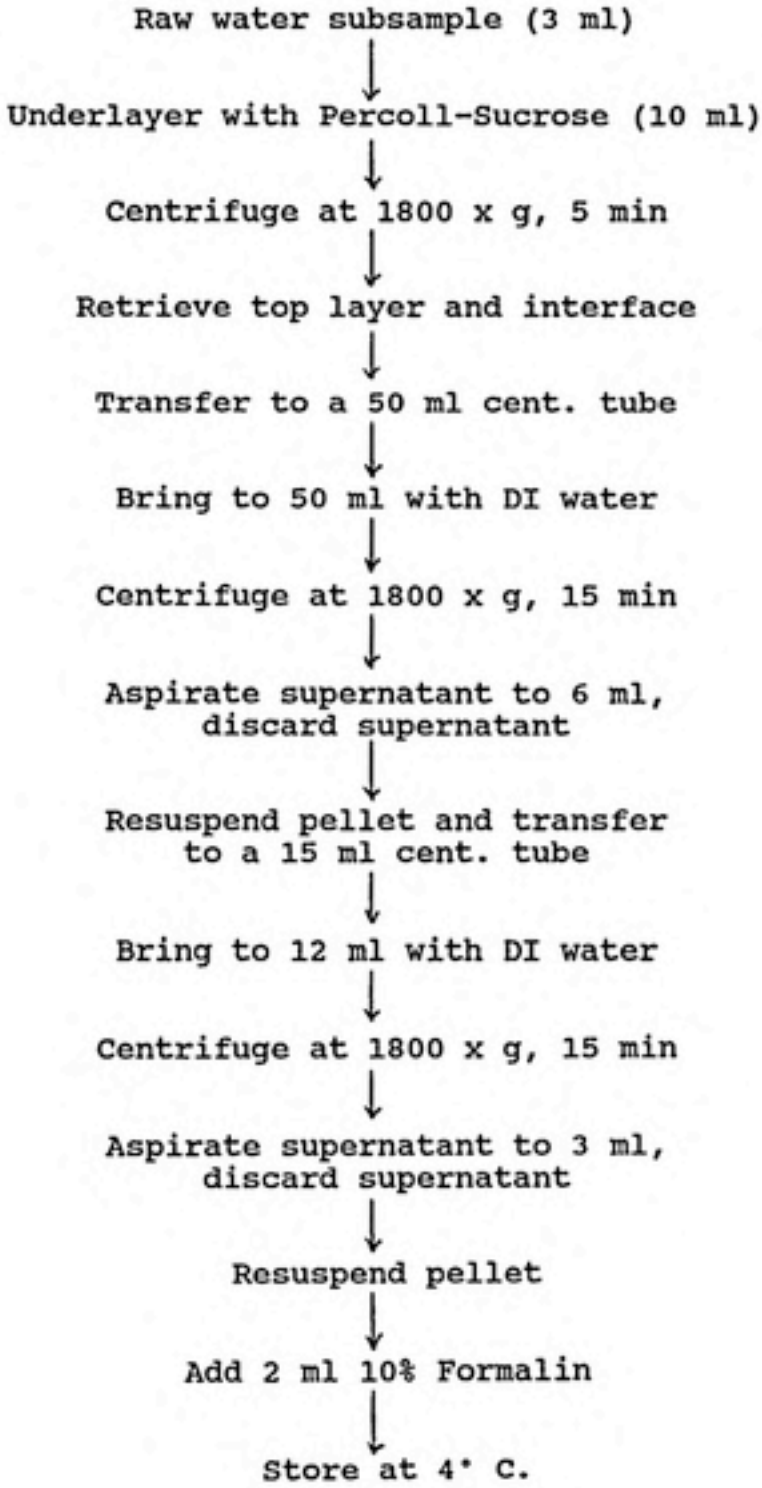
Raw water subsamples received another flotation (figure 4) (Trok, 1987). Each 3 ml solution of pellet and supernatant was underlayered with 10 ml of Percoll-Sucrose (same density as above) and spun as above. The top layer and interface were transferred to a 50 ml centrifuge tube, filled to 50 ml and spun as above. The supernatant was aspirated off to 6 ml and the pellet was resuspended, placed in a 15 ml cent. tube, filled to 12 ml and spun as above. The supernatant was aspirated off to 3 ml and the pellet resuspended and stored with 2 ml 10% Formalin at 4° C.

Detection, Identification and Quantitation

G. lamblia cysts and C. parvum oocysts were counted using Merifluor(tm) indirect immunofluorescent procedures. Monoclonal anti-giardial and anti-cryptosporidial antibodies were added to

FIGURE 4

RAW WATER SUBSAMPLE PURIFICATION



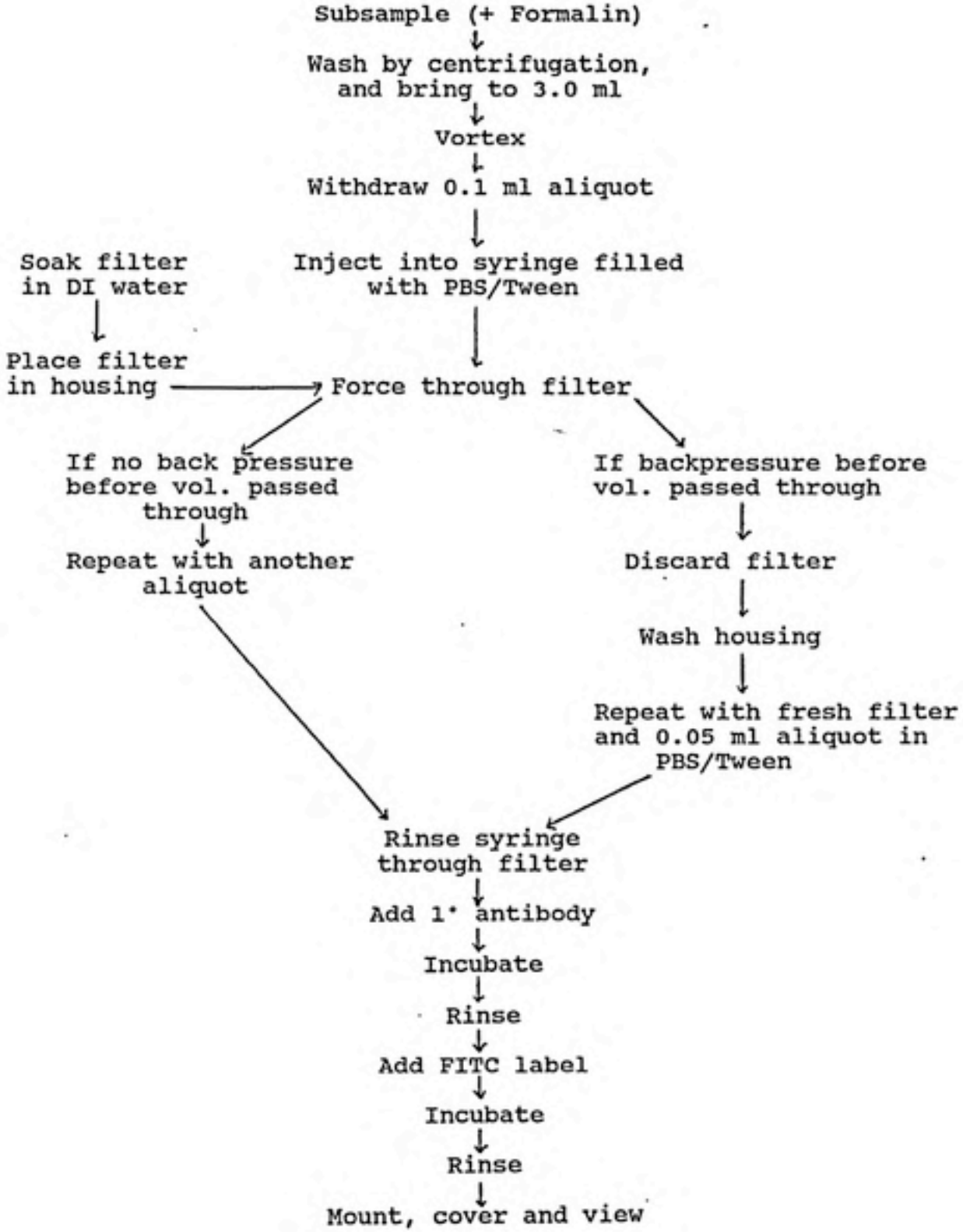
the samples and these would bind to the cyst or oocyst walls. Cysts and oocysts with bound antibodies were then reacted with a fluorescein isothiocyanate (FITC)-conjugated anti-species immunoglobulin solution. Cysts and oocysts that were fluorescein-labeled on their outer walls were viewed under a fluorescent microscope.

Microscopic detection, identification and quantitation of the three aliquots of each subsample were done on membrane filters (Nuclepore: 13 mm polycarbonate, 2.0 µm porosity, black) (figure 5). The filters were briefly soaked in DI water, then placed while damp in in-line stainless steel filter housings (Millipore, Swinnex, 13 mm). Subsamples were washed once with DI water by centrifugation (1800 x g for 5 min.). The supernatant was aspirated off to 3.0 ml and the pellet vortexed (Trok, 1987). A 3.0 ml syringe (3.0 ml Luer-lok) was filled with 1.0 ml 0.05% PBS/Tween 80. A micropipette (Pipetteman) was used to draw a 0.1 ml aliquot of subsample which was injected into the syringe. This mixture was forced through the filter. The syringe was then rinsed with 3 ml 0.05% PBS/Tween 80 and this wash was forced through the filter. If there was no back-pressure on the filter the above subsample filtration steps were repeated until back-pressure occurred. If back-pressure occurred before the full volume was forced through the filter, the filter was discarded, the housing throughly washed and the procedure was repeated with a fresh filter and a 0.05 ml aliquot.

Primary antibody (Meridian Diagnostics Merifluor kit) was

FIGURE 5

DETECTION AND IDENTIFICATION



diluted 1:10 with PBS from the kit, 0.15 ml was added to the filter and the filter assembly was sealed with parafilm and incubated at room temperature (RT) for 30 min.. The filter was washed with 10 ml 0.05% PBS/Tween 80, then treated with 0.15 ml FITC-anti-species antiserum, also diluted 1:10. The filter assembly was incubated at RT for 30 min., and then washed as above.

The filter was removed and mounted on a glass slide (Meridian Merifluor kit) with fluorescent mounting medium and covered with a clear glass slide which acted as a cover slip. This thickness of glass was necessary because the objective used (Leitz, Fluotar Phaco 2) was designed to view through 0.6-1.2 mm of glass. The slide was viewed at 400 x under a fluorescent microscope (Leitz Ortholux II).

Cysts and oocysts glow bright apple green under the ultraviolet light of the microscope. Presumptive criteria for G. lamblia counts were: definite bright-apple green cyst wall, ovoid shape, 8-12 μm long. Confirmative criteria were: 2-4 nuclei and well defined cyst wall visible under bright field light (Trok, 1987). Criteria for C. parvum oocysts were: spherical to slightly ovoid shape, 4-6 μm, suture visible and definite oocyst walls staining bright apple green (Rose, 1988)

Bacteria

Raw waters, raw waters diluted 1:10 and finished waters were

analyzed by membrane filtration for two bacterial indicators of fecal contamination: fecal coliforms (APHA, 1985) and Enterococci (U.S.EPA, 1985) (figure 6).

The 1:10 raw water was diluted by mixing 100 ml raw water into 900 ml of 0.1% peptone water. Eight 100 ml aliquots of each sample were passed through separate 47 mm 0.45μm porosity filters (Gelman) by vacuum. Four replicate filters from each sample were placed on mFC agar and incubated at 37° C for 24 hrs. Shiny, blue colonies were scored as positive colony forming units (CFUs). Four replicate filters from each sample were placed on mE agar and incubated at 41.5° C for 48 hrs. Filters with shiny, dark red colonies were considered presumptive positives and placed on plates of esculin iron agar substrate (EIA) and incubated at the same temperature for 1 hr. Confirmation as enterococcus was a black ring around the colony from esculin hydrolysis and iron reduction.

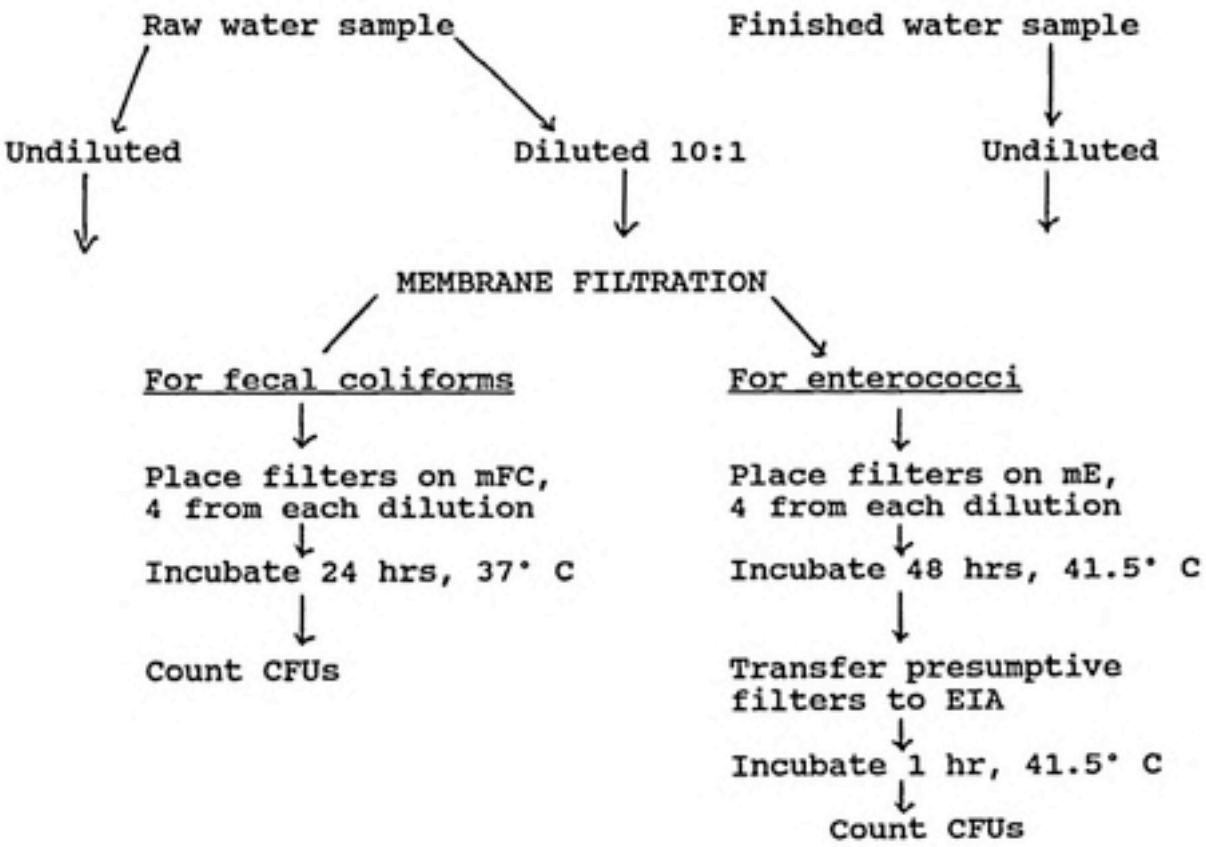
CFU counts for each group of four replicate filters were averaged to compute the number of fecal coliforms and enterocci per 100 ml.

Methods of Identifying Cyst and Oocyst sources in Watersheds

Wastewater treatment plants (WWTPs) can be a significant source of G. lamblia cysts and C. parvum oocysts (Rose et al., 1987). To assess possible contamination by WWTPs, map and record searches were performed to determine: WWTPs in each watershed, distance from WWTP discharge to WTP intake, average daily volume

FIGURE 6

PROCESSING FOR FECAL COLIFORMS AND ENTEROCOCCI



of effluent from WWTPs, and average daily flow volume of receiving waters.

Methods to Evaluate Cyst and Oocyst Recovery Procedures Using Seeded Samples

Seeded sample studies were done with raw water collected from Durham on 7/5/88, a water considered representative. Although some studies have done seeded runs with each water sampled (Ongerth and Stibbs, 1987; Ongerth et al., 1988), in this present study it was not possible to do that due to time and material constraints.

Samples of the selected water were seeded with known quantities of G. lamblia cysts and C. parvum oocysts and then processed through the recovery and detection steps used for environmental samples (see Methods) to determine: (1) mean cyst and oocyst recovery efficiencies; (2) recovery efficiency variance; (3) and representativeness of 20% subsamples relative to each other and to the entire sample.

Counting Sample Seeds of Cysts and Oocysts

The seed cyst and oocyst preparation was 2.5 ml of a solution of contaminated calf feces (Meridian Diagnostics, Merifluor kit) containing an unknown concentration of G.lamblia cysts and C. parvum oocysts. To determine the mean cyst and oocyst concentrations and variances of this preparation, six aliquots of the preparation (four of 0.10 ml and two 0f 0.05 ml) were passed through membrane filters using the same methodology as for field samples (see identification and detection in Methods). To avoid double counting, which might occur during a

complete scan of the filter, 100 random fields were viewed at 400x and all cysts and oocysts identified in each field were counted. Cyst and oocyst concentrations were estimated by statistical means (appendix A).

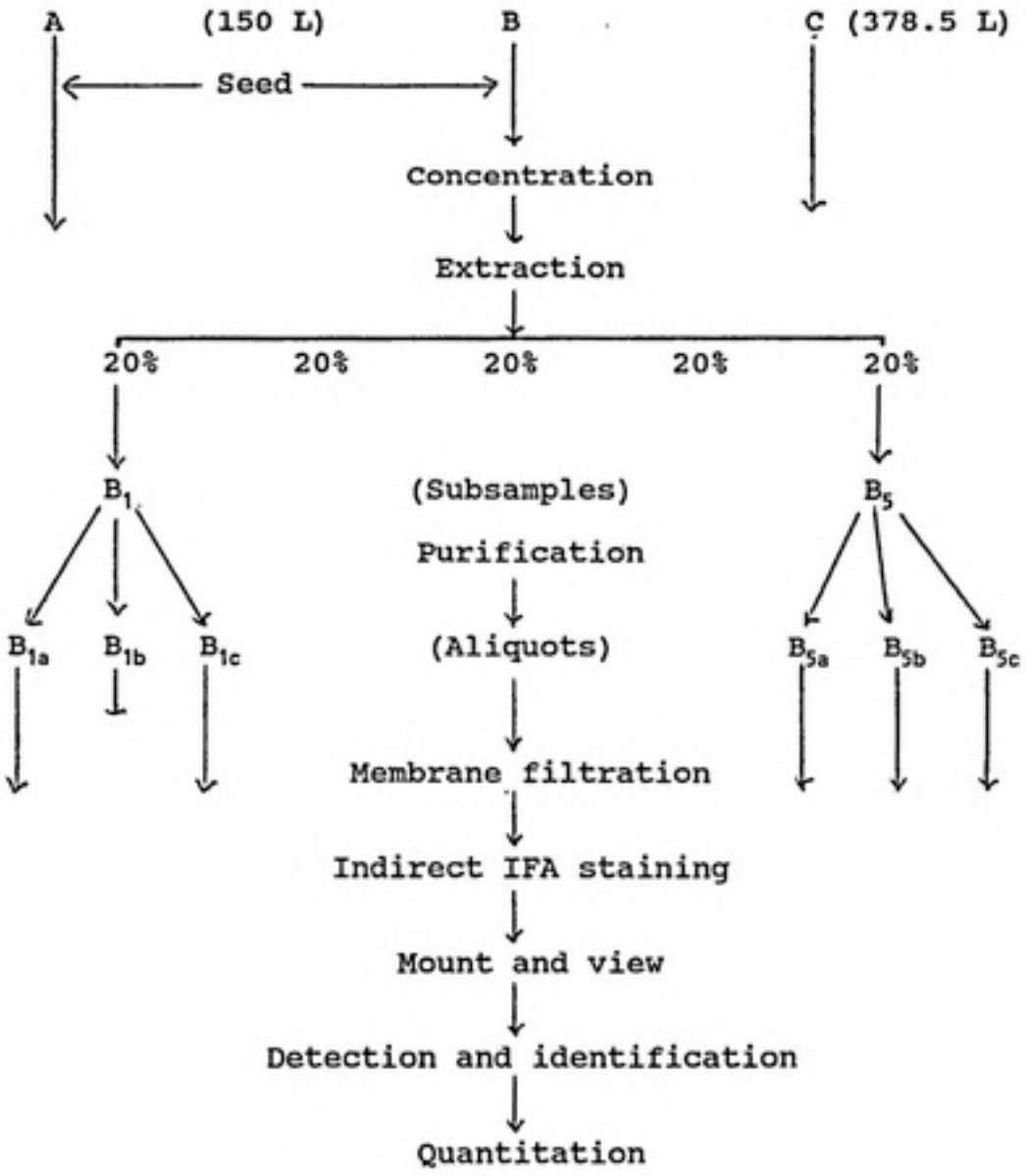
To determine cyst and oocyst recovery efficiencies from raw water, two 150 L (40 gal) field samples (A and B) collected from the Durham raw water supply on 7/5/88 were each seeded with 0.6 ml of seed solution (figure 7). Cysts were seeded at a concentration of 117 cysts/gal (33 cysts/L). Oocysts were seeded at a concentration of 5.05×10^4 oocysts/gal (1.33×10^4 oocysts/L). To avoid contamination, equipment separate from that used for field sampling was used for the seeded samples (Musial et al., 1987; Rose, 1988) A 378.5 L (100 gal) unseeded raw water sample (C) was collected simultaneously as a control. A, B and C were then processed through all the assessment steps in the methods section.

After extraction, A, B and C were vortexed and two 20% subsamples, by volume, were withdrawn from each sample for further processing. To test for reproducibility of subsamples, each sample was transferred to another 50 ml centrifuge tube and the first and last subsamples of each sample (A_1, A_5, B_1, B_5, C_1, and C_5) were processed through the purification steps. Aliquots of these subsamples were then processed through the detection, identification and quantitation steps.

From the control, 6 aliquots, representing 2 gal (2% of the total) each, were labeled and counted, three from each 20%

FIGURE 7

SEEDED SAMPLE PROCESSING



subsample (C_{1a}, C_{1b}, C_{1c}, C_{5a}, C_{5b}, and C_{5c}). From A and B, five filters, representing 1 gal (2.5% of the total) each, were counted; two from the first subsample and three from the second. To determine the reproducibility of the aliquots, the entire subsample volume was transferred to a new 15 ml centrifuge tube and the first, middle and last aliquots were filtered (A_{1a}, A_{1b}, A_{1c}, A_{5a}, A_{5b}, A_{5c}, B_{1a}, B_{1b}, B_{1c}, B_{5a}, B_{5b}, and B_{5c}). Aliquot flow through the filter housings holding A_{1b} and B_{1b} short-circuited and the results were discarded.

Recovery Efficiency Results and Discussion

The results were statistically analyzed using a components of variance approach (appendix B). An Analysis of Variance (ANOVA) of the data showed that the variance between each sample occurred mostly in the last step, the membrane filtration, not in the concentration/extraction or purification steps. This means that (1) there is reproducibility between samples; (2) there is reproducibility between 20% subsamples; and (3) there is not reproducibility between aliquots. On the basis of this analysis it was decided to withdraw, label and count the first, middle and last aliquot of each subsample.

Recovery efficiency was determined on only one raw source water due to time and material constraints. G. lamblia cyst recovery efficiency was 46.4% $\pm$ 26.6% std. err.. C. parvum oocyst recovery efficiency was 37.6% $\pm$ 13.6% std. err..

In comparison, the range of recovery efficiencies for C. parvum oocysts from five studies in the literature is 9-59%, with only one of the analyses being done with untreated water (Ongerth and Stibbs, 1987); the rest of the seeded sample analyses were done with tap water (Rose, 1988). Ongerth and Stibbs seeded with 10^3-10^4 oocysts/L and attained 5-22% recovery efficiency. This is less than the recovery efficiency of this present study, despite the fact that waters in Washington are generally much less turbid than those in North Carolina. Reasons for this result may be: (1) this present study seeded only one water which had very low turbidity (2.5 NTU) for North Carolina Piedmont waters (table 1R) and there may not have been as high a concentration of masking particulates; (2) this method may be more efficient in general; and (3) the sample size may be too small to be considered representative.

G. lamblia cyst recovery efficiency reported in the literature ranges from 10-85% in a compilation of studies (Hibler, 1987) and from 5-44%, mean 21.8%, in another study (Ongerth et al., 1988). In this present study recovery is in the mid-range of Hibler's compilation and on the high side of the range of Ongerth et al. (1988). The reasons for higher G. lamblia recovery efficiency than Ongerth et al. may be the same as noted above for C. parvum oocyst recovery.

Results and Discussion

Treatment used to remove/inactivate the above protozoans and bacterial indicators varies little from treatment plant to treatment plant (table 5). All of the WTPs in this study prechlorinate, coagulate, sediment, filter and disinfect their source waters. The only major difference is in the coagulation step where OWASA coagulates with alum, caustic and polymer, the Johnson plant in Raleigh uses just alum and the rest of the plants use mostly alum; using caustic if necessary to control pH.

Raw waters

G. lamblia cysts and/or *C. parvum* oocysts were detected in raw water samples from 3 WTPs: OWASA, Durham and Winston-Salem (table 6). No cysts or oocysts were detected in raw water samples from Raleigh, Burlington or High Point. Turbidity (as NTUs) ranged from 1.0 to 45, with one value of questionable validity from Raleigh on 7/28/88 (table 6). This sample gave the most turbid pellet after purification processing, yet it had a reading of only 1.0 NTU on the WTP laboratory turbidimeter.

All WTPs had raw water fecal coliform and enterococci concentrations of > 1/100 ml in at least one sample. One sample each from Raleigh (3/18) and OWASA (3/23) had fecal coliforms and enterococci concentrations of < 1/100 ml (table 6). Fecal coliform ranges were from 0.25-97 /100 ml, and enterococci concentrations ranged from 0-79 /100 ml. One sample (OWASA

TABLE 5

TREATMENT USED BY THE SIX WATER TREATMENT PLANTS IN THIS STUDY

Plant	Source water	P.C.	Coag.	Sed.	Filt.	Dis.
OWASA	Univ. Lake	Y	C	S	A	G
Neilsen (W-S)	Yadkin R.	Y	B	S	A	G
Johnson (Ral.)	Neuse R.	Y	A	S	A	G
Williams (Dur.)	Lake Michie	Y	B	S	A	G
H. P.	Deep R.	Y	B	S	A	G
Ed Thomas	Stoney Ck.	Y	B	S	A	G

P.C. = Prechlorination: Y = yes
Coag. = Coagulation: A = Alum
B = Alum and Caustic
C = Alum, Caustic and Polymer.
Sed. = Sedimentation rate: S = Standard rate
Filt. = Filtration: A = rapid sand/anthracite
Dis. = Disinfection: G = Chlorine gas

TABLE 6

CONCENTRATIONS OF PROTOZOANS, BACTERIA AND TURBIDITY IN RAW WATERS

Sample	Date	Analyte and Concentration*				
		G.l. cysts/ L	C.p. oocysts/ L	FC CFU/ 100 ml	E CFU/ 100 ml	NTU
OWASA	1/11/88	0.63 (0.90)	ND	97 (NA)	79 (NA)	16.7
HP	1/15/88	ND	ND	12.8 (9.4)	22.2 (5.0)	18
Dur.	2/15/88	ND	(0.03) (0.09)	17.8 (14.1)	6.8 (1.9)	45
Burl.	2/19/88	ND	ND	2.5 (3.8)	1.2 (3.8)	33
W-S	3/04/88	0.09 (0.15)	ND	37.5 (25.2)	3.0 (2.3)	6.0
Ral.	3/18/88	ND	ND	0.25 (1.0)	ND	5.0
OWASA	3/23/88	0.26 (0.92)	0.26 (0.92)	1.0 (1.6)	0.25 (1.0)	4.4
Dur.	7/05/88	0.04 (0.14)	0.05 (0.14)	4.0 (4.3)	1.5 (3.5)	2.5
W-S	7/18/88	0.02 (0.08)	0.02 (0.08)	40.8 (13.1)	48.5 (4.2)	27
HP	7/18/88	ND	ND	7.8 (4.7)	8.2 (4.4)	9.0
Ral.	7/28/88	ND	ND	23.3 (15.7)	5.0 (2.8)	1**
Burl.	7/28/88	ND	ND	9.0 (8.7)	0.75 (1.9)	18

****

* - Mean concentration above, (x) = 2 std. dev.

** = Probably instrumental error

1/11/88) gave bacterial colony counts that were too-numerous-to-count (TNTC) in the undiluted sample and much too low in the 10^{-1} dilution. Using a technique derived by Haas (1987), approximate values for fecal coliforms and enterococci concentrations were determined (table 6).

Because recovery efficiencies for both protozoans was less than 100% some adjustments must be made to correctly interpret their concentrations in raw water samples (table 7). Concentrations determined by counting cysts and oocysts were divided by the appropriate mean recovery efficiency to correct for inevitable losses during processing. Corrected cyst concentrations ranged from 0-1.36 cysts/L (0-5.2 cysts/gal) and corrected oosyst concentrations ranged from 0-0.70 oocysts/L (0-2.6 oocysts/gal). This compares to results of previous studies giving 0-15 cysts/L (Monzingo et al., 1986; Ongerth et al., 1988; Rose et al., 1988; Sykora et al., 1987) and 0-240 oocysts/L (Ongerth and Stibbs, 1987; Rose, 1988; Rose et al., 1988)

Raw water samples in which no cysts or oocysts were detected should not be considered free of protozoans. A more accurate interpretation of the results would be that about 46% and 38% of the time, cysts and oocysts, respectively, would be found if present. Limits of detection were calculated for each sample in which no protozoans were detected (table 7), these range from $< 0.03 - < 1.76$ cysts or oocysts / L in raw water samples.

The portion of each total sample analyzed depended on the final pellet turbidity and available time for analyses. Studies

TABLE 7

CORRECTED CONCENTRATIONS AND LIMITS OF DETECTION OF PROTOZOANS IN RAW WATER SAMPLES

		Analyte and Concentration*	
Sample	Date	G.l. cysts/ L	C.p. oocysts/ L
OWASA	1/11/88	1.36	< 0.17
HP	1/15/88	< 0.28	< 0.35
Dur.	2/15/88	< 0.03	0.07
Burl.	2/19/88	< 0.30	< 0.37
W-S	3/04/88	0.19	< 0.12
Ral.	3/18/88	< 0.57	< 0.70
OWASA	3/23/88	0.57	0.70
Dur.	7/05/88	0.10	0.17
W-S	7/18/88	0.05	0.06
HP	7/18/88	< 0.57	< 0.70
Ral.	7/28/88	< 1.42	< 1.76
Burl.	7/28/88	< 0.57	< 0.70

****

* = if cyst or oocyst concentration was below the limits of detection, the value is given as less than (<) the detection limit

in Arizona examined at least 10% of the total sample volumes (Musial et al., 1987). In Washington entire 20L samples were examined for cysts and oocysts (Ongerth and Stibbs, 1987; Ongerth et al., 1988). In general, "(s)ample size and frequency have been based on practical and economic considerations."(Craun and Jakubowski, 1987). In this case the practical consideration was the amount of time it took to scan each IFA labelled 13 mm membrane filter. Average time per filter was 1-1.5 hrs. Thus, each set of 3 filters took 3-4.5 hrs. With very turbid samples, examining 10% of the total could take a week or more.

The worst case was the Raleigh sample of 7/28/88. Using the established protocol a set of three filters was examined, but this comprised only 0.4% of the total sample. To examine 10% of the total would have taken 75-112 hrs of microscope time and 38-50 hrs of processing time for a total of 113-162 hrs for this one sample. This amount of time was not available. Considering time limitations and the recovery efficiency of the processing protocol, it was decided to process and count as many samples as time allowed.

Statistical Analyses

Cursory examination of the data for associations among G. lamblia and/or C. parvum, fecal coliforms or enterococci, and turbidity revealed no apparent relationships (fig. 9-10). Therefore, various statistical methods were used to examine associations of the parameters in question and to determine

Figure 9.

G. lamblia with fecal coliforms and enterococci

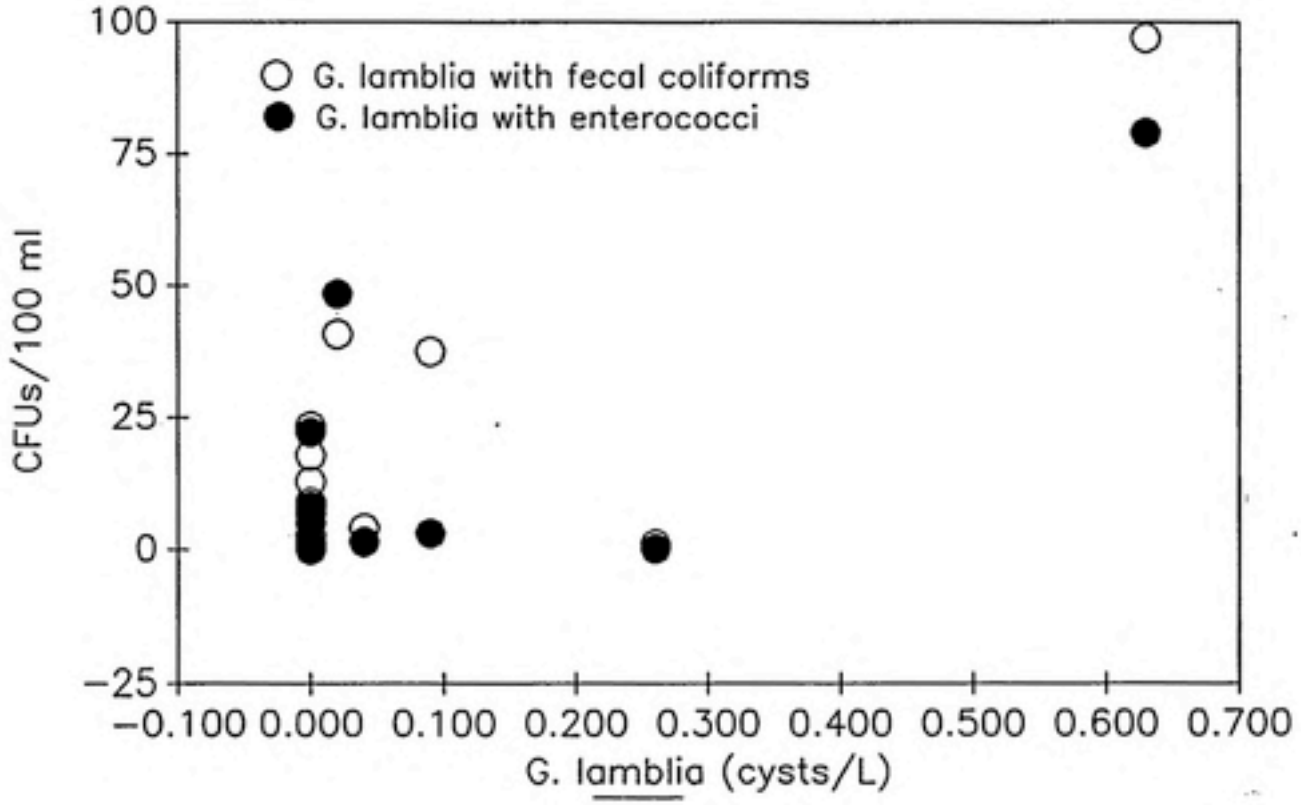
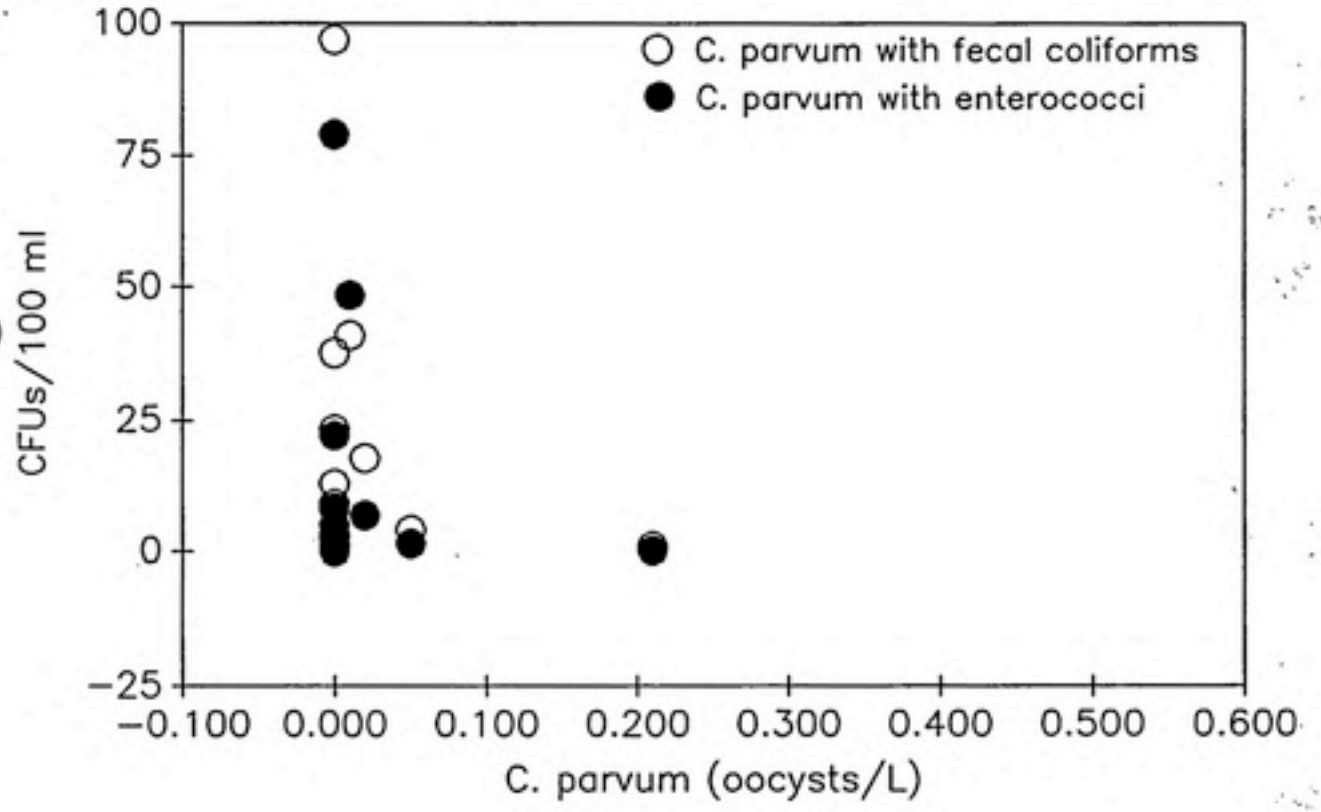


Figure 10.

C. parvum with fecal coliforms and enterococci



whether or not the % of total sample analyzed had any effect on the ability to detect protozoan cysts or oocysts.

A Pearson correlation matrix (table 8) showed good correlations of G. lamblia cysts with fecal coliforms ($r = 0.771$) and enterococci ($r = 0.710$). These correlations were highly significant because at $p = 0.05$, $r \geq 0.576$ (Neave, 1978). However, an examination of the data (table 6) and scatterplots (fig. 9-10) shows an outlier (OWASA 1/11/88). This would strongly affect the test because the test does not correct for outliers. There are two additional problems with using Pearson correlation coefficients for these data. This analysis requires bivariate normal distributions; which these are not, and the sample size is very small.

Jacknife analysis, a nonparametric computer intensive resampling technique that provides asymptotically valid tests of significance, was also performed (Sokal and Rohlf, 1981; Wu, 1986). The chief drawbacks of Jacknife are that it works best for samples $n>50$ and it is affected by outliers. Its strength is that it is robust to violations of most distributional assumptions (i.e. it doesn't require bivariate normal distribution). This analysis showed no significant association between cysts or oocysts and either fecal coliforms or enterococci. However, there was a significant association of fecal coliforms and enterococci ($t = 2.709$; if $t \geq 2.201$, $p \leq 0.05$) (table 9).

The next two methods do not need linearity for association.

TABLE 8

PEARSON CORRELATION MATRIX OF RAW WATER SAMPLES

	G.l.	C.p.	F.C.	E.	NTU	%
G.l.	1.000					
C.p.	0.256	1.000				
F.C.	0.771*	-0.265	1.000			
E.	0.710*	-0.200	0.892	1.000		
NTU	-0.116	-0.238	0.098	0.205	1.000	
%	-0.085	-0.061	0.189	0.173	0.688*	1.000

* = Significant correlation (if $r \geq 0.576$, $p \leq 0.05$)

TABLE 9

JACKNIFE SIGNIFICANCE TESTS FOR CORRELATION OF RAW WATER SAMPLES

Dependent var.	Independent var.	t
G.l.	C.p.	0.395
G.l.	F.C.	0.964
G.l.	E.	0.888
G.l.	NTU	-0.804
G.l.	%	-0.396
C.p.	F.C.	-0.686
C.p.	E.	-1.041
C.p.	NTU	-0.813
C.p.	%	-0.302
NTU	F.C.	0.417
NTU	E.	0.565
NTU	%	2.467*
F.C.	E.	2.709*
F.C.	%	0.371
E.	%	0.268

* = Significant correlation (if $t \geq 2.201$, $p \leq 0.05$)

They examine whether the parameters measured increase or decrease together (Bhattacharyya and Johnson, 1977). The Spearman matrix of correlation coefficients (table 10) shows a significant relationship between fecal coliforms and enterococci only (r_{sp} = 0.804, for $p \leq 0.01$, $r_{sp} \geq 0.708$). The weak point with the Spearman analysis is that the minimum n = 10 and the n = 12 of this study is near this minimum

The final nonparametric method employed was the Kendall tau-B matrix of coefficients (table 11). This analysis showed a significant association of fecal coliforms with enterococci (r_{tau} = 0.636; for $p \leq 0.05$, $r_{tau} \geq 0.455$ and for $p \leq 0.01$, $r_{tau} \geq 0.576$). There was no significant association of cysts or oocysts with either bacterial indicator system or with turbidity.

Other studies have examined associations of fecally associated bacterial indicators with G. lamblia cysts and C. parvum oocysts in surface water samples. Associations of cysts with total coliforms were not significant (Akin and Jakubowski, 1986; Craun and Jakubowski, 1987; Lippy and Logsdon, 1984; NPDWR, 1987); and associations of cysts with fecal coliforms were not significant (Craun and Jakubowski, 1987; Rose et al., 1988). Finally, associations of oocysts with fecal coliforms (D'Antonio er al., 1985; Rose et al., 1988) and with total coliforms (Rose et al., 1988) were not significant. Thus, this present study agrees with other studies in finding fecally associated bacterial indicator systems to not be adequate predictors of G. lamblia cyst or C. parvum oocyst presence in surface waters.

TABLE 10

MATRIX OF SPREARMAN CORRELATION COEFFICIENTS OF RAW WATER SAMPLES

	G.l.	C.p.	F.C.	E.	NTU	%
G.l.	1.000					
C.p.	0.399	1.000				
F.C.	0.304	-0.179	1.000			
E.	0.156	-0.125	0.804*	1.000		
NTU	-0.297	-0.079	0.235	0.340	1.000	
%	0.329	0.430	0.434	0.452	0.497	1.000

* = Significant correlation (if $r_{sp} \geq 0.708$, $p \leq 0.01$)
(if $r_{sp} \geq o.576$, $p \leq 0.05$)

TABLE 11

MATRIX OF KENDALL TAU-B COEFFICIENTS OF RAW WATER SAMPLES

	G.l.	C.p.	F.C.	E.	NTU	%
G.l.	1.000					
C.p.	0.387	1.000				
F.C.	0.239	-0.160	1.000			
E.	0.128	-0.120	0.636*	1.000		
NTU	-0.203	0.000	0.168	0.229	1.000	
%	0.192	0.356	0.318	0.350	0.368	1.000

* = Significant correlation (if $r_{tau} \geq 0.576$, $p \leq 0.01$)
(if $r_{tau} \geq 0.455$, $p \leq 0.05$)

To test whether or not sample volume had any effect on cyst or oocyst detection, the correlations and associations of cysts and oocysts with the percent of the total sample counted (%) were examined. None of the these tests showed significant correlations or associations (tables 8-11).

Finished Waters

No G. lamblia cysts or C. parvum oocysts were detected in any of the finished water samples (table 12). Cysts and oocysts were found in a sample from Durham (2/15/88), but careful examination of 2 other 20% subsamples found none. It was determined that contamination of the membrane filters in the first aliquots examined occurred by way of the tweezers used in handling them as these tweezers were also used to handle the membrane filters used for determining cyst and oocyst concentrations in the seed preparation. This points out the need for very thorough cleaning of all equipment used in these studies.

Limits of detection of cysts and oocysts were calculated for all of the finished water samples (table 13). These ranged from $< 0.005 - < 0.034$ cysts or oocysts / L. Because these calculations were based on the recovery efficiencies of cysts and oocysts from the raw water in the seeded sample methods section, the actual limits of detection are probably much lower.

No fecal coliforms or enterocci were detected in any of the finished water samples (table 12). This may be because of an

TABLE 12

CONCENTRATIONS OF PROTOZOANS, BACTERIA AND TURBIDITY IN FINISHED WATER SAMPLES

Sample	Date	Analyte and Concentration*				
		G.l. cysts/ L	C.p. oocysts/ L	FC CFU/ 100 ml	E CFU/ 100 ml	NTU
OWASA	1/11/88	ND	ND	ND	ND	0.31
HP	1/15/88	ND	ND	ND	ND	0.20
Dur.	2/15/88	ND	ND	ND	ND	0.05
Burl.	2/19/88	ND	ND	ND	ND	0.44
W-S	3/04/88	ND	ND	ND	ND	0.20
Ral.	3/18/88	ND	ND	ND	ND	0.07
OWASA	3/23/88	ND	ND	ND	ND	0.07
Dur.	7/05/88	ND	ND	ND	ND	0.05
W-S	7/18/88	ND	ND	ND	ND	0.43
HP	7/18/88	ND	ND	ND	ND	0.23
Ral.	7/28/88	ND	ND	ND	ND	0.17
Burl.	7/28/88	ND	ND	ND	ND	0.07

* = ND indicates none detected

TABLE 13

LIMITS OF DETECTION OF PROTOZOANS
IN FINISHED WATER SAMPLES

Sample	Date	Analyte and Concentration* G.l. cysts/ L	C.p. oocysts/ L
OWASA	1/11/88	< 0.005	< 0.008
HP	1/15/88	< 0.005	< 0.008
Dur.	2/15/88	< 0.005	< 0.005
Burl.	2/19/88	< 0.029	< 0.034
W-S	3/04/88	< 0.005	< 0.008
Ral.	3/18/88	< 0.013	< 0.018
OWASA	3/23/88	< 0.005	< 0.008
Dur.	7/05/88	< 0.005	< 0.008
W-S	7/18/88	< 0.005	< 0.008
HP	7/18/88	< 0.005	< 0.008
Ral.	7/28/88	< 0.005	< 0.008
Burl.	7/28/88	< 0.005	< 0.008

****

* = these limits of detection are probably high as recovery efficiency assessments were performed on raw, more turbid, waters.

oversight by the investigator wherein no sodium thiosulfate was added to the finished water samples to inactivate the chlorine disinfectent. However, water treatment plant operators reported no total coliforms in the same waters and it is probably reasonable to assume that if there were no total coliforms that there would have been no fecal coliforms and perhaps no enterocci present.

This study shows that this type of "conventional" treatment (NPDWR, 1987) is adequate to remove/inactivate G. lamblia cysts, C. parvum oocysts, fecal coliforms and enterococci to below the limits of detection.

Wastewater Treatment Plant Locations

Wastewater treatment plants (WWTPs) discharging into the watersheds studied were identified from Division of Environmental Management (DEM) maps and files which were compiled from NPDES permits. WWTPs discharge into the source waters of three of the community water systems in this study: Winston-Salem, Raleigh and Burlington. One WWTP in the Winston-Salem watershed and one in the Burlington watershed discharged into surface waters other than the ones sampled by this study (table 13).

This present study shows no strong association of WWTP discharge into surface waters with cyst or oocyst presence. Only 2 source waters sampled had WWTPs upstream and in only one (Winston-Salem) were cysts or oocysts detected (table 14). The concentrations of cysts and oocysts found in Winston-Salem raw

TABLE 14

WASTEWATER TREATMENT PLANTS IN WATER TREATMENT PLANT WATERSHEDS

WTP	WWTP Name	Receiving stream	Miles downstream to WTP
Ral.	Durham-Eno R.	Eno R.	60
	D. Little Lick Ck	Little Lick Ck.	35
	D. Northside	Ellerbe Ck.	50
	Hillsborough	Eno R.	85
W-S	Kernersville Salem Ck.*	Salem Ck.	10
	Wayne poultry**	Chapman Ck.	80
	Dobson	Cody Ck.	80
	Elkin	Yadkin R.	80
	Jonesville	Sandberry Ck.	80
	Boonville	Tanyard Ck.	65
	Yadkinville	Haw Branch	30
	King Sanitary Dist.	Yadkin R.	35
	N. Wilkesboro	Yadkin R.	120
	Wilkesboro	Cub Ck.	125
	Warrior Ck. Park (new)	Yadkin R.	150
	Warrior Ck. Park (old)	Yadkin R.	150
	N. Wilkesboro (not built)?	Mulberry Ck.	120
Burl.	Gibsonville*	Cedar Ck.	15

* = Not in the watershed tested, though does feed into distribution system via another WTP

** = May contribute C. meleagridis oocysts

waters were not higher than the concentrations found in waters not receiving WWTP discharge (table 15) suggesting that WWTP discharges did not have a detectable impact on cyst or oocyst concentrations at the time of sampling.

There is not enough data to determine what influence WWTP discharges had on the presence of cysts and/or oocysts in these waters. All that can be said at present is (1) WWTPs may not be discharging detectable quantities of cysts and/or oocysts into surface waters, (2) WWTPs may be discharging cysts and/or oocysts, but input to them may not be constant, thus discharge may not be constant and these events may have been missed by the limited sampling schedule, and (3) cysts and/or oocysts may be being discharged in detectable quantities, but they may be destroyed or inactivated over time and distance to the extent that they are not detectable.

Other Possible Cyst of Oocyst Inputs

All of these watersheds have agricultral impacts including dairy farms which may directly and/or indirectly contaminate surface waters. Also, all of these watersheds are inhabited by beaver, muskrat and many other mammals which may act as reservoirs. No systematic studies have been done on either of these possible cyst or oocyst sources in these areas.

TABLE 15

CONCENTRATIONS OF PROTOZOANS AND BACTERIA IN RAW WATERS WITH WASTE WATER TREATMENT PLANT PRESENCE

		Analyte and Concentration				
Sample	Date	G.l. cysts/ L	C.p. oocysts/ L	FC CFU/ 100 ml	E CFU/ 100 ml	WWTP upstream (Y/N)
OWASA	1/11/88	0.63	0	97	79	N
HP	1/15/88	0	0	12.8	22.2	N
Dur.	2/15/88	0	0.03	17.8	6.8	N
Burl.	2/19/88	0	0	2.5	1.2	N*
W-S	3/04/88	0.09	0	37.5	3.0	Y
Ral.	3/18/88	0	0	0.25	0	Y
OWASA	3/23/88	0.26	0.26	1.0	0.25	N
Dur.	7/05/88	0.04	0.05	4.0	1.5	N
W-S	7/18/88	0.02	0.02	40.8	48.5	Y
HP	7/18/88	0	0	7.8	8.2	N
Ral.	7/28/88	0	0	23.3	5.0	Y
Burl.	7/28/88	0	0	9.0	0.75	N*

****

* = one WWTP does discharge into Burl. source waters, but not into the water tested

From the NC Dept. of Nat. Res. and Com. Dev., Div. of Env. Mng., 198

Conclusions

Based on the limited analyses conducted in this study:

1) *G. lamblia* cysts and *C. parvum* oocysts are present at low concentrations in selected surface waters of the North Carolina Piedmont.
2) Conventional water treatment, including prechlorination, coagulation, sedimentation, rapid sand/anthracite filtration and disinfection with chlorine gas, reduces cyst, oocyst fecal coliform and enterococci concentrations to undetectable levels.
3) In raw waters, levels of cysts or oocysts detected were not significantly associated with levels of fecal coliforms or enterococci.

Recommendations

1) Develop a standard method for detection and quantitation of *infectious* cysts and oocysts.
2) Survey southeastern surface drinking water sources:
    a) to determine cyst and oocyst concentrations,
    b) if found, to determine what the major contamination sources are: point or non-point sources.
3) Explore other predictor systems attempting to find one(s) that will exhibit a consistent, positive association with infectious cyst and/or oocyst presence.
4) Determine recovery efficiencies of cysts and oocysts with each water sampled.

APPENDIX A

Seed Concentration Determination

The following is the stastical method used to determine the concentration of cysts and oocysts in the preparation from Meridian Diagnostics.

List of variables and definitions

K = 2.5 ml = the total volume of the (oo)cyst contaminated preparation provided by Meridian Diagnostics.

k = 0.5 ml = the volume of the preparation analyzed to determine average (oo)cyst concentrations. This was done in 6 aliquots.

$j = 1,\ldots,6$ = aliquot numbers. Each aliquot was filtered through a membrane filter, labeled, mounted, viewed, and (oo)cysts in each field were counted.

$n = 1,\ldots,100$ = number of each field.

s = standard deviation of counts of (oo)cysts in 100 fields.

Γ = actual (oo)cyst concentration as #/ml.

$V_{j,}$ = volume of aliquot analyzed spread over 13 mm filter (1290 fields).

$V_j \approx$ volume of aliquot / field = $V_{j,}$ / 1290 fields

$\overline{X_j}$ = mean count of (oo)cysts per field, from 100 fields.

X_j = ($V_{j,}$ / 1290 fields) * Γ (oo)cysts, actual number of (oo)cysts per field.

$X_{j,n}$ = actual counts of (oo)cysts / field.

$X_{j,1}, X_{j,2}, \ldots, X_{j,100}$ $[(V_{j,}/1290) * \Gamma_{,j}^{2}]$, and $V_j = V_{j,}/1290$

By the Central Limit Theorem: $j = 1,\ldots,6$

$X_j \sim N(V_j \Gamma, \sigma_j^2/100)$

The estimator of (oo)cyst concentration Γ is

$$\hat{\Gamma} = \overline{X_j}/V_j \quad = \text{mean \# (oo)cysts / volume}$$

with variance $V^{\wedge}(\Gamma) = \sigma_j^2/(V_j^2/100)$

Now we pool the $\hat{\Gamma}_j$s for each filter j = 1,...,6

$\bar{X}_j/V_j = \hat{\Gamma}_j \sim N\ (\ \Gamma,\ \sigma_j^2/V_j^2 * 100)$, use s_j^2 for σ_j^2

and, $V^*(\hat{\Gamma}_j) = \sigma_j^2/V_j^2 * 100)$

After weighting the mean by the inverse of the variance we get:

$$\hat{\Gamma} = \frac{\sum^{6} V_j \bar{X}_j/s_j^2}{\sum^{6} V_j^2/s_j^2}$$ as an estimate of the true [(oo)cyst]

and:

$$V^*(\hat{\Gamma}) = [100 \sum^{6} V_j^2/s_j^2]^{-1}$$

95% confidence intervals for $\hat{\Gamma}$:

$$\hat{\Gamma} \pm 2\sqrt{V^*(\hat{\Gamma})}$$

Because of the limited size of the initial preparation it is possible to use a Finite Sample Adjustment (FSA) to get a more accurate estimate of the distribution, variance and the 95% CI:

$V^*(\hat{\Gamma})_{FSA} = (1-k/K)(V^*(\hat{\Gamma}))$ thus, $\hat{\Gamma} \sim N(\ \Gamma,\ V\ (\hat{\Gamma})_{FSA})$

and the 95% CI:

$$\hat{\Gamma} \pm 2\sqrt{(1-k/K)(100 \sum^{6} V_j^2/s_j^2)^{-1}}$$

For G. lamblia

$\hat{\Gamma}$ = 4668 cysts / ml

$V^*(\hat{\Gamma})_{FSA}$ = 106,980 cysts / ml

$s\ (\hat{\Gamma})_{FSA}$ = 327 cysts / ml

95% CI = (4014, 5322)

For C. parvum

$\hat{\Gamma} = 2.02 \times 10^6$ oocysts / ml

$V^*(\hat{\Gamma})_{FSA} = 7.94 \times 10^7$ oocysts / ml

$s\ (\hat{\Gamma})_{FSA}$ = 8,909 oocysts / ml

95% CI = $(2.003 \times 10^6,\ 2.039 \times 10^6)$

APPENDIX B

Recovery efficiency determination

The estimate of % recovery is based on the seeded concentration Γ; estimated as $\hat{\Gamma} \pm \sqrt{V(\hat{\Gamma})}$. Then % recovery is given by μ/Γ x100, where μ is the overall mean count of cysts or oocysts on each filter, and % recovery is estimated by:

$\hat{\mu}/\hat{\Gamma}$ x 100, where $\hat{\mu}$ and $\hat{\Gamma}$ are independent.

The estimated standard deviation of recovery as a % is:

$$100\sqrt{\hat{V}(\hat{\mu}/\hat{\Gamma})} \approx 100/\hat{\Gamma}\sqrt{V(\hat{\mu}) + (\hat{\mu}^2/\hat{\Gamma}^2)V(\hat{\Gamma})}$$

Values for Giardia lamblia:

$\hat{\mu}$ = estimate of mean recovered cyst concentration

= 26 cysts/0.8 gal = 32.5 cysts/gal

$\hat{\Gamma}$ = estimate of cyst concentration in seed

= 4668 cysts/ml seed

= (4668 cysts/ml seed) x (0.6 ml seed) x (1/40 gal)

= 70 cysts/gal

$V(\hat{\Gamma})$ = variance of cyst concentration in seed

= 106,980 cysts/ml seed

= (106,980 cysts/ml seed) x (0.6 ml seed) x (1/40 gal)

= 1605 cysts/gal

$V(\hat{\mu})$ = 0.748 cysts^2/gal^2, from Components of Variance (Snedecor and Cochran, 1967)

Recovery efficiency

$\hat{\mu}/\hat{\Gamma}$ = [(32.5 cysts/gal)/(70 cysts/gal)] x 100 = 46.4 %

Standard deviation as %

$$100/\hat{\Gamma}\sqrt{V(\hat{\mu}) + (\hat{\mu}^2/\hat{\Gamma}^2)V(\hat{\Gamma})} =$$

$= 100\ \text{gal}/\ 70\ \text{cysts} \quad 0.748\ \text{cysts}^2/\text{gal}^2 + (1056.3/4900)(1605)$

$= 26.6\ \%$

Values for Cryptosporidium parvum

$\hat{\mu}$ = estimate of mean recovered oocyst concentration

= 91.3 oocysts/0.8 gal = 114.1 oocysts/gal

$\hat{\Gamma}$ = estimate of oocyst concentration in seed

= 2.02×10^4 oocysts/ml seed

= $(2.02 \times 10^4$ oocysts/ml seed)x(0.6 ml seed)x(1/40 gal)

= 303 oocysts/gal

$V(\hat{\Gamma})$ = variance of oocyst concentration in seed

= 7.94×10^5 oocysts/ml seed

=$(7.94 \times 10^5$ oocysts/ml seed)x(0.6 ml seed)x(1/40 gal)

= 11,910 oocysts/gal

$V(\hat{\mu})$ = 0.23.77 $\text{oocysts}^2/\text{gal}^2$, from Components of Variance (Snedecor and Cochran, 1967)

Recovery efficiency

$\hat{\mu}/\hat{\Gamma}$ = [(114.1 oocysts/gal)/(303 oocysts/gal)] x 100 = 37.6%

Standard deviation as %

$$100/\hat{\Gamma}\sqrt{V(\hat{\mu}) + (\hat{\mu}^2/\hat{\Gamma}^2)V(\hat{\Gamma})} =$$

$=100\text{gal}/303\ \text{oocysts} \quad 23.77\text{oocysts}^2/\text{gal}^2 + (12966/91809)(11,910)$

= 13.6 %

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