Assessment of methods for determining viability of *Giardia spp.* in freshwater and seawater.

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Abstract.

The determination of viability of *Giardia spp.* cysts is difficult.
At present the problem is that once detected it is not known whether this cyst is viable and therefore potentially infective for humans.
This study assesses two methods; Nomarski Differential Interference Contrast Microscopy (Nomarski DIC) and Fluorogenic Dyes (Fluorescein Diacetate and Propidium Iodide) as they compare to the current benchmark for viability Excystation.

In vivo Giardia muris and *Giardia intestinalis* cysts were assessed for viability at time intervals and different temperatures in two separate inactivation systems; chlorine at standard municipal treatment levels and seawater with a view to their use in routine viability testing of cysts detected in environmental samples. *G. muris* was trialled as it was thought it may prove to be a good model for *G. intestinalis*.

The effect of seawater as an environmental inactivation system is important due to current domestic waste disposal practises ie. sea disposal of treated waste. These effects were assessed in this study by Excystation, Nomarski DIC and Fluorogenic Dyes.

Seawater has a cysticidal effect on *Giardia* cysts. This is due mainly to osmotic and alkaline nature of seawater.
Nomarski DIC when compared to Excystation, has a limited capacity for determining viability of cysts from freshwater, seawater and chlorine inactivation systems. Fluorogenic Dyes seem more suited to determination of viability of cysts isolated from fresh and untreated waters.

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Contents.

List of Figures.

List of Tables.

List of Plates.

Chapter One The Organism.

1.1 Discovery.

Van Leeuwenhoek an amateur lens maker from Delft, Holland, was the first person to describe the microbial world. Using a microscope with only a single lens, he discovered a wide variety of micro-organisms in materials ranging from hay infusions to tooth scrapings. His success in seeing cells as small as bacteria can be attributed to the exceptionally high quality of his lenses, his technique of mounting and lighting the specimen and his insatiable curiosity. It was probably this curiosity that prompted him to examine his own diarrhoeic stool.

He described many microorganisms, and in a letter dated 4 November 1681, he stated (in translation)

> "Their bodies were somewhat longer than broad, and their belly which was flat like, furnisht with sundry little paws, where with they made such a stir in the clean medium and among the globules, that you might e'en fancy you saw a woodlouse running up a wall; and albeit they made such a commotion with their paws, yet for all that they made but slow progress."
> (Dobell 1932)

From this statement many scientists today believe Van Leeuwenhoek to have been the first to observe *Giardia* trophozoites.

Vilem Lambl, a Czech physician is credited with the discovery of the organism, which he named Lamblia. Van Leeuwenhoek and Lambl saw and described *Giardia* trophozoites, but there is no evidence that either man saw or recognised the relationship of the trophozoite to the cyst form of the parasite. *Giardia* cysts were first noted by Grassi. He first thought they might be

coccidia, but in 1888 he concluded that they represented the flagellated form of the organism (Grassi 1888).

1.2 Taxonomy.

The taxonomy and nomenclature of organisms in this group presented problems in the late 19th and early 20th century. Two generic names were applied to these protozoa. Kunstler (1882), isolated *Giardia* from the tadpole and Blanchard (1888), Lamblia from mammals. The organism these scientists described were of the Genus as demonstrated by Hegner (1922).
Today the name *Giardia* is given to all representatives of this group and the name giardiasis given to the disease they cause.

To date the assignment of *Giardia* isolates to a specific species has undergone much review. Until the middle of this century it was common practise to assign *Giardia* species names on the basis of host specificity (Woo et al 1984), and on the differences in various trophozoite dimensions (Filice 1952). Kulda and Nohynkova (1978) were able to list references to more than forty *Giardia* "species", demonstrating that representatives of this genus appear to exist intestinally in virtually every mammalian species examined. Filice (1952) took a more objective view and concluded that the use of host specificity and body measurement was an unreliable method of species classification. His work assigned *Giardia* from different sources to a given species on the basis of median body shape. This he thought made use of "constant biological differences" which are visible in all trophozoite isolates of *Giardia*. His classification put *Giardia* of the median body type that parasitise humans and other mammals into the species *Giardia* duodenalis. The median bodies of this type are paired, lie transversely across the trophozoite body, and resemble the claw of a hammer, hence the description "claw shaped median bodies".

The median bodies of *Giardia muris* are small and rounded, and *Giardia agilis* teardrop shaped. This thesis has determined to use the name *Giardia*

intestinalis put forward by Boreham et al (1984) to describe species demonstrated as infecting humans and non-rodent animals. Since the proposal of this classification 23 of the 40 "species" listed by Kulda and Nohynkova in their 1978 review have been classed as *Giardia intestinalis* having claw shaped median bodies.

1.3 Biology of *Giardia.*

Giardia is a flagellated protozoan belonging to the classification:

Class Zoomastigophorasidan,
Order Diplomonadida,
Family Hexamitidae.

It is found in two morphologically distinct forms: in fresh faeces as the infective cyst and in the intestine of infected humans and animals as the reproductive trophozoite.

The trophozoite found living in the duodenum, is responsible for the pathogenesis of the disease. They are bilaterally symmetrical organisms with rather a pear shape. Trophozoites have four pairs of flagella and attach to the microvilli of the duodenum and jejunum by means of an anteriorly situated ventral sucking disc. Possession of this disc differentiates *Giardia* from other members of the family Hexamitidae (Feely et al 1984).

The cyst form is oval in shape and ranges in size from 8-12 μm long and 7-10 μm wide. They consist typically of four nuclei, intracytoplasmic axonemes and median bodies encased within a hyaline cyst wall. The cyst forms during passage through the jejunum (Burke 1977; Lambert 1970), and passes out in the faeces. This is predominant in the faeces, as the cyst is the means of transmission and survival outside the body.

The trophozoite and cyst forms of *Giardia* do share some internal organelles.

-Axonemes,

-median bodies, and

-nuclei are often immediately obvious as being common, with the adhesive disc and flagella being the exclusive property of the trophozoite.

1.4 The Disease.

1.4.1 Introduction.

G. intestinalis is the etiological agent of giardiasis. This was not immediately clear due to the range of conditions the disease manifests, ranging from asymptomatic carriage through to chronic diarrhoeic malabsorption. But now as stated by Kulda and Nohnykova (1978),"there is no doubt that the organism is capable of causing disease in man". This was concluded on the basis of symptoms such as malabsorption, and histopathology. Further, reports of epidemic outbreaks of giardiasis particularly in the case of Aspen, Colorado, gave foundation to these claims. Here during the 1965-66 ski-season, 123 out of 1094 skiers tested, developed symptoms characteristic of Giardiasis, and on pathological examination of all those infected revealed no other pathogens apart from *G. intestinalis*.

1.4.2 Pathogenesis

At the outset, giardiasis begins as an acute disease. This is preceded by a prepatent period of between nine and twenty days (Brodsky et al 1974, Rebdtorff 1954, Walzer et al 1971). Prepatent periods shorter than this and followed by similar symptoms can often be attributed to Cryptosporidiosis (Jokipii et al 1985).

The acute infection lasts only a few days. During this phase there is dense colonisation of the jejunal epithelium with adherence to columnar epithelial cells at the base of the villi (Owen et al 1979). Trophozoites are also commonly detected in the duodenum of infected patients. Trophozoites attach but do not normally penetrate the intestinal epithelium. However if this does

occur then it is usually due to necrosis or mechanical trauma (Owen et al 1979) of the mucosa.

The trophozoite is normally adherent to the epithelial cell surface by means of its ventral adhesive disc and cysts are passed in the faeces in large numbers (in the order of 10^8/gram faeces/day). The acute phase usually resolves spontaneously to give rise to either a sub-acute or chronic stage. Cysts may disappear from faeces and the patient becomes an asymptomatic cyst-passer; others may have periodic brief recurrences of the acute symptoms.

Pathologically the sheer number of trophozoites colonising the intestine causes a problem in itself. Wright et al (1977) found evidence of Vitamin B12 malabsorption in 20 of 40 patients with giardiasis. This can also cause absorption of fats to be inhibited and a reduced surface area means a much lower level of fat is uptaken.

1.4.3 The Symptoms

A variety of symptoms may signal the onset of giardiasis; these include nausea, anorexia, a feeling of queasiness in the upper intestine, malaise, and perhaps low grade fever and chills. Then in the acute phase usually the sudden onset of explosive watery foul smelling diarrhoea, associated with foul flatulence and regular abdominal cramps. Foul belching often called "purple burps" may also occur. Mucus is rarely found in the stool and blood if present is only indicative of anal irritation from the diarrhoea. If acute infection is prolonged as is the case in many immuno-compromised persons and small children, then the patient may suffer from malabsorption, steatorrhoea, debility and weight loss (Meyer 1990).

1.4.4 Diagnosis

The term Giardiasis as an infection is often used to wrongly describe any

infection that causes the above symptoms. Chronic diarrhoea caused by giardiasis must be differentiated from that caused by *Cryptosporidium*, *Dientamoeba fragilis*, inflammatory bowel disease and irritable colon. Giardiasis should be suspected in the absence of blood or mucus in the stool, and in the presence of upper abdominal cramps, distention and foul smelling flatulence and faeces.

The unequivocal diagnosis of giardiasis involves the examination of a series of at least three stool specimens taken over a period of one week. This is due to the intermittent nature of excretion of *Giardia* cysts. Cysts are detected most commonly by a direct faecal smear and suspension in saline or Lugol's Iodine may be sufficient to see cysts if they are particularly numerous.

If diagnosis by this means fails to find parasites, and symptoms are indicative of a *Giardia* infection, then a small bowel biopsy or intestinal fluid examination may prove to be more effective (Meyer 1990).

1.4.5 Treatment

A number of drugs are available for treatment of patients with giardiasis. These include Quinacrine, the Nitroimidazoles Metronidazole and Tinidazole, and Furazolidone.

Quinacrine is a highly effective agent for treatment of giardiasis when given for a 5-10 day period. However gastro-intestinal side effects are common, and rarely toxic psychosis and haemolysis in glucose-6-phosphate-dehydrogenase-deficient patients may occur. It is thought that Quinacrine's action is due to action on the flavoprotein and quinone components of respiration (Paget et al 1989). The nitroimidazoles are highly effective for the treatment of giardiasis. They have a broad spectrum of

activity against anaerobic bacteria and protozoans.

Metronidazole (Flagyl™) is widely used in the USA and New Zealand for treatment, and is more than 90% effective when given for a 5-day course. Side effects such as nausea and general malaise are common during therapy, and serious side effects are rare. Use of Metronidazole is effective but it has been shown to be a carcinogen in mice and is suspected to be similar in humans (although not proven).

Tinidazole (Fasigyn™) is effective when given as a single dose. A single 2 gram dose, is usually effective in clearing infection in an otherwise healthy individual. This drug is well tolerated and its use in New Zealand is now common place, although in the USA it has not been approved for use.

Furazolidone, although less effective than Quinacrine and Metronidazole, is used in small children as the bitter taste and gastro-intestinal side effects of quinacrine make its use less than suitable. It is not known to be a carcinogen of man, but like metronidazole it is a carcinogen in rats.

Patients who fail to respond to treatment usually respond to a second course of treatment with the same drug or with a different treatment. In the case of asymptomatic giardiasis, treatment is often conducted on the ground of general public health. This person obviously poses a risk to the well being of those surrounding them with the risk of transmission; either fecal-oral or water-borne becoming more probable.

1.5 Transmission of Giardiasis.

Historically diseases of man such as *Vibrio cholera* and Hepatitis A were spread as a result of the poor hygiene standards of the time. The common factor in many outbreaks such as these is that there is usually gross fecal-oral transmission compounded by contaminated water supplies. Giardiasis similarly

is transmitted in the same way; directly by the fecal-oral route and indirectly by waterborne means.

1.5.1 Direct/Fecal-Oral Transmission.

The transmission of *Giardia* by the fecal-oral route is a well documented means of *Giardia* transmission. This is particularly so in persons living/working in close confines such as at day care centres and institutions (Brown 1948, Black et al 1977, Woo and Patterson 1986). The level of infection in day care centres has been seen to be about 17-55% in some cases (Bartlett et al 1985, Black et al 1977, Boreham and Shepherd 1984, Keystone et al 1978, , Pickering 1986).

This mode of transmission can also implicate domestic animals. Meyer (1979), showed this by infecting dogs and cats with a human strain of *Giardia*. This addresses the issue of whether *Giardia* is a zoonoses. Faubert (1988); and Bemrick and Erlandsen (1988) proposed that wild animals and perhaps birds which do not contain more than a single morphological or serological type within the G. intestinalis group, may occasionally have the potential to serve as a reservoir of human giardiasis in nature, but when this happens they are simply functioning as biological intermediaries for the human *Giardia*, having obtained their original infections from man. One should perhaps consider wild and domestic animals to be potential human parasitic reservoirs rather than sources of a zoonoses. Marino et al (1992), showed that *Giardia* was indeed endemic in populations of domestic animals such as ship rats and opossums.

1.5.2 Indirect/Waterborne Transmission.

Waterborne giardiasis contrary to public opinion accounts for a much lower proportion of giardiasis cases than fecal-oral transmission. Media coverage of waterborne outbreaks has been seen to emphasize this mode of transmission

(eg. Paekakariki, Linton outbreaks), and often when the case is examined epidemiologically many cases are found to be due to fecal-oral transmission.

Giardia cysts are deposited in fresh faeces into water by infected animals or humans. The number of cysts excreted may be of the order of 10^8/gram of faeces/day. Research into the sources of contamination of water has indicated in the United States of America (USA) that animals and humans infected with *Giardia* using the catchment as their natural habitat, or for recreational uses, are responsible for contamination. In some areas of the USA the beaver is responsible. The beaver has its habitat high in the hills which may be the water catchment for a local town. The species of *Giardia* beavers maintain has been shown to be infective for dogs and cats (Jakubowski 1988, Meyer 1979), and is thought to be infective for humans (Davies and Hibler 1979).

Humans using the catchment area for activities such as tramping climbing and skiing, are at risk of acquiring giardiasis as well as being a potential contaminator (asymptomatic or symptomatic). These activities by their very nature mean use of natural fresh-water supplies for daily use is unavoidable. The risk that this poses to these people is reduced when fresh-water is taken above the beaver line in the USA (Hibler and Hancock 1989). In New Zealand is thought to be a risk in many rivers.

1.6 *Giardia* Cysts in Drinking Water.

1.6.1 Incidence.

Giardia cysts are not normally found in drinking water supplied by local authorities. Fresh-water sources are more likely to be contaminated if they originate from surface waters than from an artesian supply. Contamination of ground waters usually results from human sewage (Craun 1985).
Obviously the presence of *Giardia* cysts in drinking water is unacceptable. Thus water treatment plants must be aware of the risks *Giardia* can pose to a community and operate treatment plants in such a way that cysts are effectively

removed or inactivated. The treatment process should consist of an effective particulate removal process (flocculation/ coagulation), and an effective disinfection process (A.W.W.A. 1985).

Evaluation of outbreaks indicates that they have been associated with one or more of the following when treating surface waters or polluted ground waters:

1. lack of or failure of the filtration process;
2. lack of or failure of the disinfection process;
3. lack of or failure of the chemical coagulation process.

These processes are required even when source waters are low in turbidity and have low levels of coliform fecal indicator bacteria. This derives from the fact that the presence of *Giardia* cysts in water sources is not correlated with either of these water quality parameters.

1.6.2 Detection

Detection of *Giardia* cysts in fresh-water sources is conducted under the guidelines set down by the U.S.E.P.A. in 1978, and was subsequently modified (Jakubowski 1979). Briefly the test involves filtering a minimum of 380 litres through a 23 cm long nylon wound 1 μm porosity cartridge filter. The filter is subsequently processed by shredding the fibres and washing thoroughly to remove cysts from fibres. The eluent is then pooled and concentrated by centrifugation or settling overnight. The supernatant is discarded and pellet resuspended in a minimum of 10% formalin. Cysts present in the suspension are concentrated by 1M Sucrose flotation (Roberts-Thomsen et al 1976), and harvested, then examination of this sample with specific immunoflourescent stains is conducted. A positive sample contains a cyst which exhibits typical apple-green fluorescence when examined under U.V. light.

In New Zealand the testing of waterways for *Giardia* has been conducted since March 1990 and is continuing at present. The distribution in New Zealand is fairly widespread as described by Brown et al (1992).

1.7 Viability.

1.7.1 Methods for Determining Viability of *Giardia*.

The problem at present is that once detected; it is not known whether this cyst is viable and therefore potentially infective for humans.

Many researchers have addressed this problem (Sauch 1988, Sauch et al 1991, Hoff et al 1985, Feely 1986, Shupp and Erlandsen 1987, Shupp et al 1988), and agree that excystation is the benchmark for viability testing of *Giardia*. This is because it mimics the in vivo conditions found in the intestine. A wide variety of excystation methods are available and all seem to have been based around a common protocol ie. acid incubation, reducing solution, excystation medium (Sauch 1988; Rice and Schaefer 1981; Rice and Schaefer 1984; Bhatia and Warhurst 1981; Buchel et al 1987; Meyer 1987; Feely 1986). This method has been used in various *Giardia* inactivation studies involving disinfectants (Leahy et al 1987; Neuwirth et al 1988; Hoff *et al.*, 1985; Rice et al 1982) drugs and different environmental treatments (Deng et al 1992).

Other methods for determining viability of *Giardia* have been used with varying success. Vital staining with Eosin (Kasprzak and Majewska 1983,1987) and Trypan Blue (Kaur et al 1986) as compared to excystation shows these stains consistently overestimate viability.

The fluorescent stains Fluorescein Diacetate and Propidium Iodide have been used to assess viability as compared to excystation. Fluorescein Diacetate (FDA) is the stain used to assess the relative viability of a cyst population.

The viable cyst actively uptakes the molecule and once internal, enzymes cleave the acetate groups off yielding the fluorescent Flourescein molecule. The reliability of this method is dependent on the integrity of the cyst wall. This is in contrast to Propidium Iodide (PI) which detects 'non-viable' cysts. Its action depends once again on the integrity of the cyst wall. The stain diffuses into the cyst through the damaged cyst wall, where it concentrates and fluoresces under UV light.

Shupp and Erlandsen (1987) showed that they were able to differentiate between viable and non-viable cysts as determined by mouse infectivity. Sauch (1988) and Labatavik et al (1991) examined the effectiveness of Propidium Iodide to determine the inactivation of *Giardia muris* in chlorine and ozone disinfection. Further results suggest that these vital stains may not be a satisfactory viability test for chemically killed cysts and that they may be more suited to an environmental inactivation system (Sauch 1991).

The use of morphological methods for viablity testing has been used by Shupp and Erlandsen (1987). Nomarski Differential Interference Contrast Microscopy or Nomarski DIC, has been used to assess viability of *Giardia* cysts. A viable cyst is characterised by having a clearly defined cyst wall and peritrophic space. The non-viable cyst has a granular appearing cytoplasm, intact cyst wall, cytoplasmic contents appear shrunken and peritrophic space enlarged (Shupp et al 1988). A good correlation between excystation and Nomarski DIC was found by these researchers.

This study will assess Nomarski DIC and Fluorogenic Dyes as they compare with excystation. The viability of *Giardia* cysts will be assessed by these three methods at time intervals in two inactivation systems,

-chlorine at normal municipal levels and

-seawater.

It is hypothesized that the fluorogenic dyes and Nomarski DIC optics be used

to test viability of cysts isolated from treated and raw water and also seawater. This thesis should address some of these questions.

1.7.2 *Giardia* viability in seawater.

Treatment of sewerage in New Zealand is a controversial topic. The lack of flat land and proximity to the coast in places like Wellington has meant a close examination of the way in which we dispose of this waste. Methods used at present remove most of the coarse unsightly material such as toilet paper, but many pathogenic microorganisms are still present at completion of treatment.

Hepatitis virus, *Escherischia coli* and cysts of Protozoa such as *Giardia spp.* are able to pass through the treatment and thus enter the environment. One such environment is the sea. Treated and sometimes untreated waste is pumped out into the sea, and it is not known if or for how long *Giardia* cysts remain viable in seawater. Three scenario's have been hypothesized with respect to freshwater:

1. it may be that the sea will act as a buffer due to the very balanced range of salts present, and therefore prolong the viability of the cyst when compared to fresh-water;

2. the seawater may have a highly corrosive and degrading effect rendering the cyst non-viable after a very short period of time. Osmotic shock may play an important part in providing a killing effect;

3. the seawater may have much the same effect that fresh-water has on cysts over time.

Chapter Two Materials

2.1 Viability Determination

2.1.1 *Giardia muris* Excystation.

1. Reducing Solution.

Hank's Balanced Salt Solution supplemented with 32 mM Glutathione (Sigma) and 57 mM L-Cysteine-HCl.

0.1 M Sodium Bicarbonate

Making a final pH of 4.7.

2. Excystation Medium.

0.5 % Proteose Peptone Solution, made by 10 fold dilution of 5 % Stock Proteose Solution in PBS.

Stock Proteose Peptone Solution (5 % w/v), made by gently boiling the solution for 10 minutes to destroy any remaining enzymes, then storing in small aliquots at 4°C until used.

2.1.2 *Giardia intestinalis* Excystation.

1. Low pH Induction Step.

Aqueous HCl (pH 2.0)

Hank's Balanced Salt Solution supplemented with 29 mM L-Cysteine-HCl and 67 mM Glutathione.

0.1 M Sodium Bicarbonate.

2. Excystment Step.

Excystment Medium.

0.5 % w/v Trypsin (1:100) dissolved in 1X Tyrodes Solution.

To prepare this, Trypsin was dissolved in the Tyrodes Solution by vigorous shaking for 30 minutes and then pH adjusted to 8.0 by addition of 7.5 % Sodium Bicarbonate.

This was stored in small aliquots at -20°C until used.

2.1.3 Vital Stains.

Both stains were made according to the method of Jones and Sneft (1985).

a) Flourescein Diacetate (FDA).

Stock solution of FDA (Sigma) made by dissolving 10 mg of anhydrous FDA powder in 1 ml of acetone. This solution was stored in the dark at 4°C until used.

Working solution was made up prior to each experiment by diluting the stock solution 1:250 (Shupp and Erlandsen 1979) in PBS.

b) Propidium Iodide (PI).

Working solution of Propidium Iodide was made by dissolving 1 mg of anhydrous PI in 50 ml PBS. This solution was stored at 4°C until used.

2.1.4 Nomarski Differential Interference Contrast Microscopy.

A Zeiss Axiophot Microscope equipped with Differential Interference Contrast Optics was used.

2.2 *Giardia* cysts.

2.2.1 *Giardia muris*.

Giardia muris cysts were obtained from Swiss mice that had been infected with a pure strain of cysts sent from U.S.A. kindly by Walter Jakubowski

(U.S.E.P.A.; Environmental Monitoring Systems Laboratory, Cincinnati). The cysts were isolated from faeces by washing faecal material three times with 0.01% Tween 20, and then 1 M Sucrose Flotation (Schaefer *et al.*, 1986). Following harvest of cysts from sucrose/water interface cysts were washed three times in 0.01% Tween 20 by centrifugation to remove sucrose. The cysts were enumerated using a Zeiss microscope equipped with phase contrast optics.

2.2.2 *Giardia intestinalis*.

These cysts were obtained from *Giardia* positive human faecal samples kindly sent by Shirley Gainsford of Valley Diagnostics Ltd. in Lower Hutt. On arrival in the laboratory samples were moistened with 0.01% Tween 20 and left overnight at 4°C to soften. The following day the sample was washed and cysts harvested in the same manner as for *Giardia muris* above.

2.3 D.P.D Colorimetric Method for chlorine determinations.

The level of free available chlorine was calculated using this very sensitive method.

Three stock solutions were needed:

a) Phosphate Buffer Solution.

b) N,N-Diethyl-p-phenylenediamine (DPD) indicator solution.

c) Standard Ferrous ammonium sulphate (FAS) titrant.

a) P.B.S.

Dissolve 24 grams of Anhydrous disodium hydrogen phosphate (Na_2HPO_4), and 46 grams of Anhydrous potassium dihydrogen phosphate (KH_2PO_4), in 100 mls of Milli-Q Water. Combine this solution with 100 mls of Milli-Q water in which 800 mg of Disodium ethylene diamine tetraacetate dihydrate (EDTA) have been dissolved. Dilute to 1 litre with Milli-Q water and add 20 mg Mercuric chloride to prevent mould growth, and to prevent interference in the

free available chlorine test caused by any trace amounts of iodide in the reagents.

b) N,N-DPD Indicator Solution.

Dissolve 1.5 grams of p-amino-N:N-diethylaniline sulphate, in Milli-Q water containing 8 mls of 1+3 sulphuric acid and 200 mg of EDTA. Make up to 1 litre, store in brown glass stoppered bottle, and discard when discoloured.

c) Standard FAS Titrant.

Dissolve 1.106 grams of Mohr's Salt, $Fe(NH_4)_2(SO_4)_2$-$6H_2O$ in Milli-Q water containing 1 ml of 1+3 sulphuric acid and make up to 1 litre with Milli-Q water. This primary standard may be used for 1 month, and the titer checked with potassium dichromate.

One ml of FAS titrant is equivalent to a chlorine concentration of 100 μg chlorine per 1.0 ml (= 1mg/l = 1 ppm)

Free Chlorine determination procedure.

Place 5 ml each of buffer reagent and DPD Indicator solution in the titration flask and mix. Add 100 ml of sample and mix.

Titrate rapidly with Standard FAS titrant until red colour is discharged.

This method is suitable for chlorine concentrations of up to 4 mg/l, above this and the sample must be diluted to a total volume of 100 ml.

2.4 General Media, Reagents and Equipment.

2.4.1 Hank's Balanced Salt Solution (HBSS).

Solution A:

1	NaCl	160 g
	KCl	8 g
	$MgSO_4.7H_2O$	2 g
	$MgCl_2.6H_2O$	2 g
	Milli-Q water	800 ml

2	$CaCl_2$	2.8g
	Milli-Q water	100 ml

Mix solutions 1 and 2 and make up tp 1000 ml with Milli-Q water. Add 2 ml chloroform and store at 4°C.

Solution B:	$Na_2PO_4.12H_2O$	3.04 g
	KH_2PO_4	1.2 g
	Glucose	20.0 g
	Milli-Q water	800ml

Mix solution B with 100 ml of 0.4% phenol red in NaOH and make up to 1000 ml with Milli-Q water. Add 2 ml of chloroform and store at 4°C.

To make working strength HBSS.
Add 100 ml of solution A to 100 ml of solution B and make up to 1 litre with Milli-Q water. Sterilise either by membrane filtration or autoclaving at 121°C for 15 minutes.

2.4.2 Phosphate Buffered Saline (Jakubowski 1990).

Prepare a 10X stock solution by dissolving:

NaCl	80 g
KH_2PO_4	2 g
Na_2HPO_4-$12H_20$	29 g
KCl	2 g

in a sufficient quantity of water to produce a final volume of 1000 ml. Dilute to 1 in 10 with water and adjust to pH 7.2 before use.

2.4.3 Tyrode's Salt Solution (Parker 1950).

To make up one litre of 1X Tyrodes dissolve the following in a small quantity of water:

$CaCl_2$-$2H_20$	0.265g
$MgCl_2$-$6H_20$ 0.214g	
KCl	0.200g
$NaHCO_3$	1.000g
NaCl	8.000g
Na_2HPO_4 (monobasic)	0.050g
Glucose	1.000g

Make up to 1 litre with water and store at 4°c until used.

2.4.4 Milli-Q-Water (Millipore Corporation™)

For all inactivation experiments Milli-Q-water was used as the control solution. This is because Reverse-Osmosis and Tap water have a chlorine concentration at levels that may affect viability of cysts in the experiment. The pH of Milli-Q-Water is 6.5.

2.4.5 Seawater.

It was decided that *in vivo* seawater would be used for all seawater inactivation experiments. A 20 litre sample was collected from a coastal spot on Tangimoana beach on the west coast of the North Island. The sample was subsequently filtered through a 5 μm porosity filter by positive pressure membrane filtration to remove large suspended matter. The seawater was then stored at 4°C until used. The pH of the seawater was between 7.85 and 8.47.

Chapter Three Methods.

3.1 Methods for Determination of Viability.

3.1.1 Excystation for *Giardia muris*

This was carried out as reported by Sauch (1988), with minor modifications. Briefly 1.0 ml of cyst suspension was transferred to clean dry 12 ml glass screw cap Kimax tubes. To this was added 5 mls of Reducing solution and 5 mls of 0.1 M Sodium bicarbonate (final pH=4.7).

This suspension was mixed by vigorous vortexing and incubated in a 37°C waterbath for 30 minutes. The tubes were then centrifuged at 600 g for 5 minutes and washed once in 5 mls of prewarmed excystation medium by centrifugation at 600 g, 5 minutes.

Cysts were finally suspended in 0.5 mls of prewarmed excystation medium and incubated at 37°C for 1.5 hours in a water bath.

Samples were then removed and counted in a Neaubauer haemocytometer, using a Zeiss phase contrast microscope.

For correlation with stains/Nomarski DIC the Excystation percentage was calculated by counting a minimum of 100 forms and calculating the % Excystment using the following formula.

% Excystation = (TET/2 + PEC)/(TET/2 + PEC + IC)X100/1

(Bingham *et al*; 1979)

where TET = Totally Excysted Trophozoite.

PEC = Partially Excysted Cyst.

IC = Intact Cyst.

For seawater inactivations the number of cysts excysting per ml was calculated, using the following formula.

(TET/2 + PEC) x 10^4= Number of Cysts Excysted per ml.

(From Naeuerbauer Haemocytometer).

3.1.2 Excystation for *Giardia intestinalis*.

This was carried out as reported by Rice and Schaefer (1981) with minor modifications. This excystation procedure involves two steps: a low pH induction phase using three separate solutions, and an excystation phase using an excystation medium.

The excystation for all samples was carried out in clean dry 12 ml glass screw capped Kimax tubes containing 1 ml of cyst suspension.
The induction step: 5 mls of prewarmed aqueous HCl
(pH 2.0), 2.5 ml of 1X HBSS supplemented with 29mM L-cysteine-HCl and 67 mM Glutathione, 2.5 mls of 0.1 M Sodium bicarbonate.
These solutions were added to the cyst suspension in this order and all were prewarmed to 37°C. Once $NaHCO_3$ is added cap the tube tightly and vortex briefly to resuspend cysts, and ensure adequate mixing of the three solutions. The tubes were incubated at 37°C in a water bath for 30 minutes to complete the induction step. Once induced the cysts were centrifuged at 600 g for 5 minutes to pellet the cysts and the supernatant discarded.

The excystation step: 5 mls of excystment medium (0.5% Trypsin in 1X Tyrodes Solution) was added to resuspend and wash the cysts. The cysts were then centrifuged for 5 minutes at 600 g and the supernatant discarded. They were resuspended in 0.5 mls of fresh excystation medium, and then incubated in a 37°C waterbath for 1 hour. Samples were then taken and number of cysts excysting per ml and % excystment calculated as follows:

(TET/2 + PEC) x 1 x 10^4= No.of cysts excysted/ml.
(From Neauerbauer haemocytometer)

3.1.3 Vital Stains.

Propidium Iodide (PI) and Fluorescein Diacetate (FDA) solutions were made as follows.
Add 10 μl of of each working solution to 50 μl of cyst suspension (Smith and Smith 1990). Cysts were stained for 10 minutes at room temperature before examination using a Reichart-Jung epifluorescent microscope equipped with epifluorescence at excitation wavelength of 455-490 nm for both FDA and PI.

Fluorescein stained cysts appear apple green and are scored 'viable', whereas Propidium Iodide stained cysts appear red-orange and are scored 'non-viable'. Viabilities by this method were determined by counting both green and red cysts as seen under epifluorescence, a minimum of one hundred forms was counted for each sample taken.

3.1.4 Nomarski Differential Interference Contrast Microscopy (NomarskiDIC).

Samples of 10 μl of cyst suspension were placed on a slide and viewed under optimum conditions using Nomarski Optics (Zeiss Axiophot microscope equipped with full complement of Nomarski prisms). A minimum of one hundred forms was counted and scored as viable or non-viable as determined by the method of Shupp et al; (1988).

A viable cyst is one showing the following characteristics; a clearly defined cyst wall and peritrophic space, hyaline appearance of the cytoplasm. A non-viable cyst has a granular appearing cytoplasm a deterioration of cellular organisation, and the peritrophic space is enlarged (see Plate 1 and Plate 2).

3.2 Seawater Inactivation.

Suspend 1 ml of 1-2 x 10^6 cysts in 9 mls of pre-chilled suspension medium (seawater or Milli-Q water as a control). Pre-chilling temperature is determined by the temperature of storage and is either 10°C or 15°C. The suspension is vortexed vigorously for 15 seconds. A 1 ml is sample immediately removed and called time 0 (zero). Subsequent 1 ml samples are taken at day 1, 3, 7, 14, 21.

The samples taken were immediately excysted by the methods of Rice and Schaefer (1981) for *Giardia intestinalis* and Sauch (1988) for *Giardia muris*. The number of cysts excysted per ml and percent excystment was calculated in each case. The experiment was conducted in duplicate.

3.3 Chlorine Inactivation.

1 ml of approxiamately 1 x 10^6 *Giardia muris* cysts was inoculated into 200 mls of cold (4°C) Milli-Q Water and 200 mls of cold (4°C) Milli-Water made 1 ppm Free Available Chlorine with stock hypochlorite (Sodium Hypochlorite 13.5% w/w, Ryan Chemicals Ltd., Palmerston North).

These conical flasks were incubated at 4°C in the absence of U.V. light for 149 minutes on a shaking water bath.
50 ml samples were removed for determination of viability by Excystation, Nomarski DIC and Fluorogenic dye assessment, into 50 ml conical centrifuge tubes. Chlorine action was stopped by addition of 0.5 ml of 0.1 M Sodium thiosulphate, and vigorous vortexing.

The samples were centrifuged for 5 minutes at 600 g in a Haraeus Christ swinging bucket centrifuge. The supernatant was discarded and the pellet quantitatively transferred to 1.5 ml Eppendorf tubes using a small amount of

Milli-Q water (less than 1 ml). Eppendorf tubes were centrifuged at 600 g to pellet cysts, and the supernatant discarded. Cysts were resuspended in 1 ml of Milli-Q water.

This was divided into three portions for viability testing.

500 μl Excystation

250 μl Fluorogenic Dye staining

250 μl Nomarski DIC

The pH and Free Available Chlorine (FAC) levels of these flasks was monitored throughout the experiment. Chlorine determinations were done using D.P.D. Colorimetric Method, and varied from 0.75 to 1.05 ppm FAC. The pH was measured using an Expandable ionAnalyzer (EA 920, Orion Research Ltd.), after temperature equilibration. Two separate and independent trials were conducted.

Chapter Four Results.

Figures 1 - 6 Graphs for *G. muris* and *G. intestinalis* incubated in freshwater and seawater at 5, 10, 15°C.

Figures 7 - 14 Log data for *G. muris* and *G. intestinalis* in freshwater and seawater at 5, 10, 15°C.

Figures 15 - 30 Graphs for *G. muris* and *G. intestinalis* showing the various methods of determining viability being trialled and their effectiveness.

Figures 31 - 34 Graphs for chlorine inactivation of *G. muris*.

Table 1 *Giardia spp.* cyst inactivation at t=0, showing difference between freshwater and seawater.

Table 2 *G. muris* log Nt/No gradients.

Table 3 Inactivation of *G. muris* using various chlorine treatments.

Figure 1 Viability of G. muris in Freshwater and Seawater at 5 C.

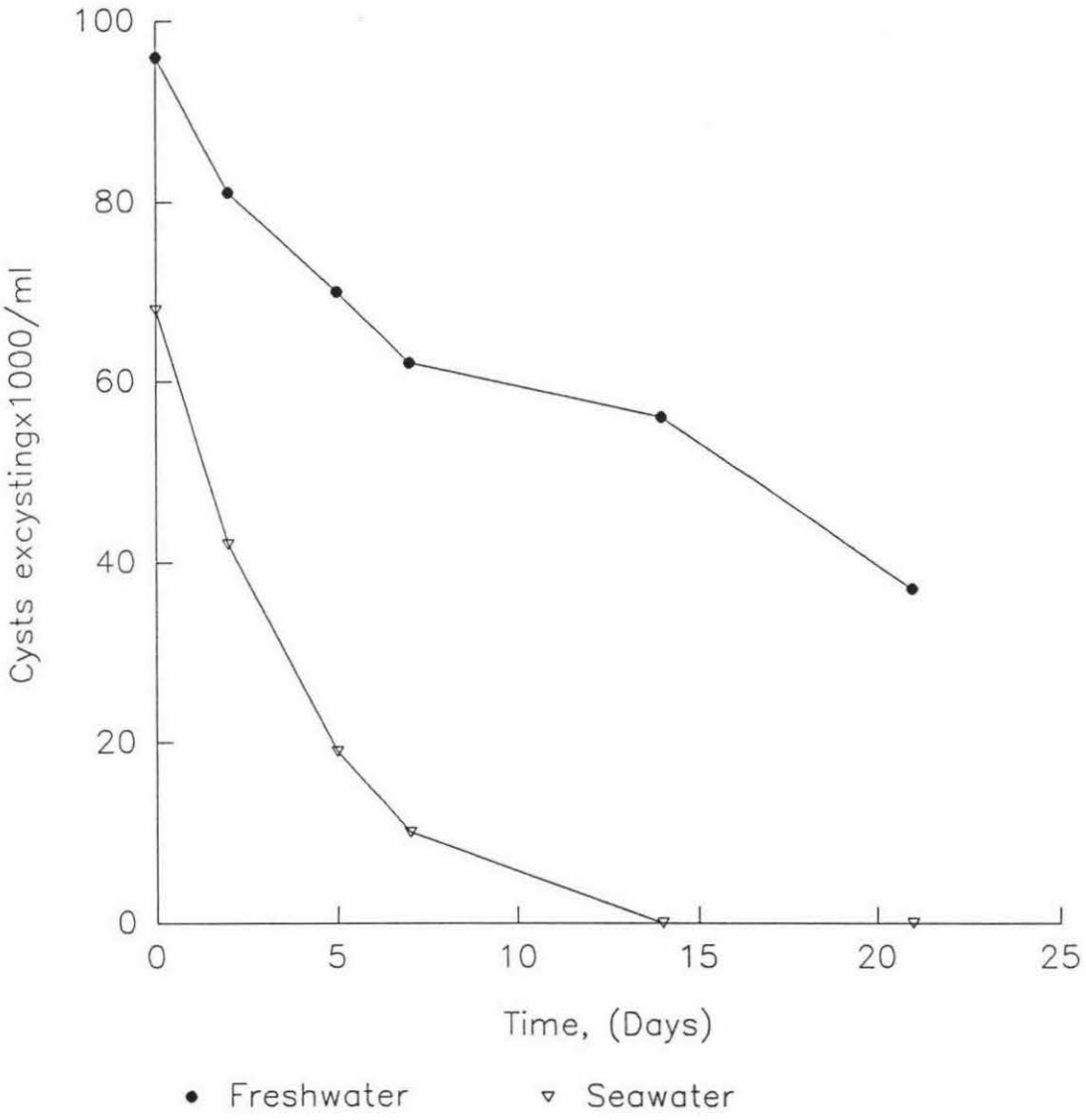


Figure 2 Viability of G. muris in Freshwater and Seawater at 10 C.

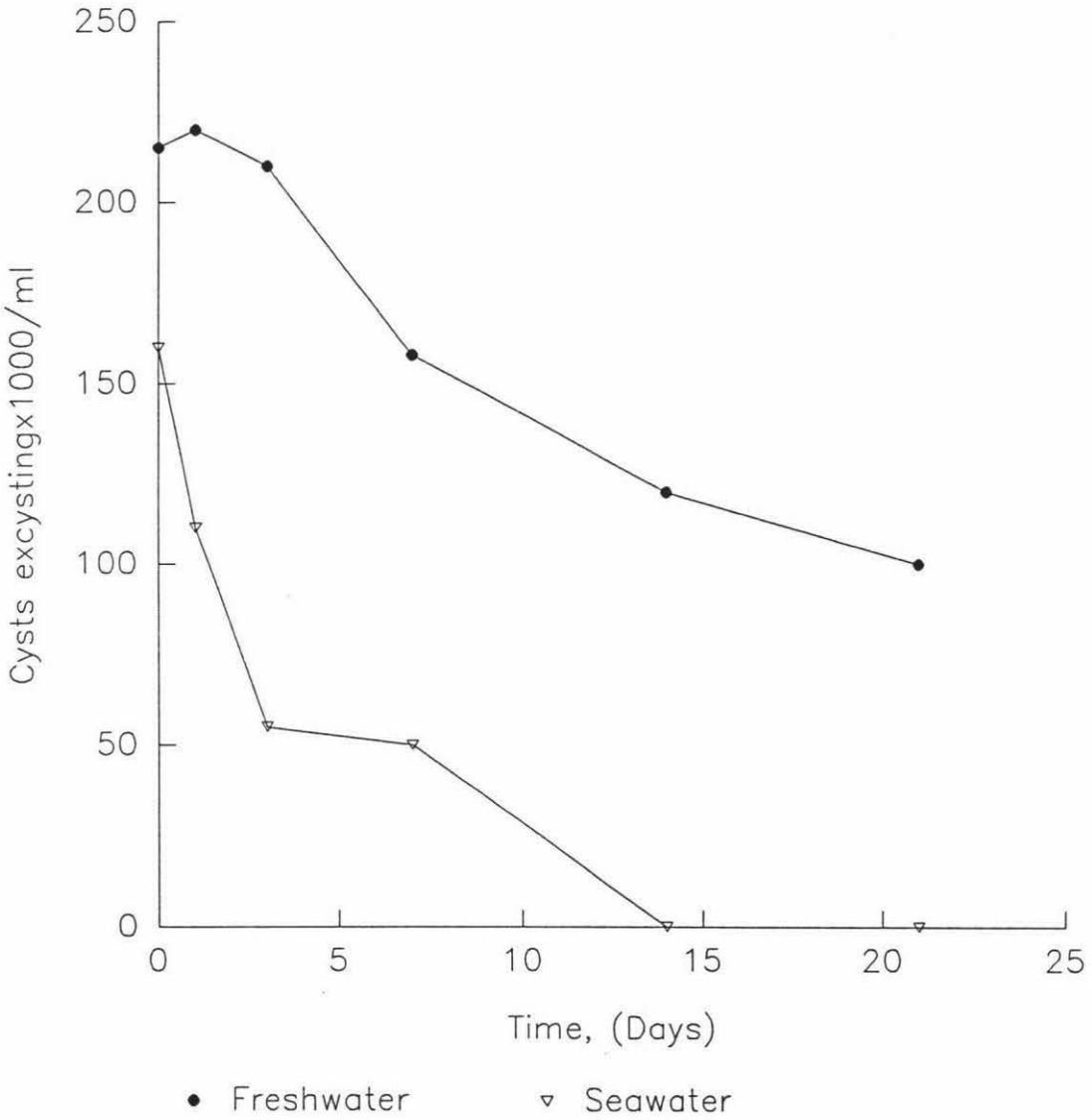


Figure 3 Viability of G. muris in Freshwater and Seawater at 15 C.

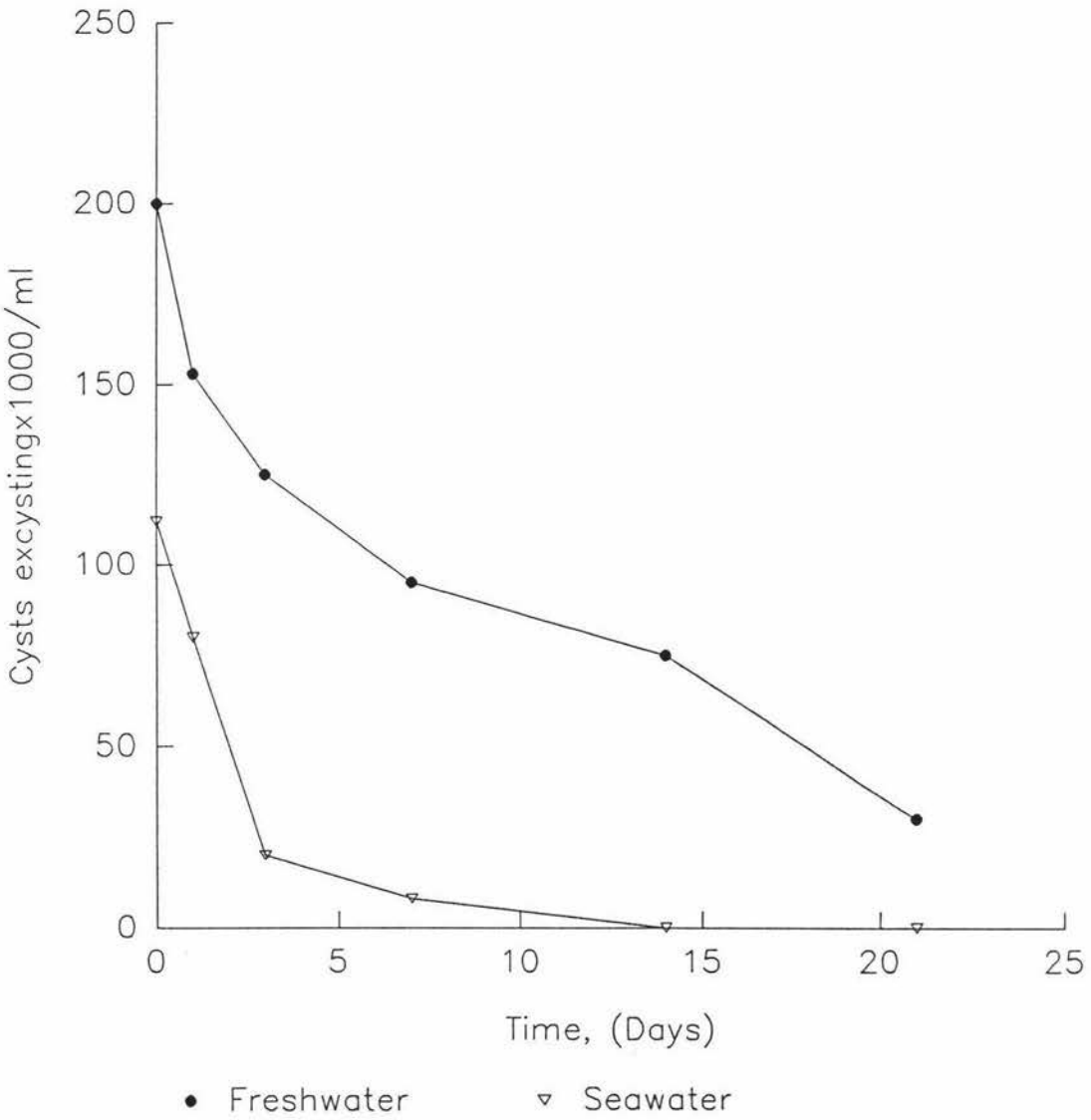


Figure 4 Viability of G. intestinalis in Freshwater and Seawater at 5 C.

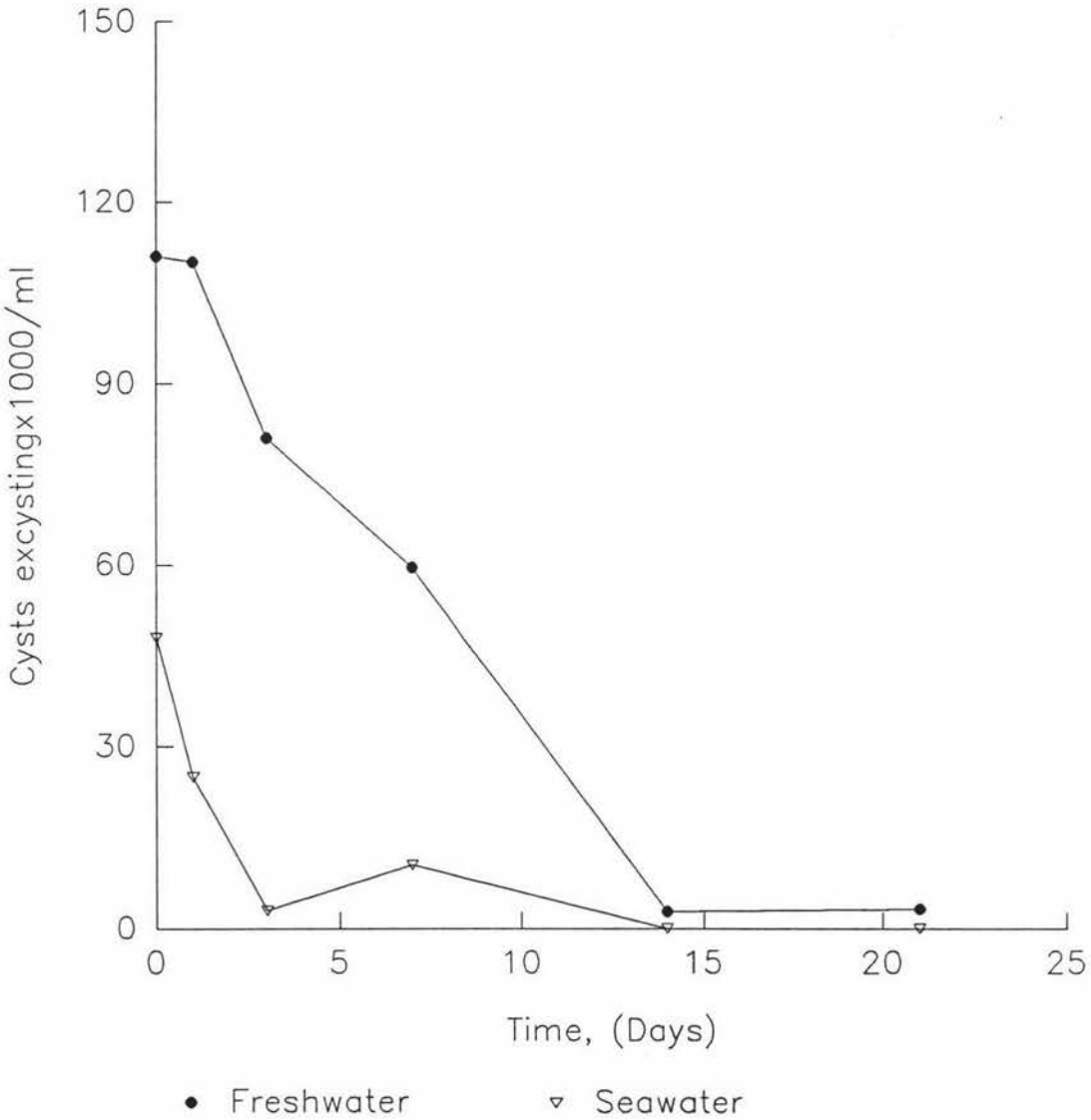


Figure 5 Viability of G. intestinalis in Freshwater and Seawater at 10 C.

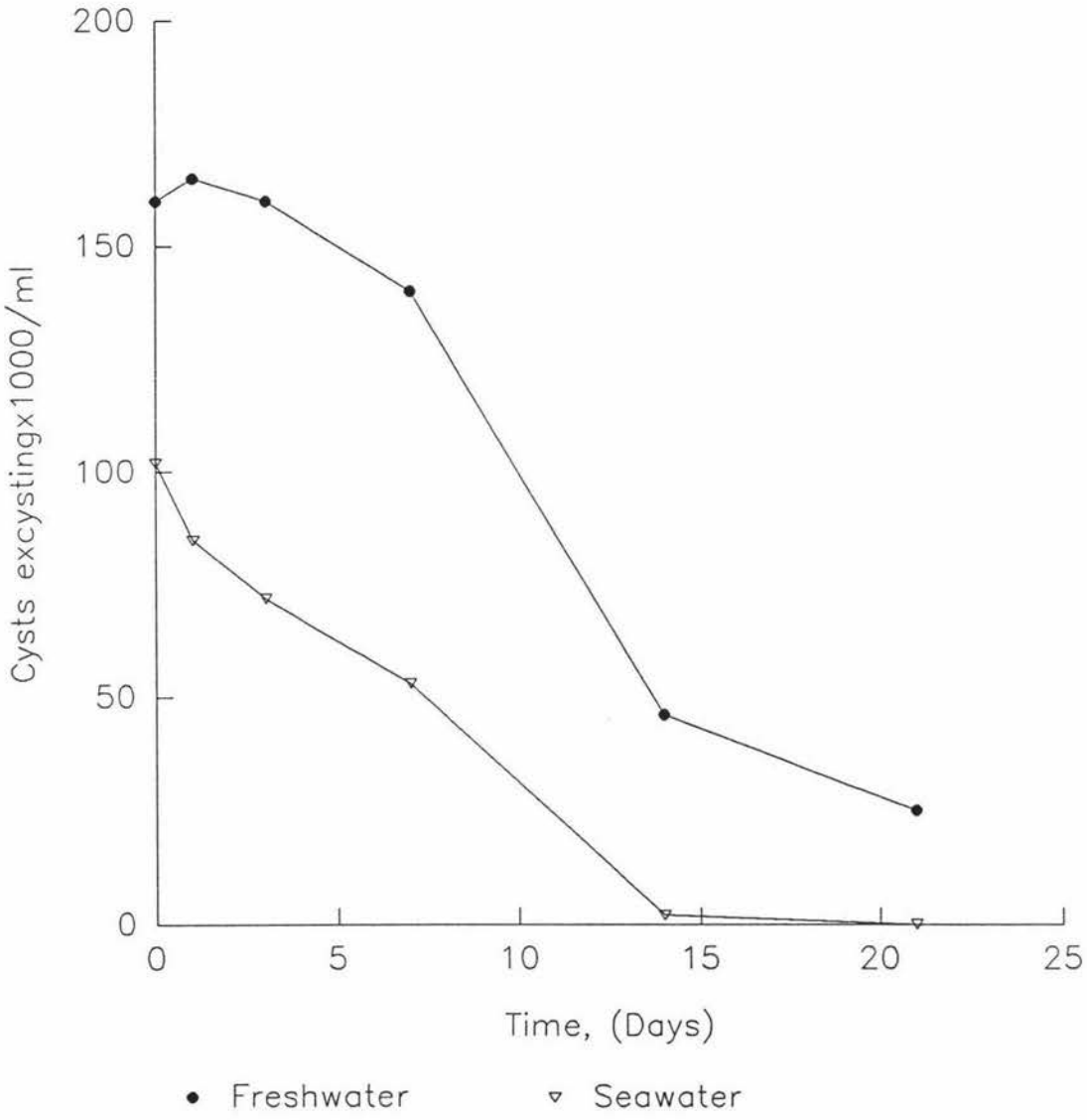


Figure 6 Viability of G. intestinalis in Freshwater and Seawater at 15 C.

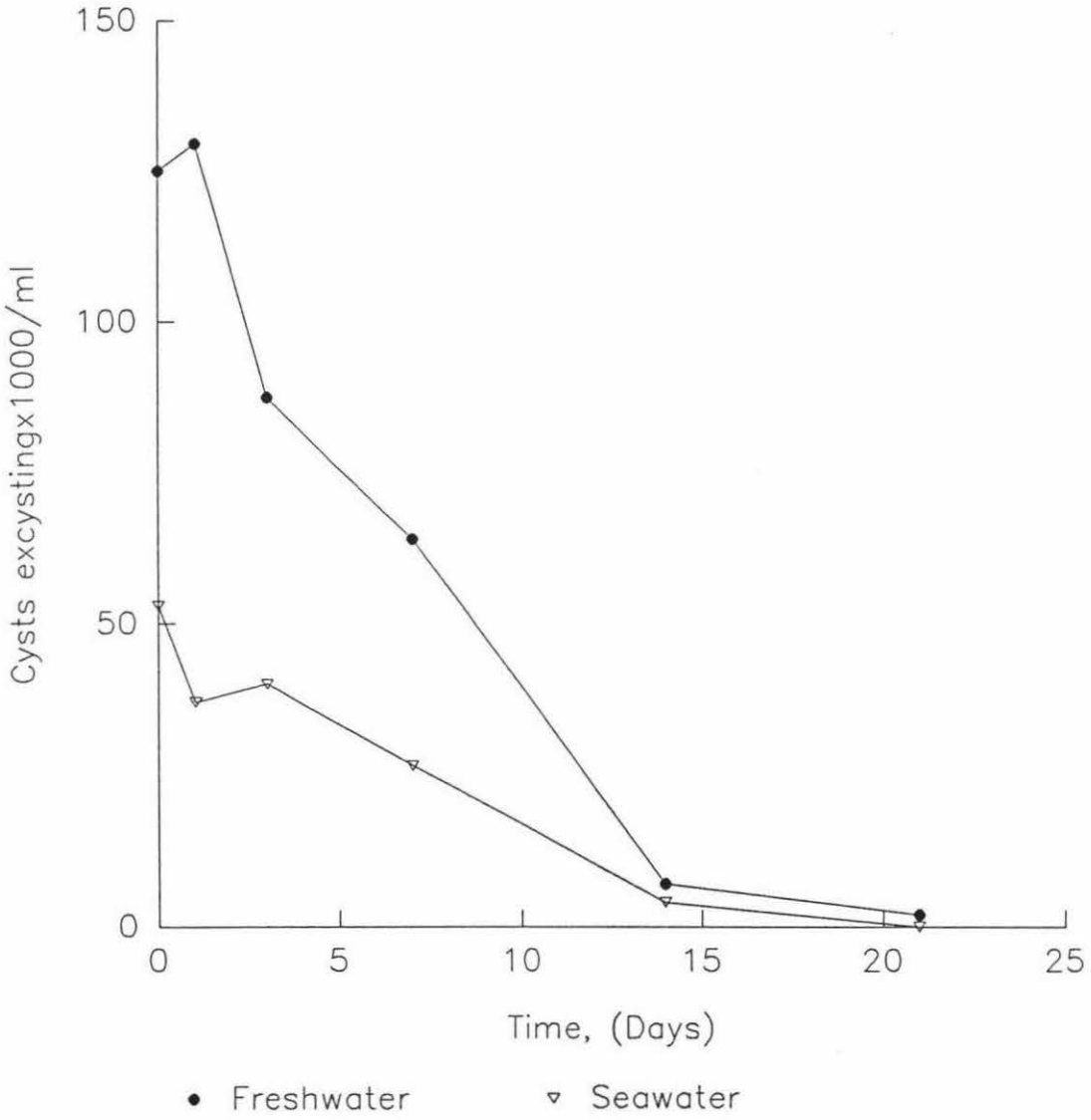


Table 1
Giardia spp. cyst inactivation at t = 0, showing differences between freshwater and seawater.

Species	Temp. (°C)	Difference (%)	Average
G. muris	5	29%	
	10	28%	
	15	43.5%	33.5%
G. intestinalis	5	57%	
	10	37%	
	15	59.5%	51%

Figure 7 Nt/No vs Time for G. muris in Freshwater and Seawater at 5 C.

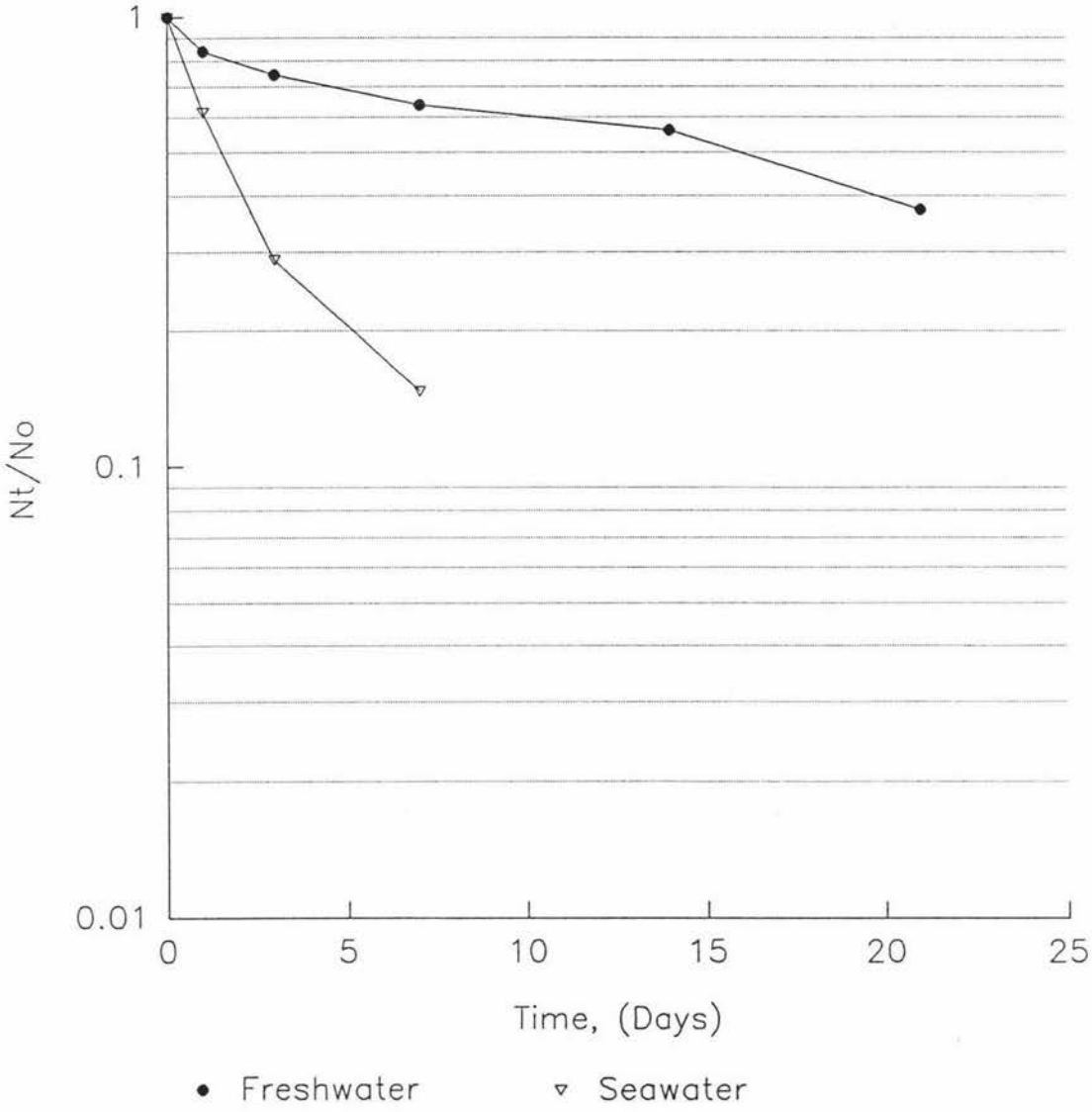


Figure 8 Nt/No vs Time for G. muris in Freshwater and Seawater at 10 C.

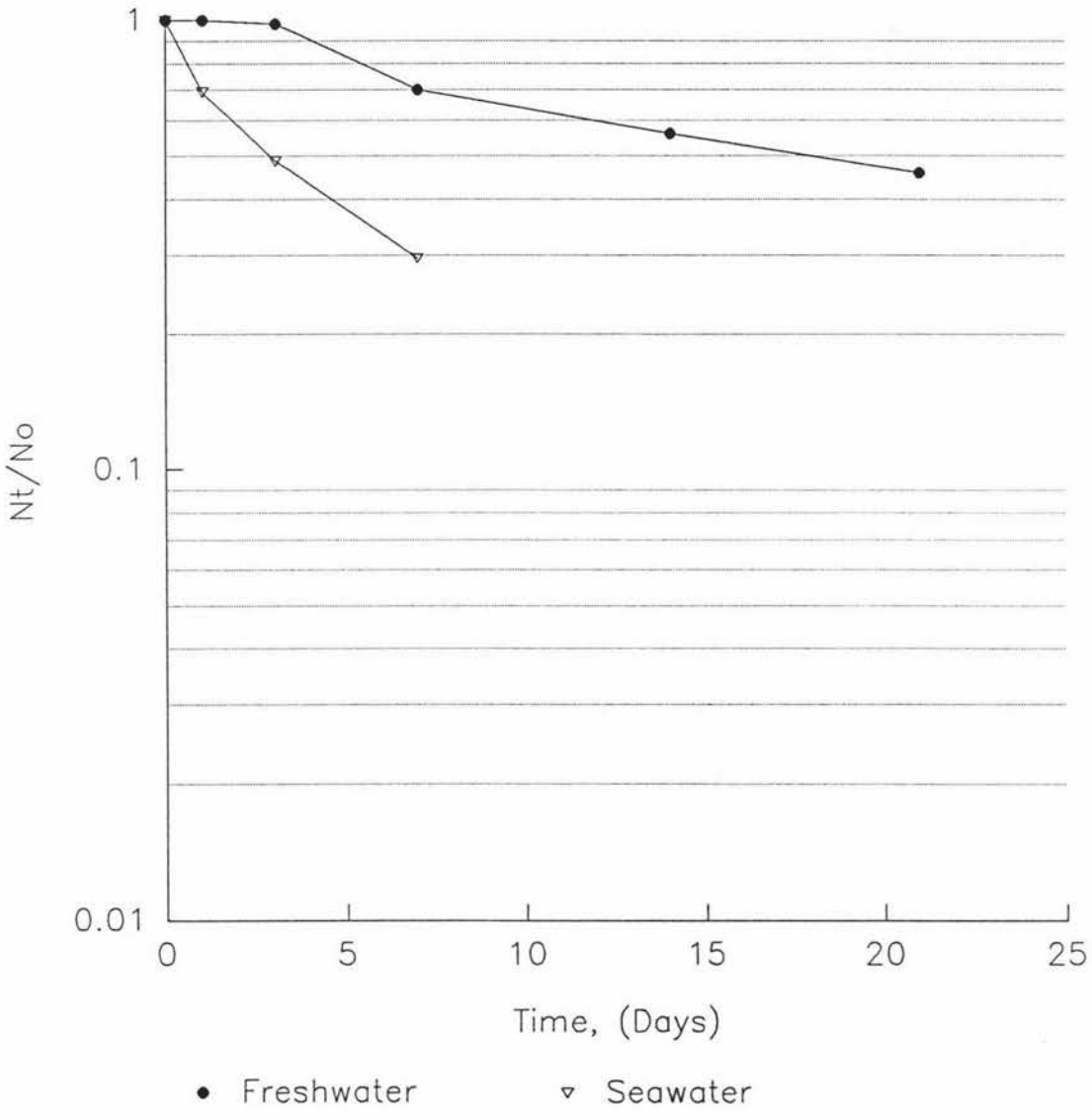


Figure 9 Nt/No vs Time for G. muris in Freshwater and Seawater at 15 C.

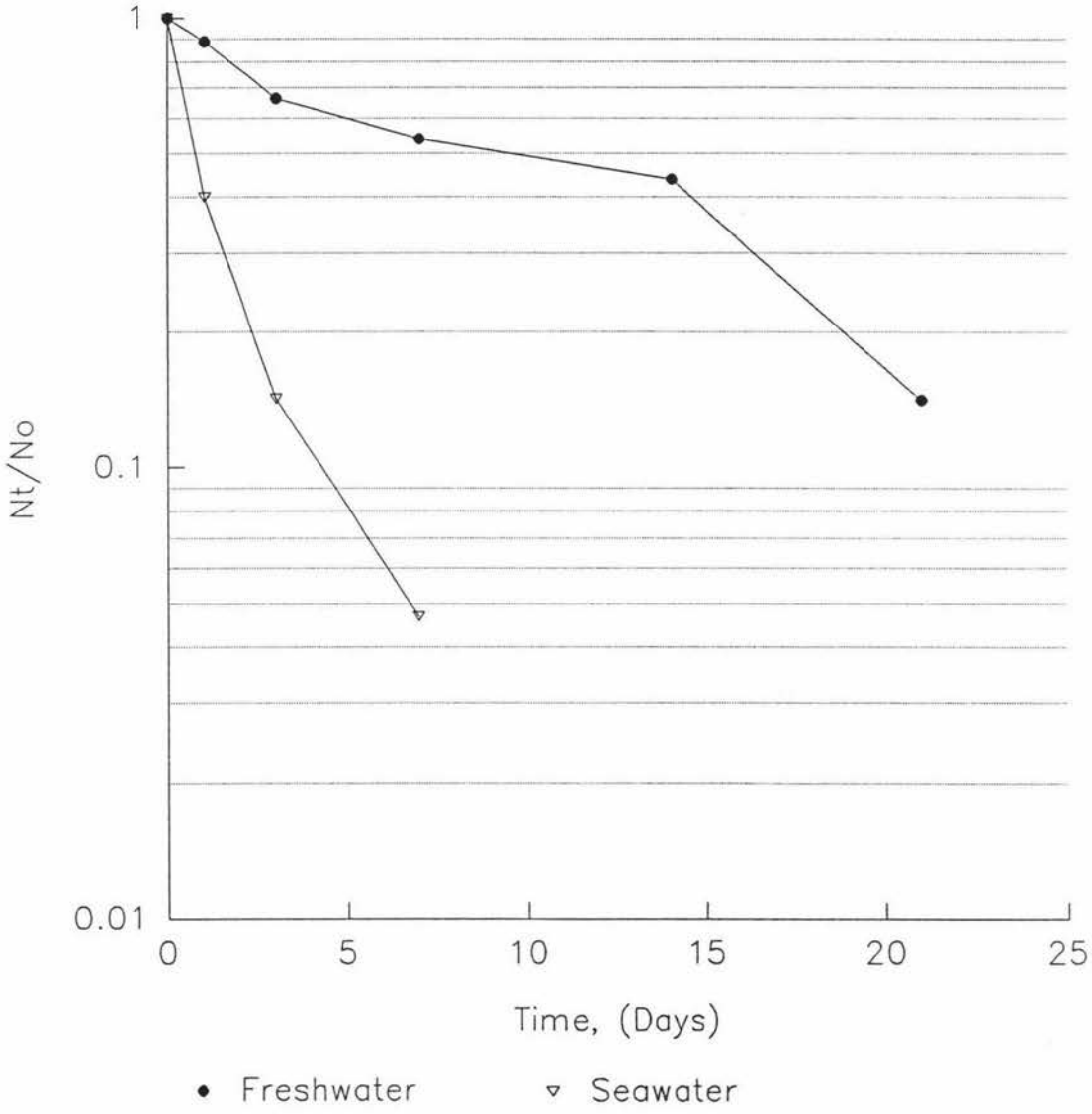


Figure 10 Nt/No vs Time for G. muris in Freshwater and Seawater at 5, 10, and 15 C.

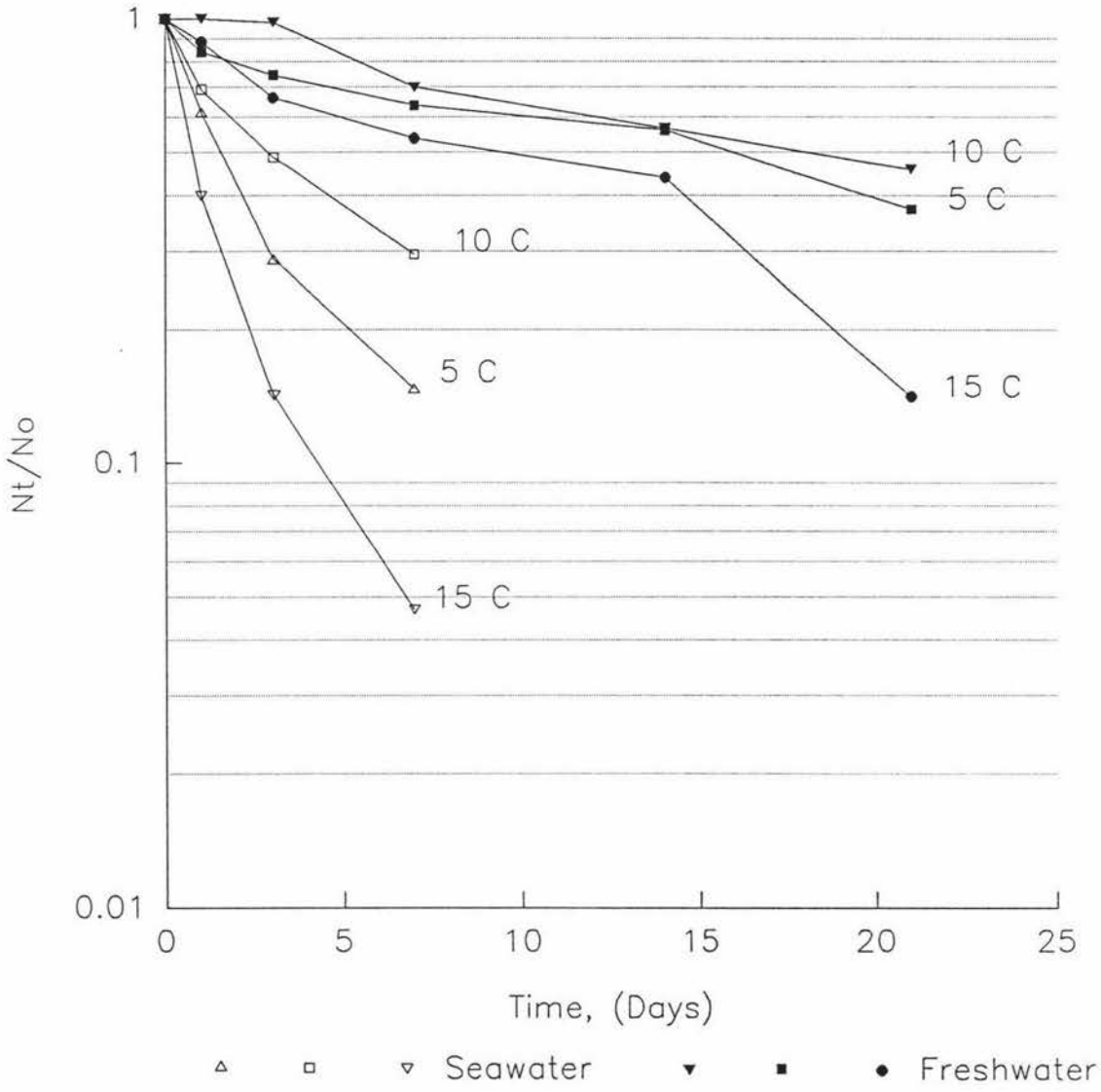


Table 2
Giardia muris Log Nt/No Gradients.

Temperature°C	Gradient Water	Gradient Seawater
5	0.0300	0.106
10	0.0257	0.0875
15	0.0404	0.180

Data for this table from Figures 7-9, and 11-13.

Figure 11 Nt/No vs Time for G. intestinalis in Freshwater and Seawater at 5 C.

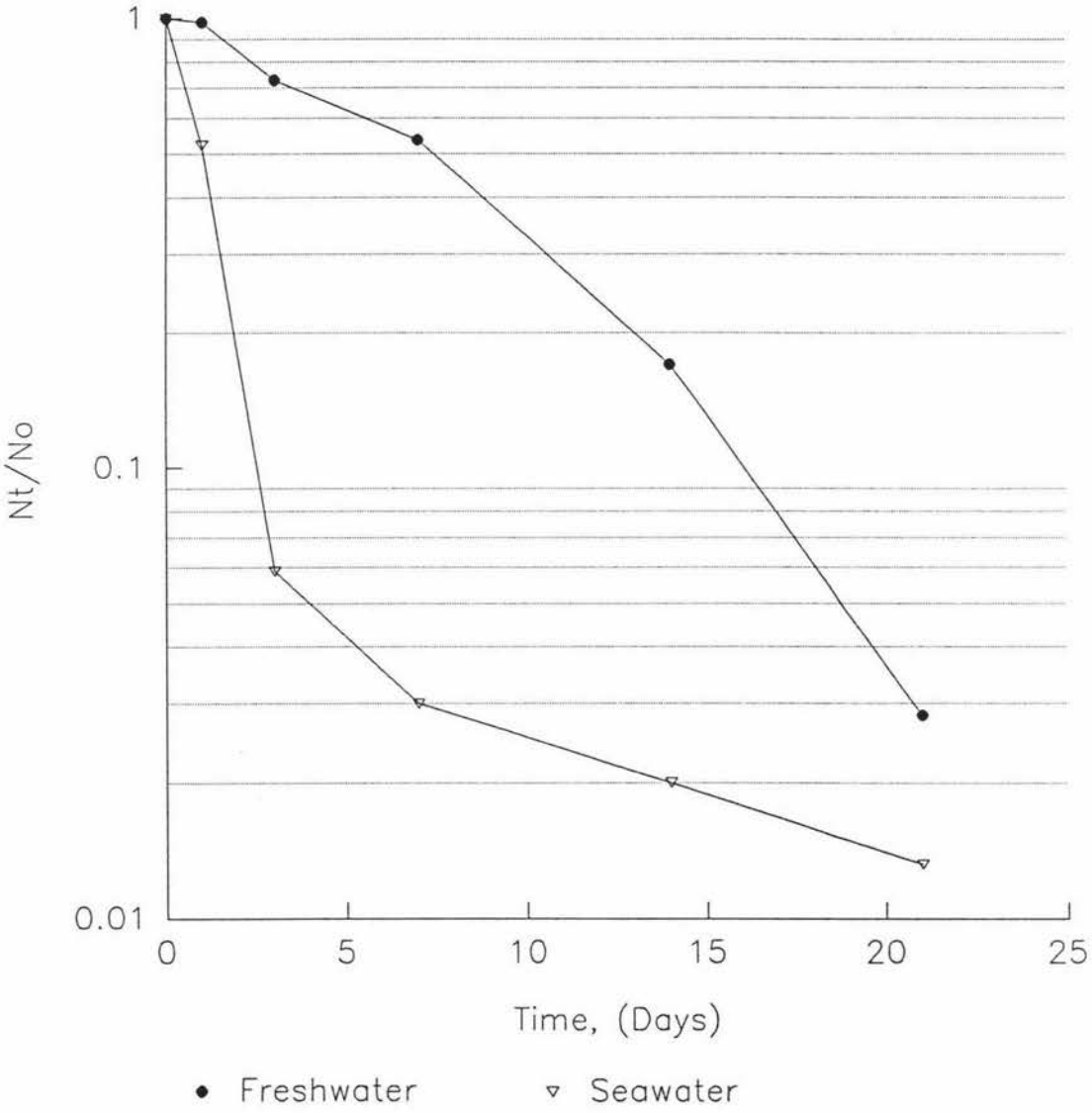


Figure 12 Nt/No vs Time for G. intestinalis in Freshwater and Seawater at 10 C.

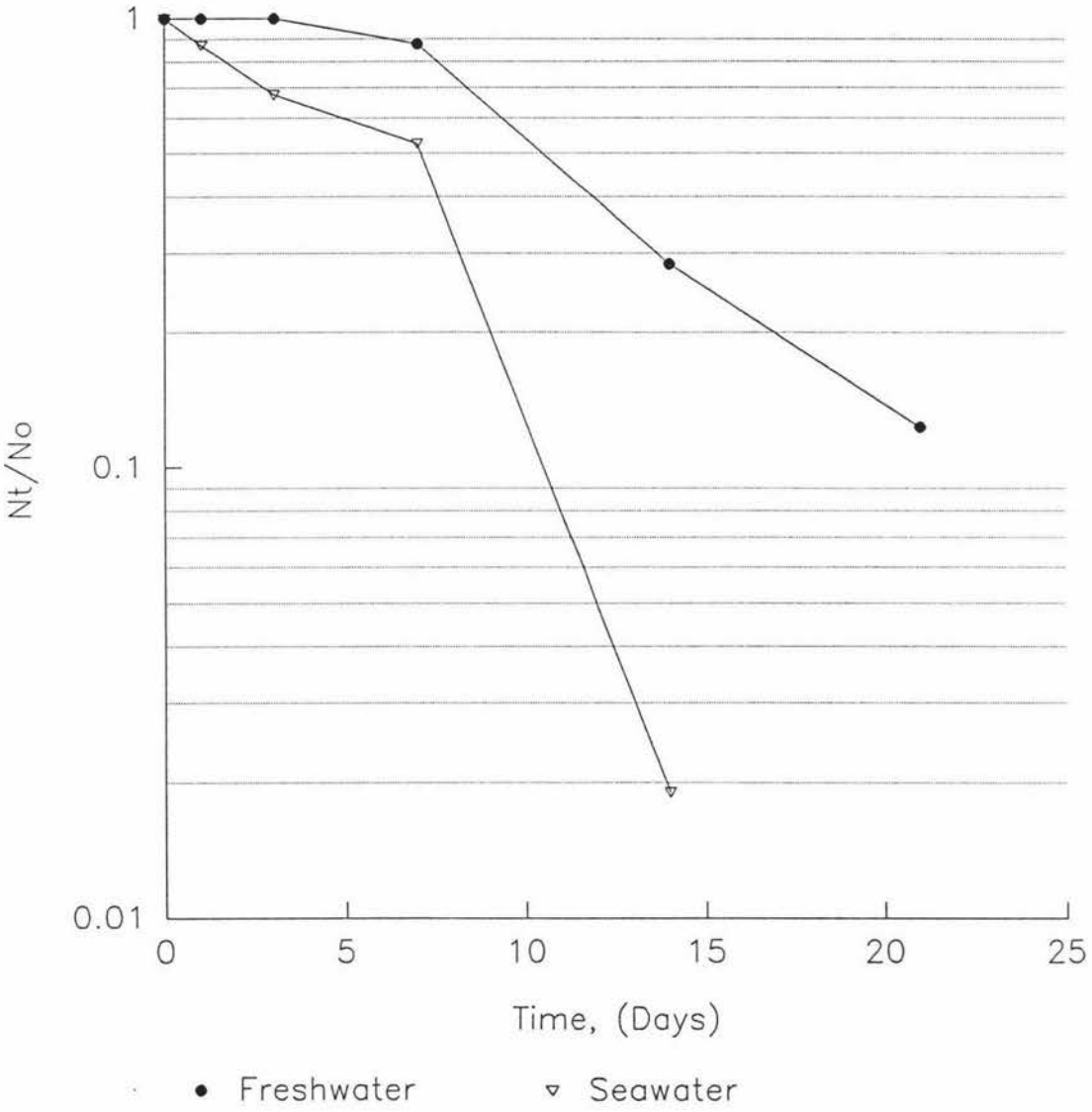


Figure 13 Nt/No vs Time for G. intestinalis in Freshwater and Seawater at 15 C.

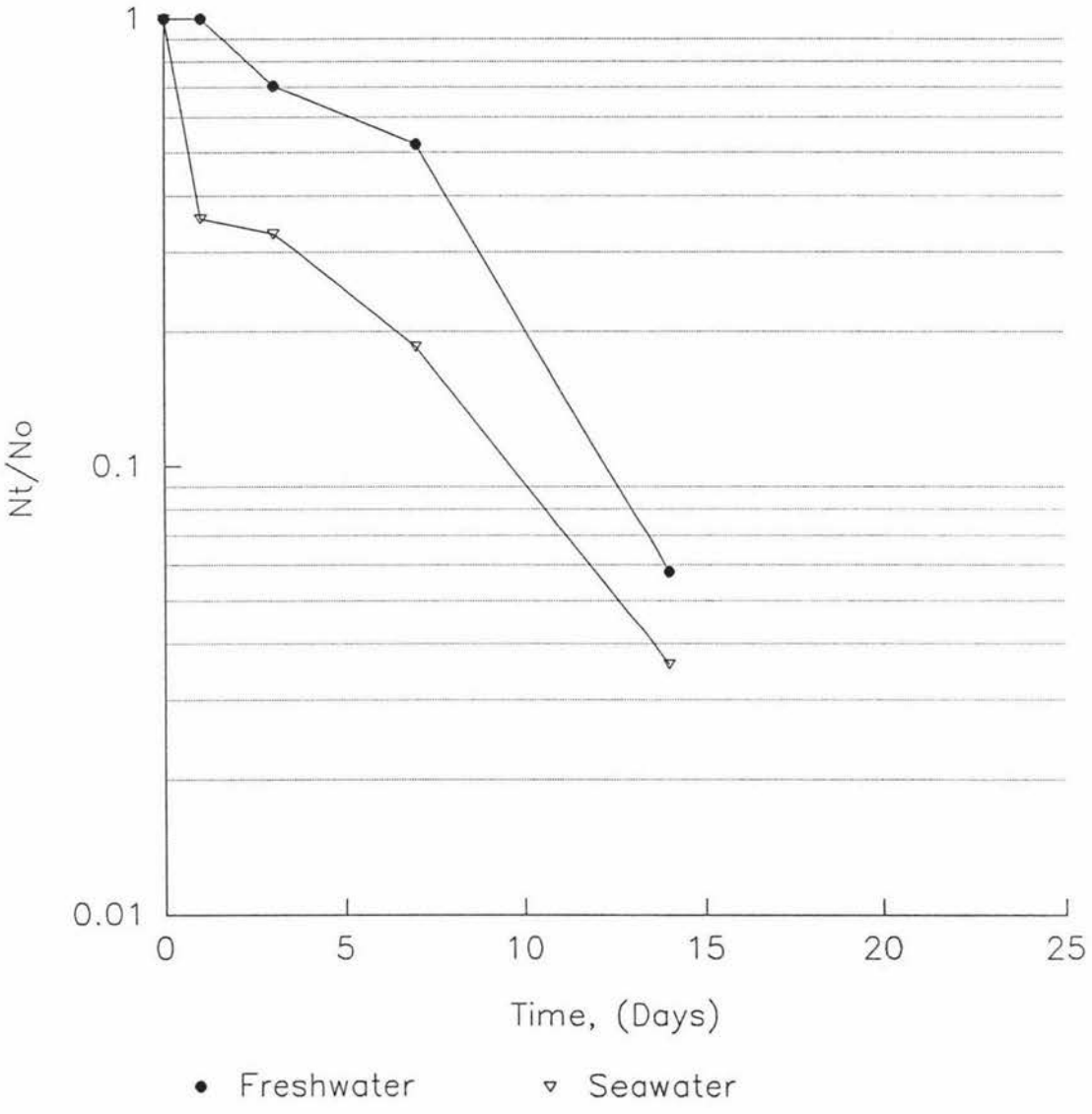
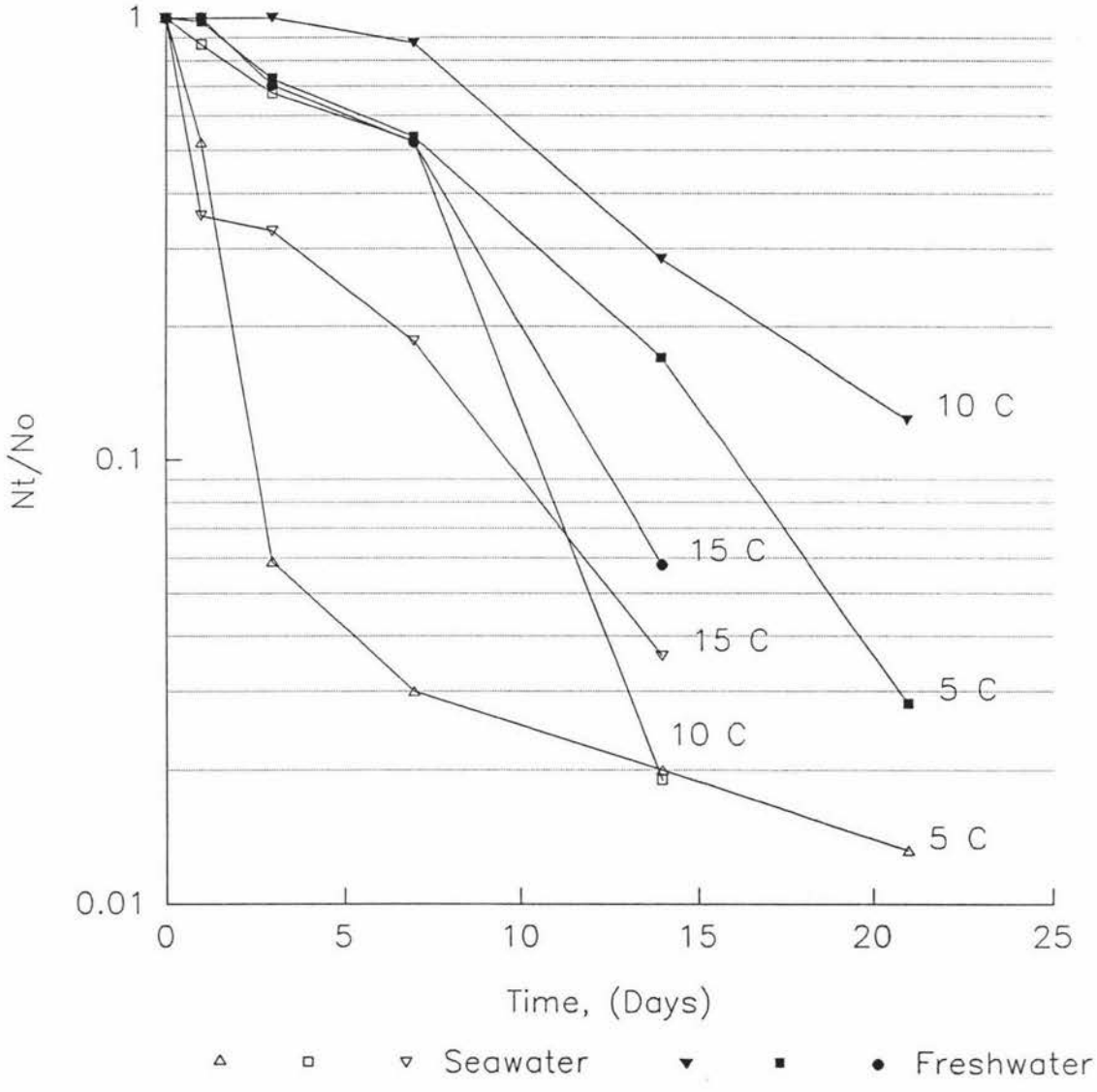


Figure 14 Nt/No vs Time for G. intestinalis in Freshwater and Seawater at 5, 10, and 15 C.



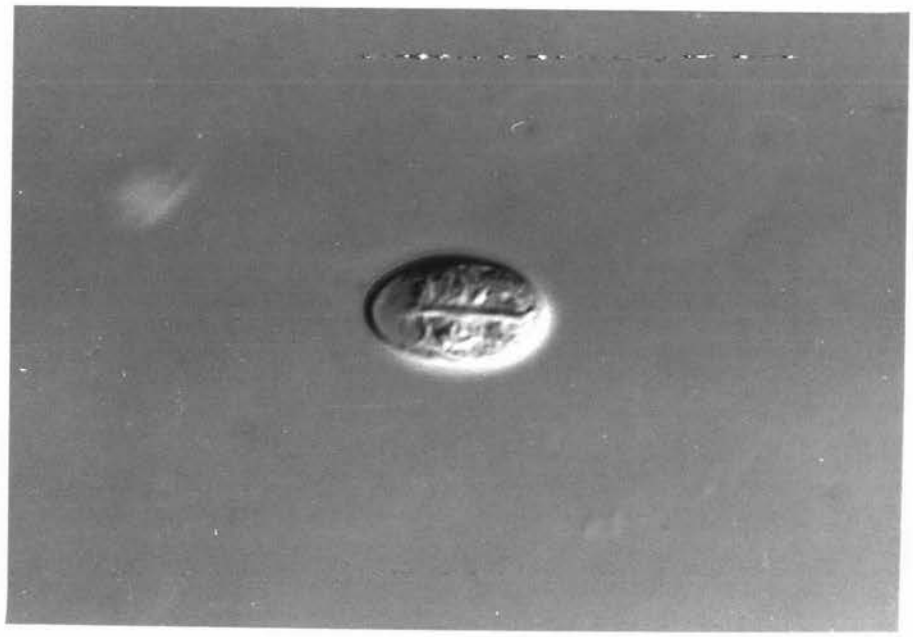


Plate 1

In vivo Giardia muris cyst (x1000), showing non-viable morphology when viewed under Nomarski DIC.

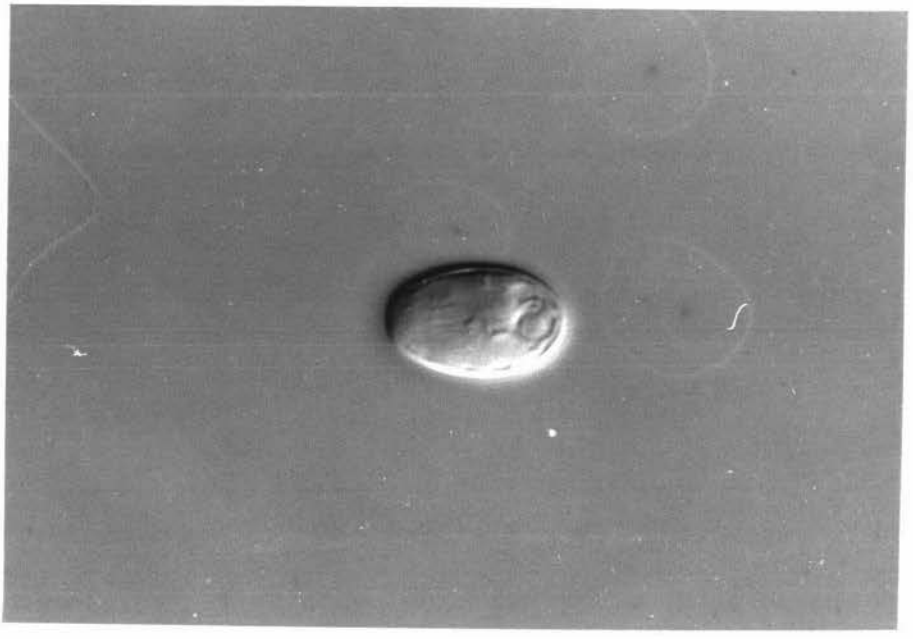


Plate 2

In vivo Giardia muris cyst (x1000), showing viable morphology when viewed under Nomarski DIC.

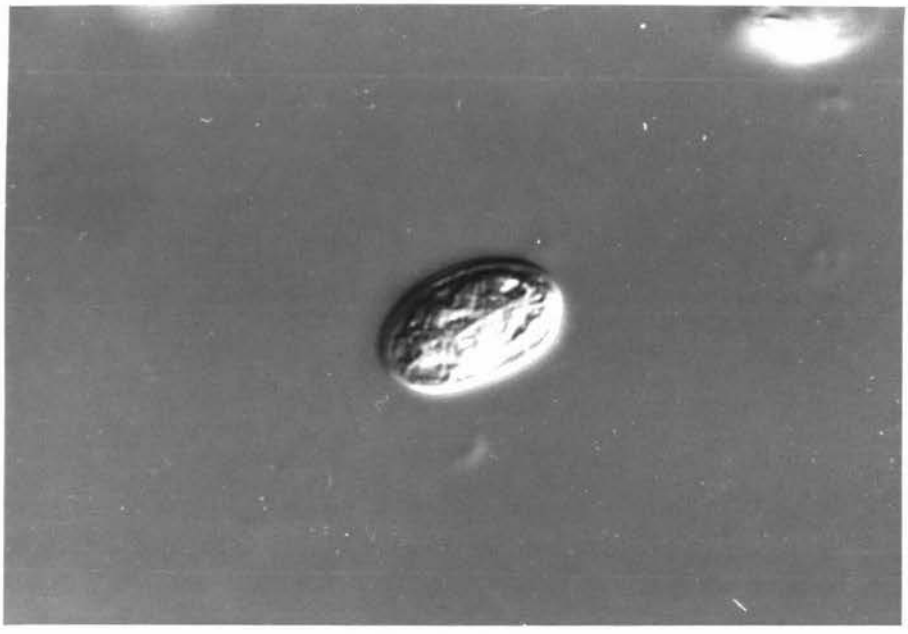


Plate 3

In vivo Giardia intestinalis cyst (x7200), showing non-viable morphology when viewed under Nomarski DIC.

Plate 4

In vivo Giardia intestinalis cyst (x7200), showing viable morphology when viewed under Nomarski DIC.

Figure 15 The percent viable cysts as determined by Nomarski DIC, FDA, PI, and Excystation for G. muris at 5 C in Freshwater.

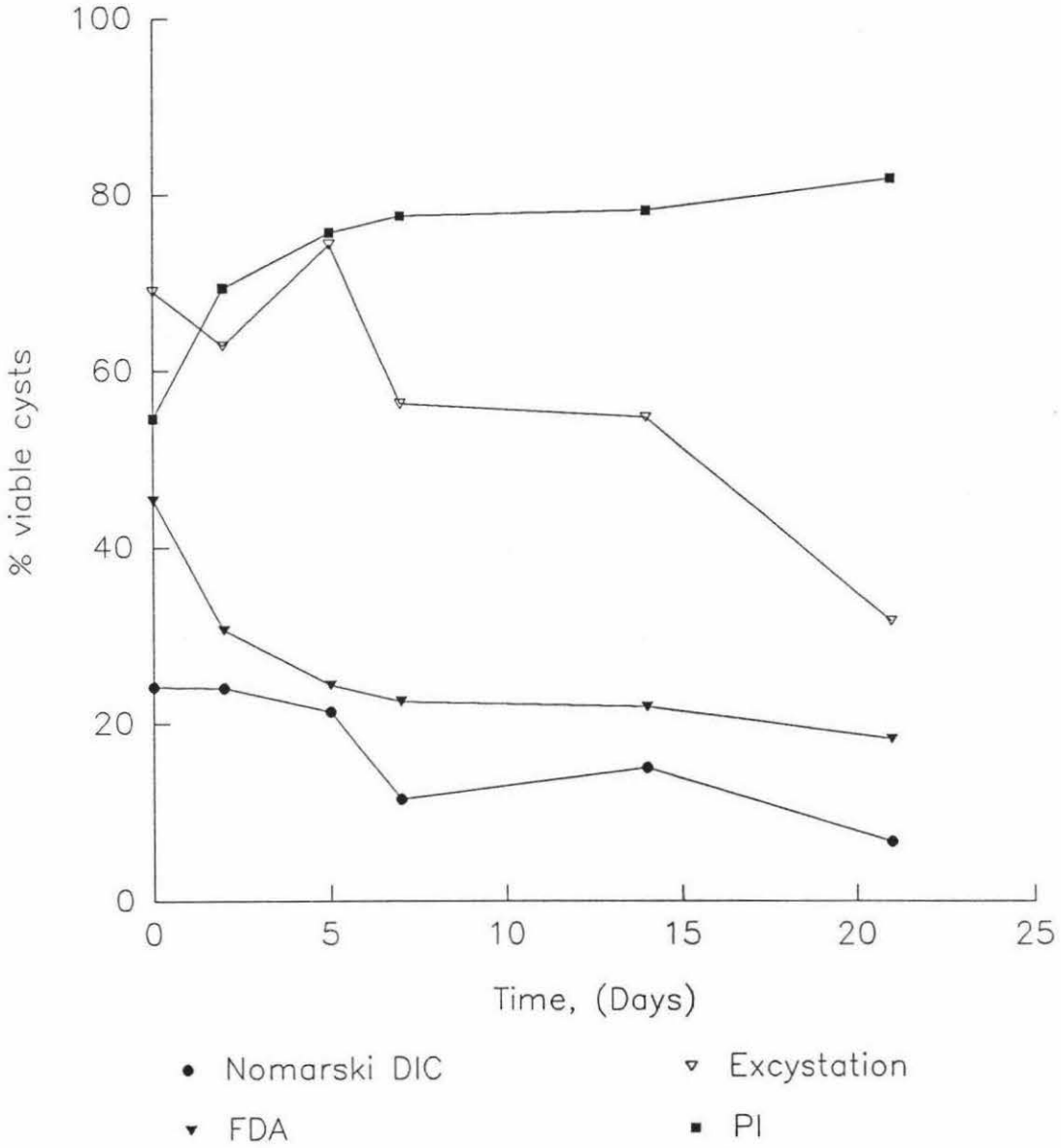


Figure 16 The percent viable cysts as determined by Nomarski DIC, FDA, PI, and Excystation for G. muris at 15 C in Freshwater.

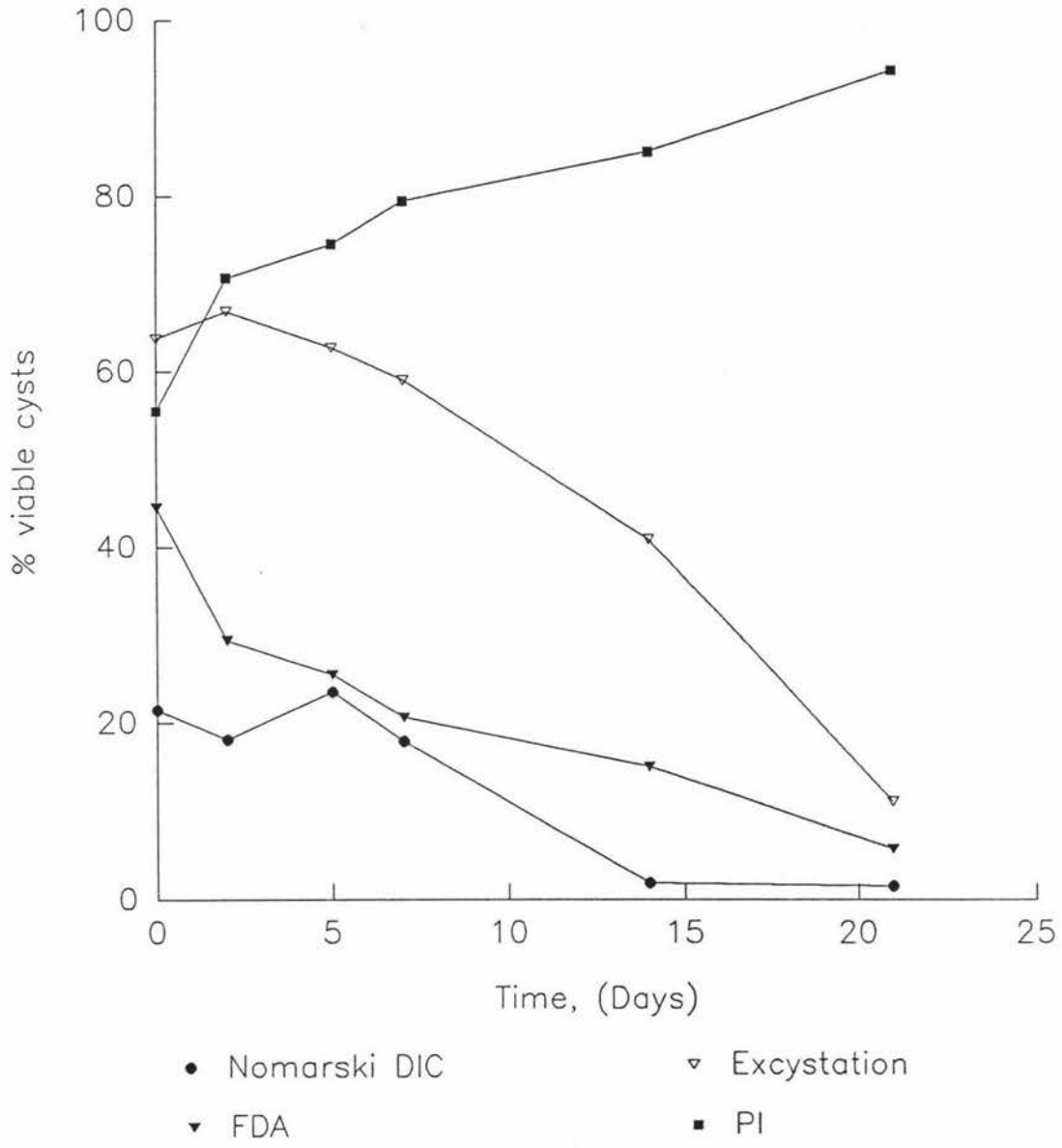


Figure 17 The percent viable cysts as determined by Nomarski DIC, FDA, PI, and Excystation for G. muris at 5 C in Seawater.

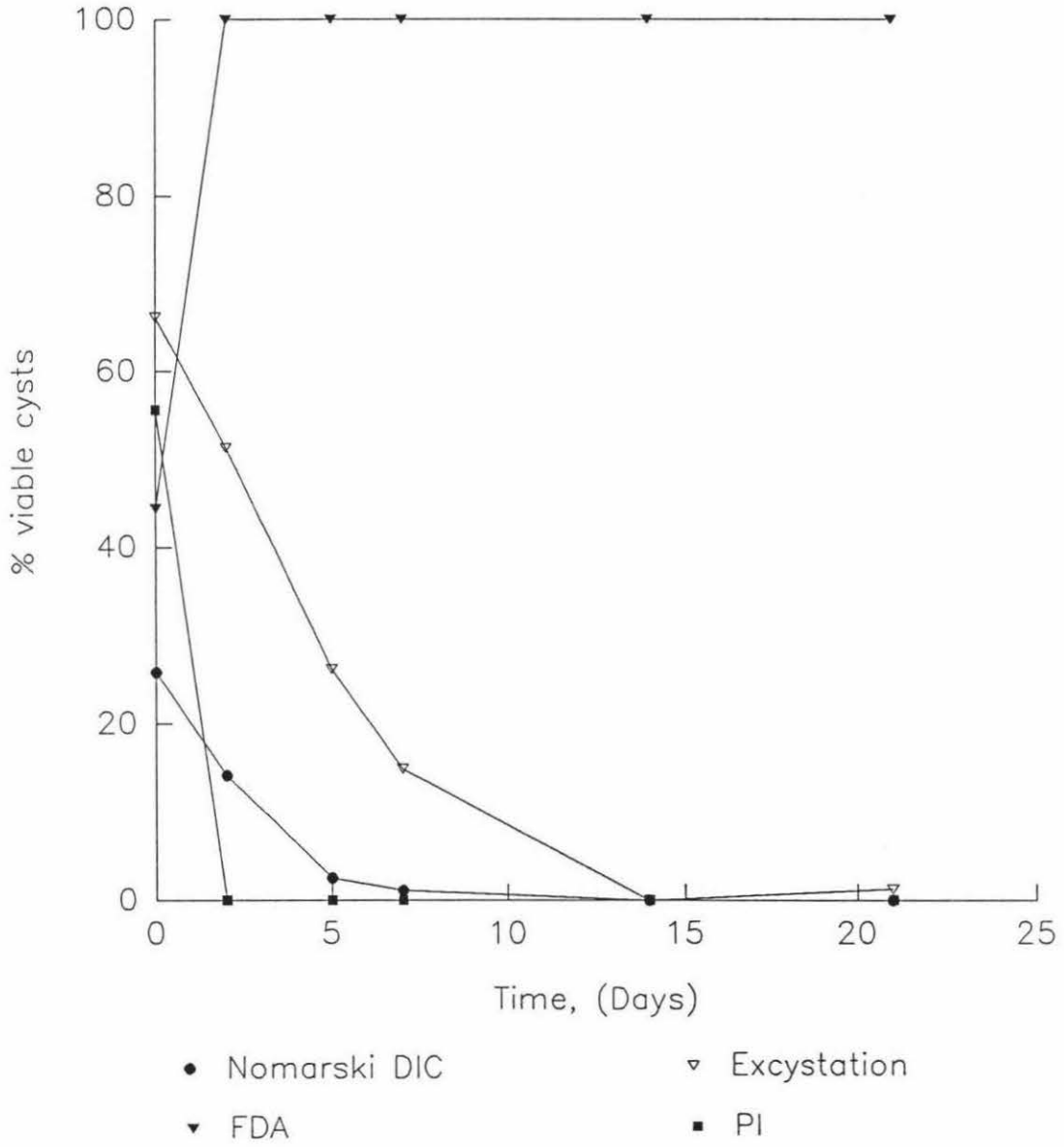


Figure 18 The percent viable cysts as determined by Nomarski DIC, FDA, PI, and Excystation for G. muris at 15 C in Seawater.

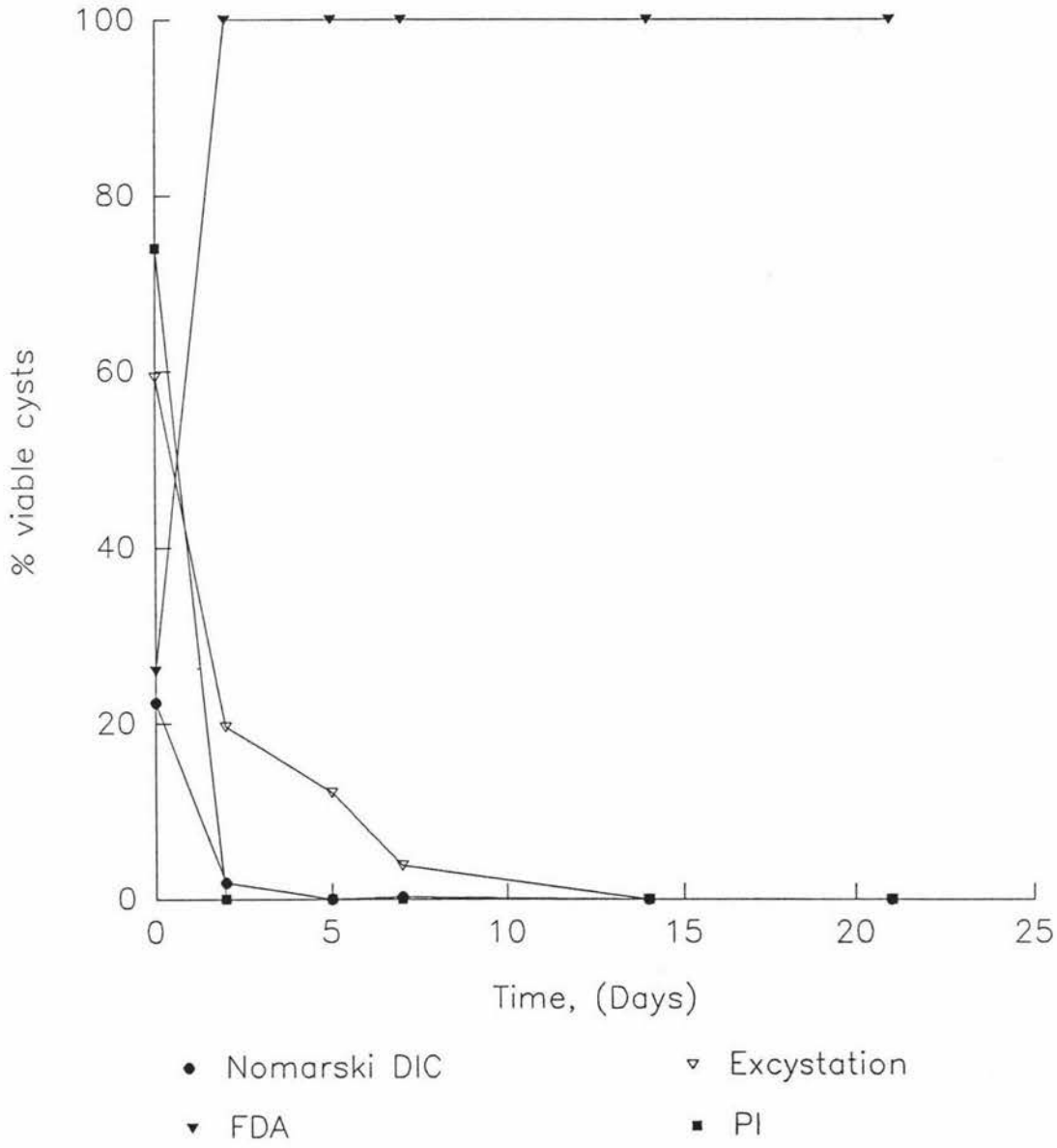


Figure 19 The percent viable cysts as determined by Nomarski DIC for G. muris showing temperature and seawater effect.

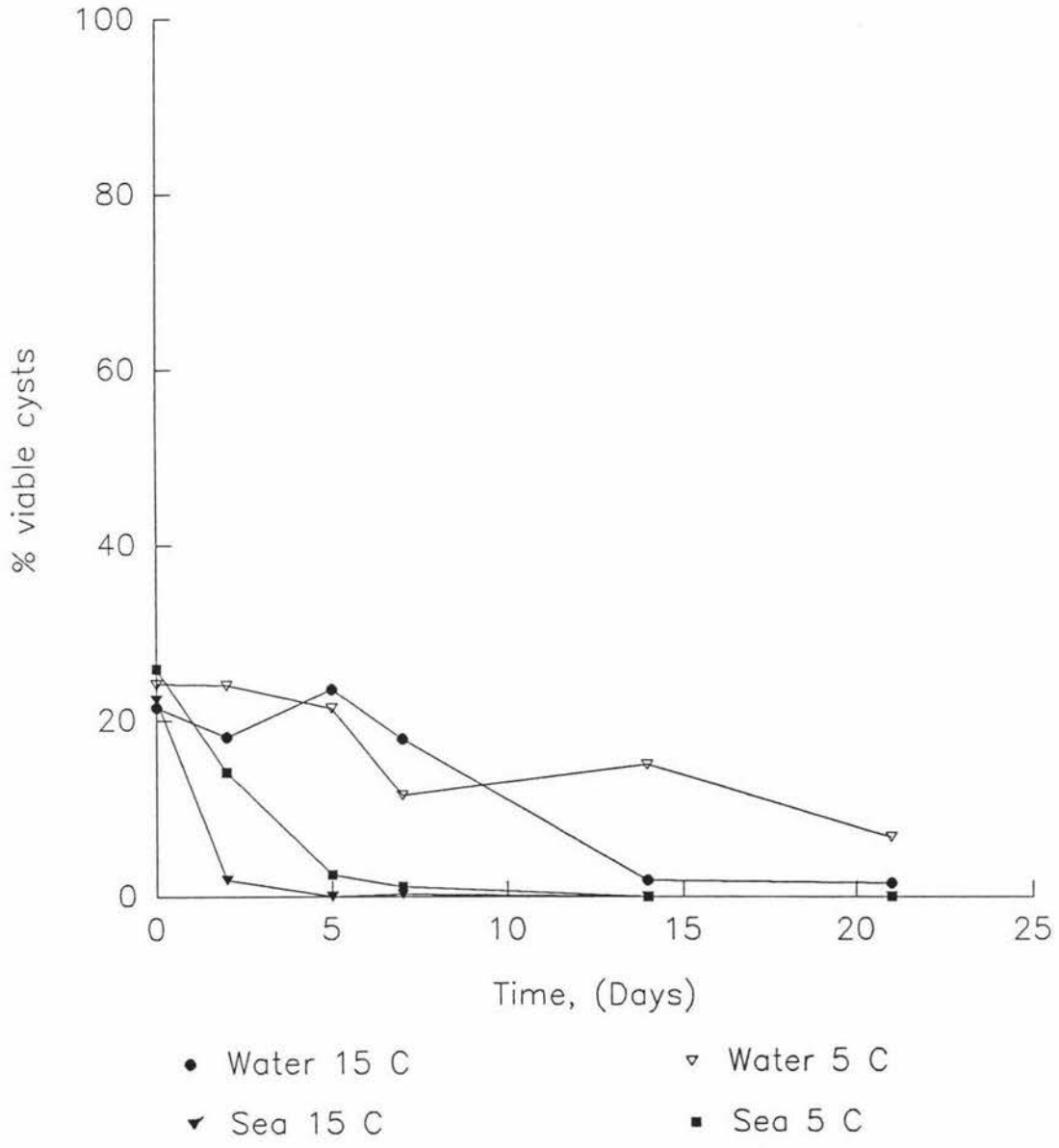


Figure 20 The percent viable cysts as determined by Excystation for G. muris showing temperature and seawater effect.

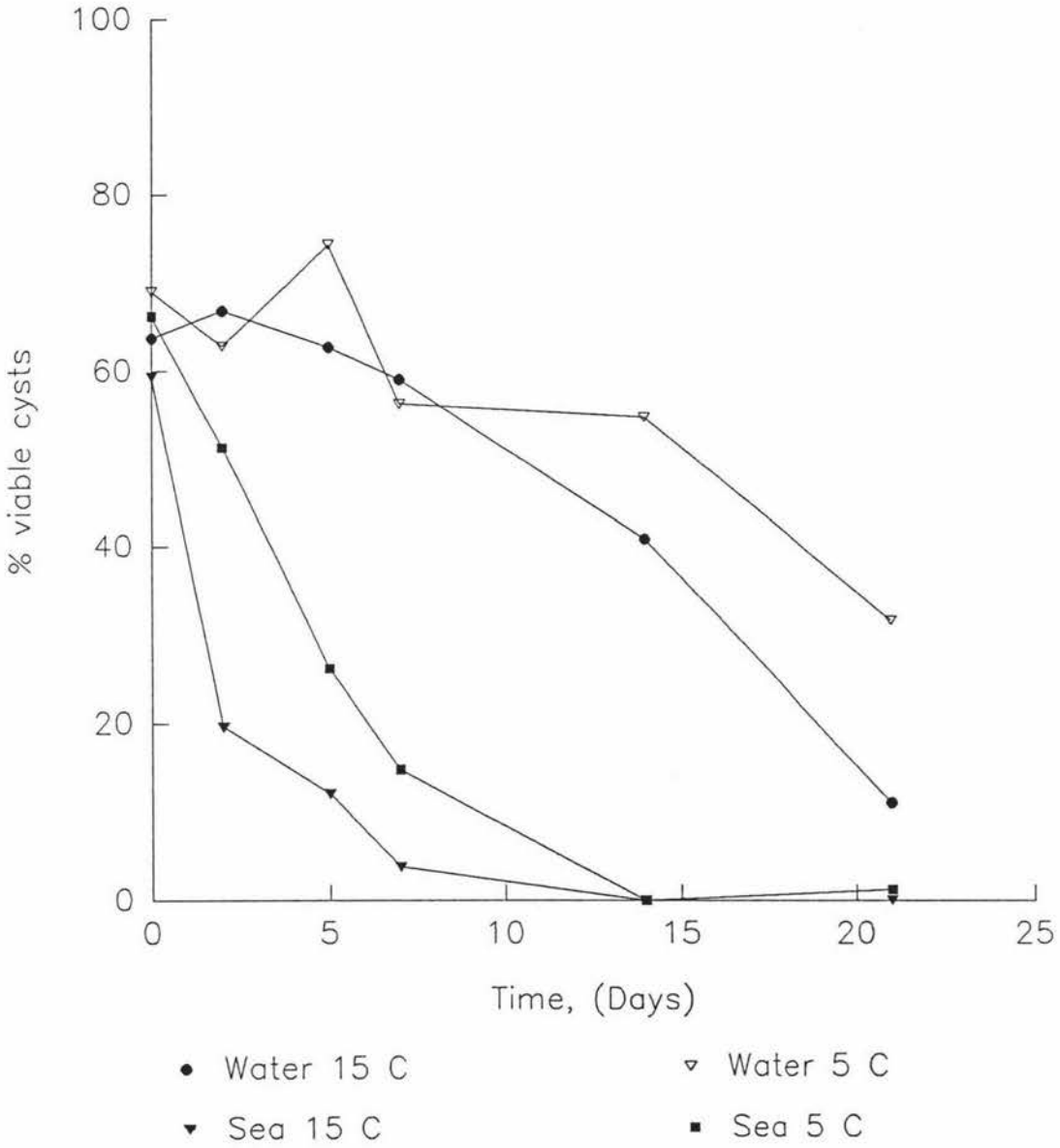


Figure 21 The percent viable cysts as determined by FDA for G. muris showing temperature and seawater effect.

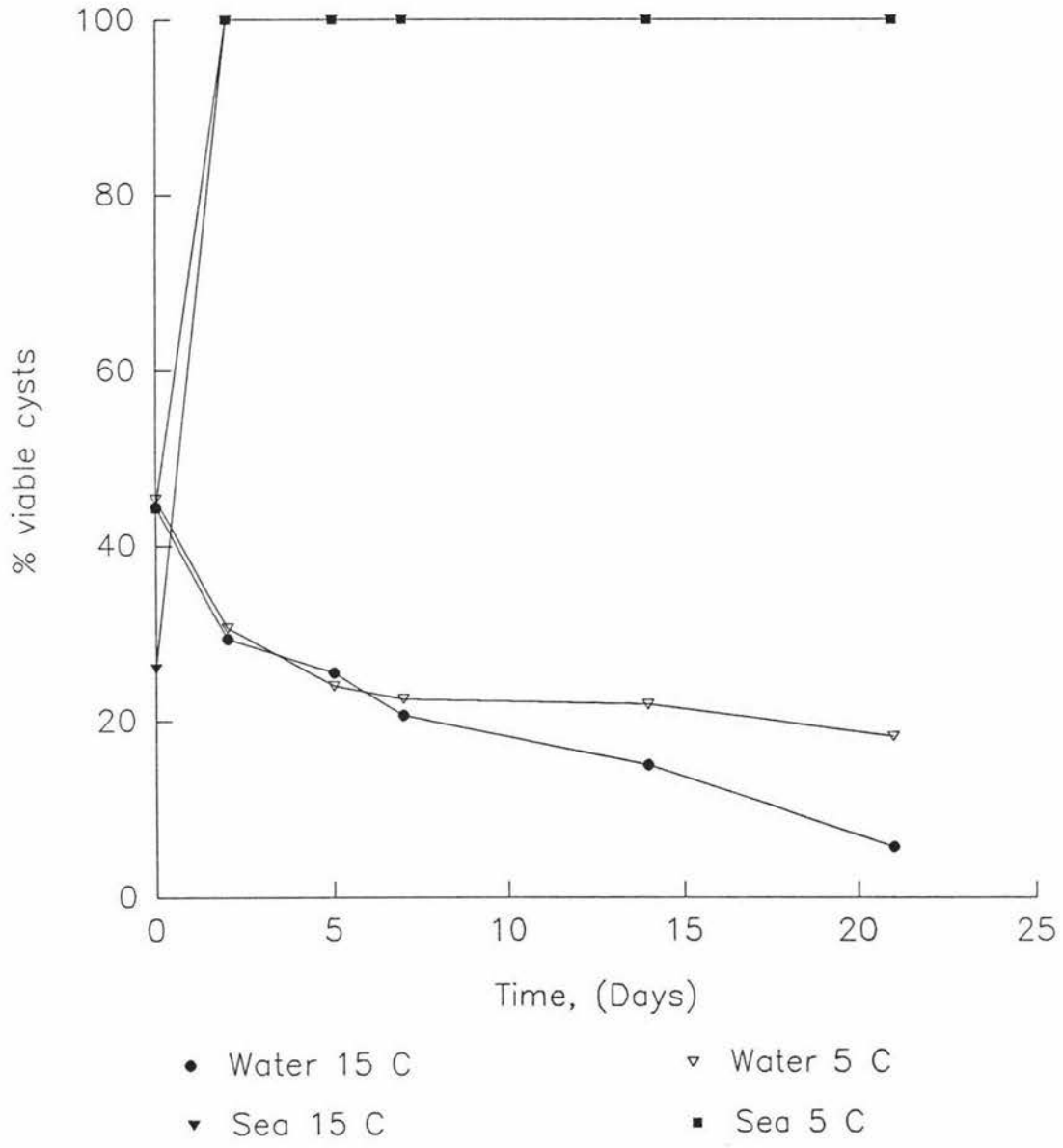
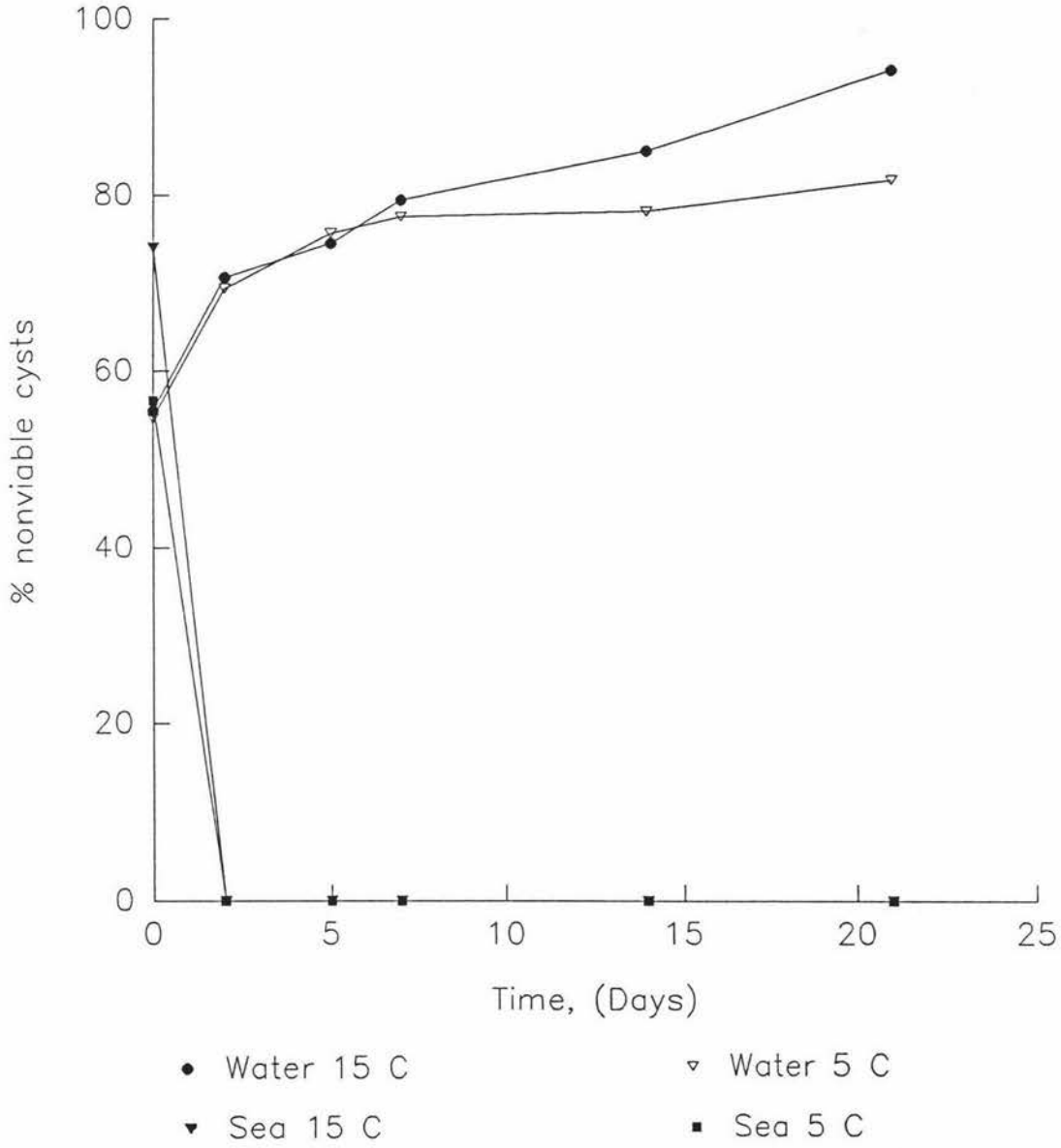


Figure 22 The percent nonviable cysts as determined by PI for G. muris showing temperature and seawater effect.



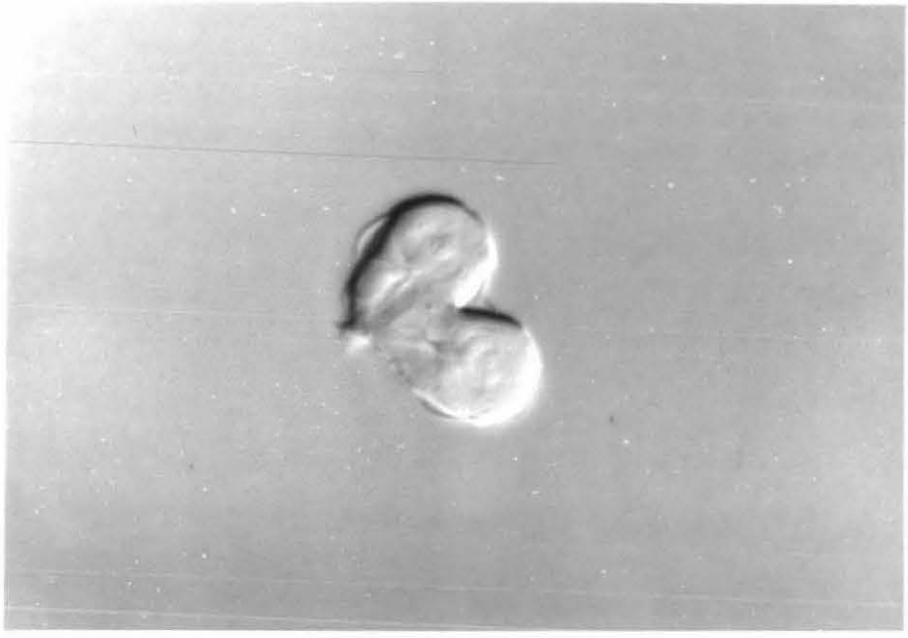


Plate 5

Fully excysted *Giardia intestinalis* trophozoites (x7200).

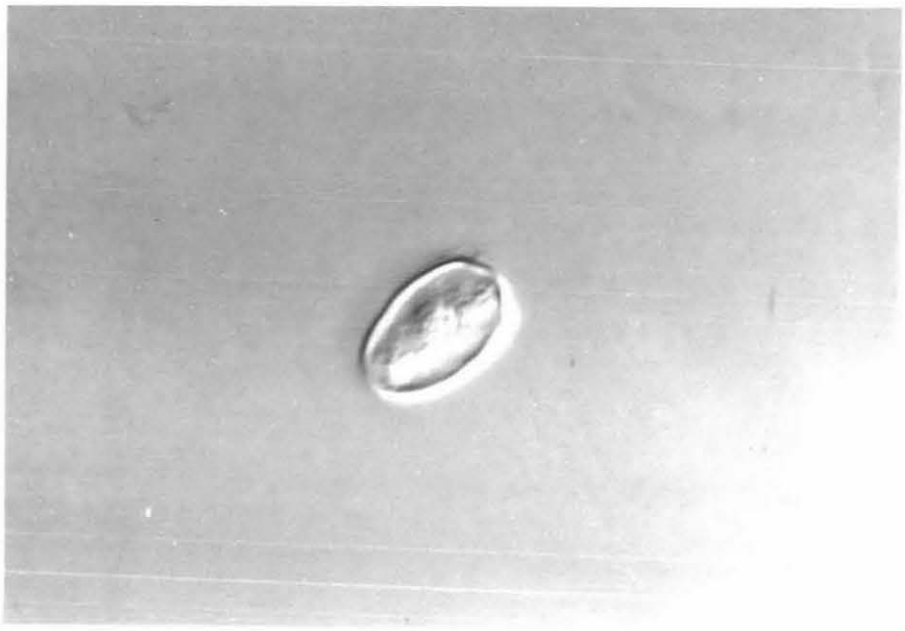


Plate 6

Seawater inactivated *Giardia* cyst (x7200).

Figure 23 The percent viable cysts as determined by Nomarski DIC, FDA, PI, and Excystation for G. intestinalis at 5 C in Freshwater.

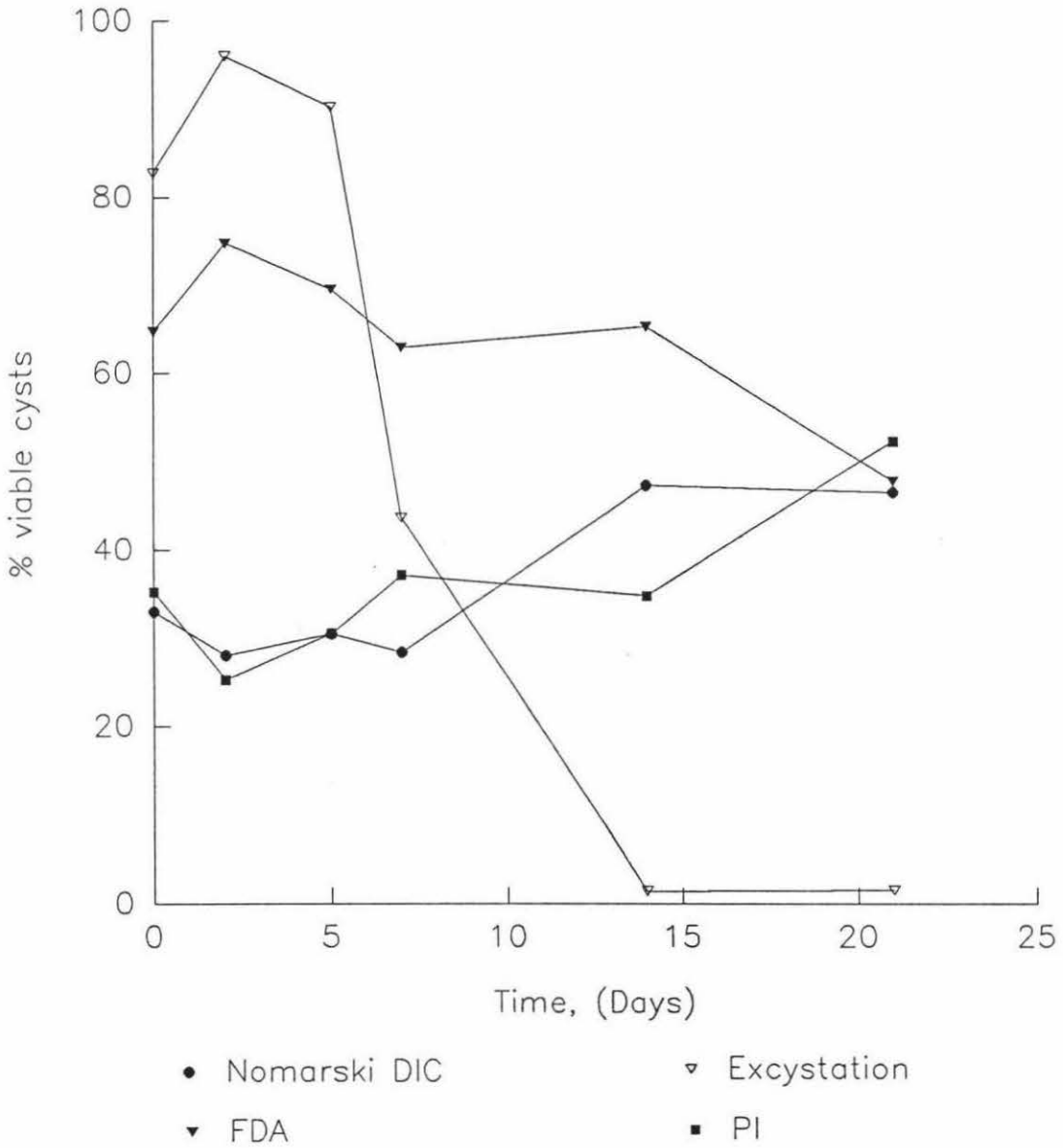


Figure 24 The percent viable cysts as determined by Nomarski DIC, FDA, PI, and Excystation for G. intestinalis at 15 C in Freshwater.

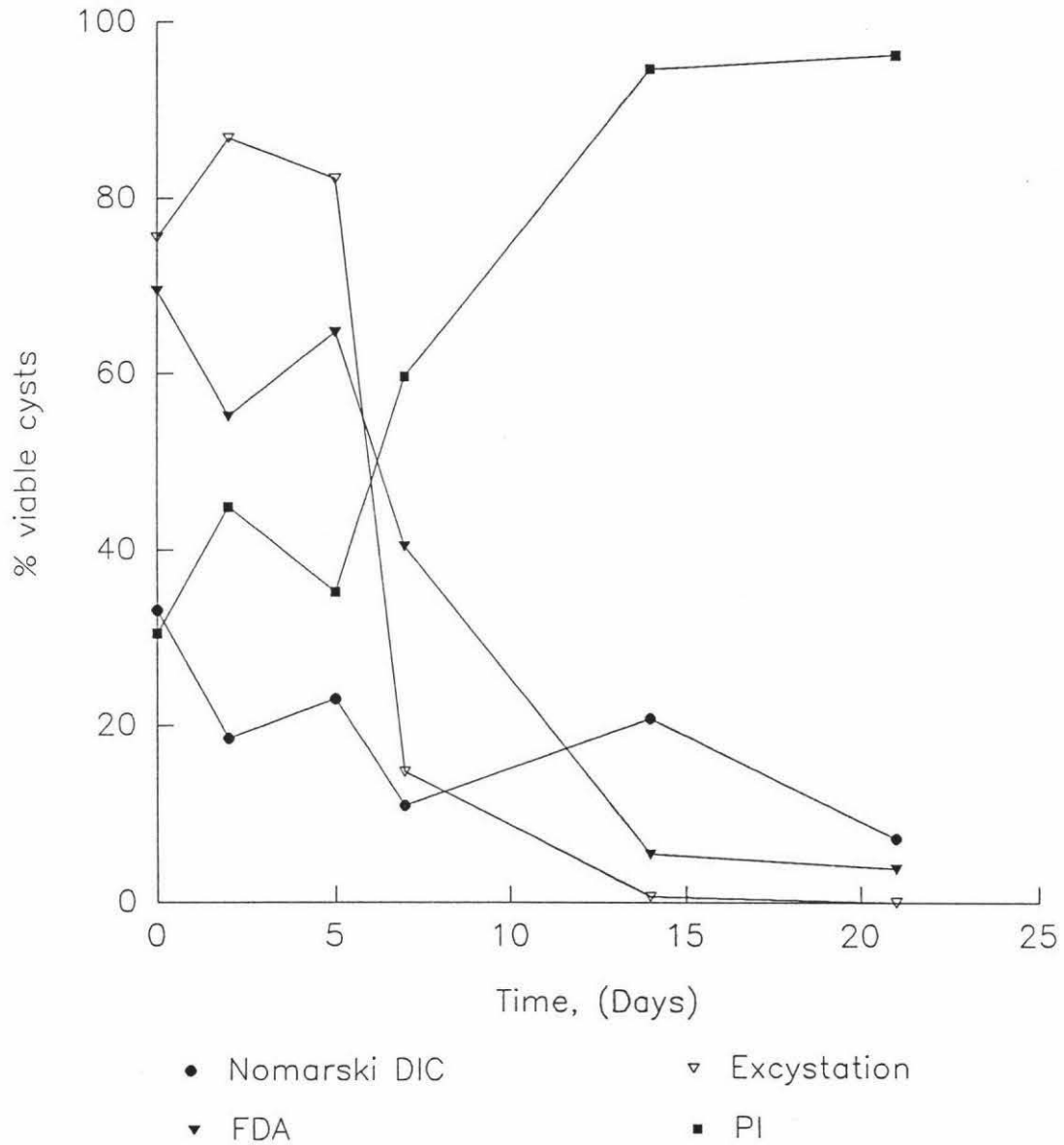


Figure 25 The percent viable cysts as determined by Nomarski DIC, FDA, PI, and Excystation for G. intestinalis at 5 C in Seawater.

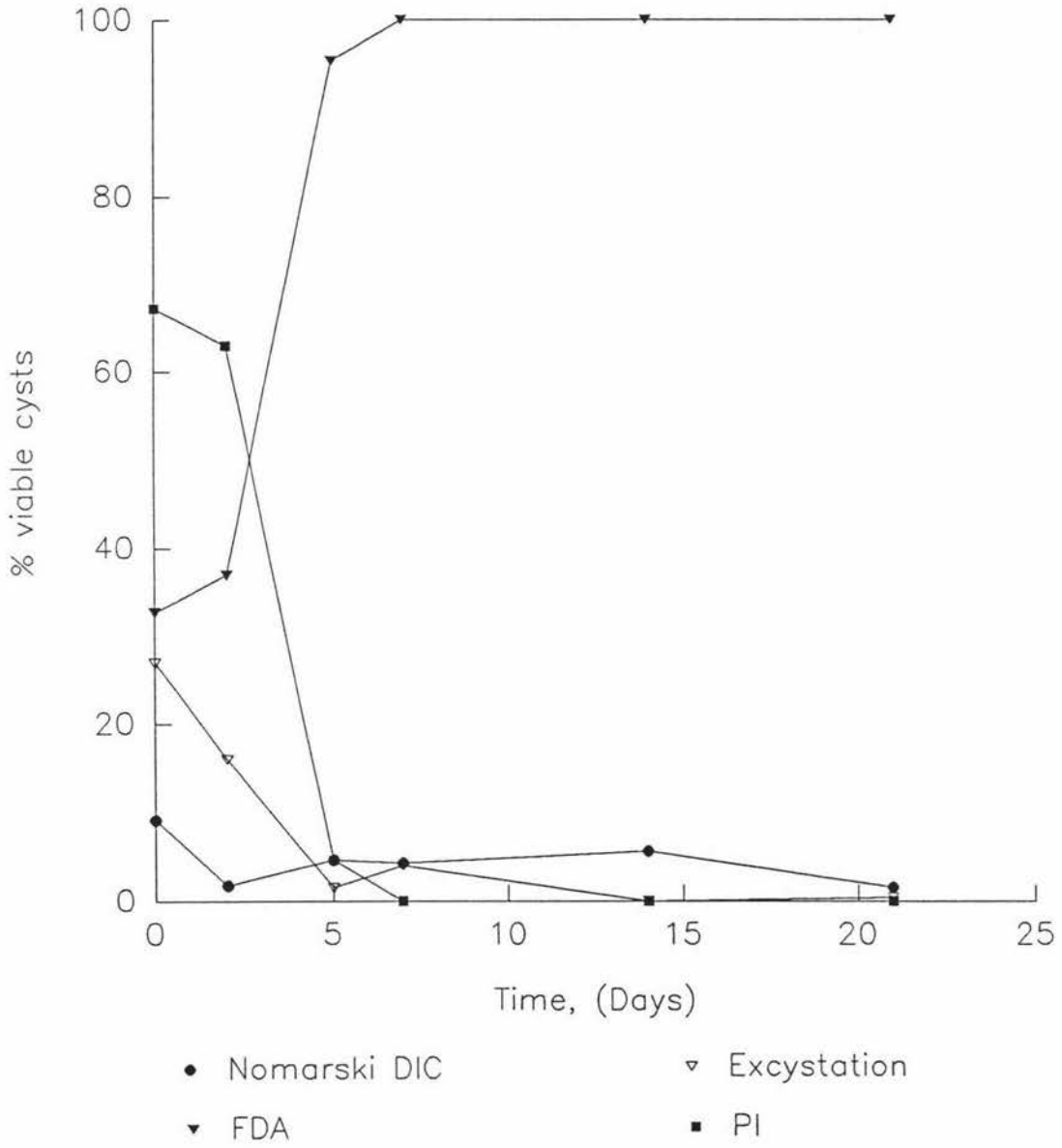


Figure 26 The percent viable cysts as determined by Nomarski DIC, FDA, PI, and Excystation for G. intestinalis at 15 C in Seawater.

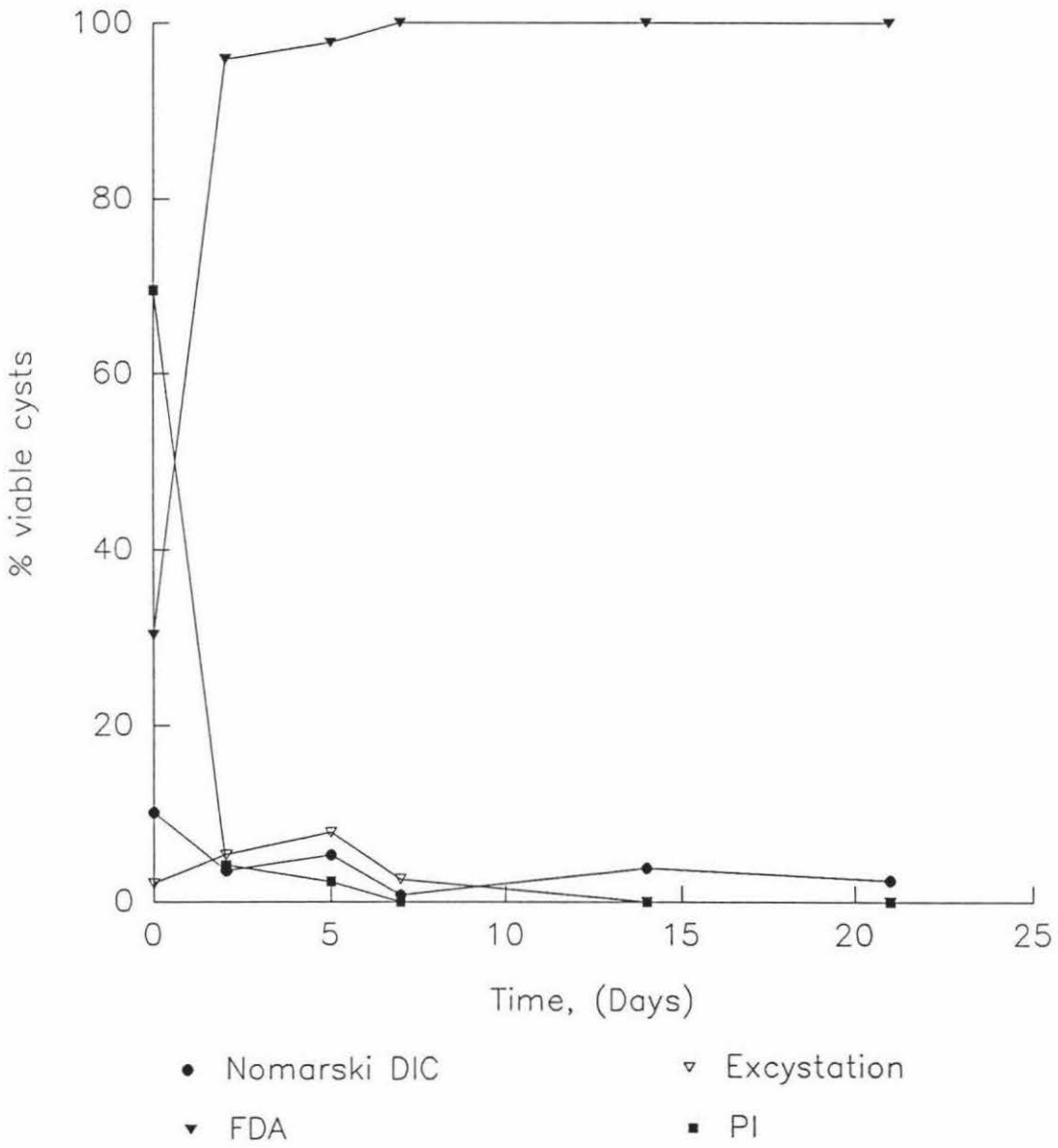


Figure 27 The percent viable cysts as determined by Nomarski DIC for G. intestinalis showing temperature and seawater effect.

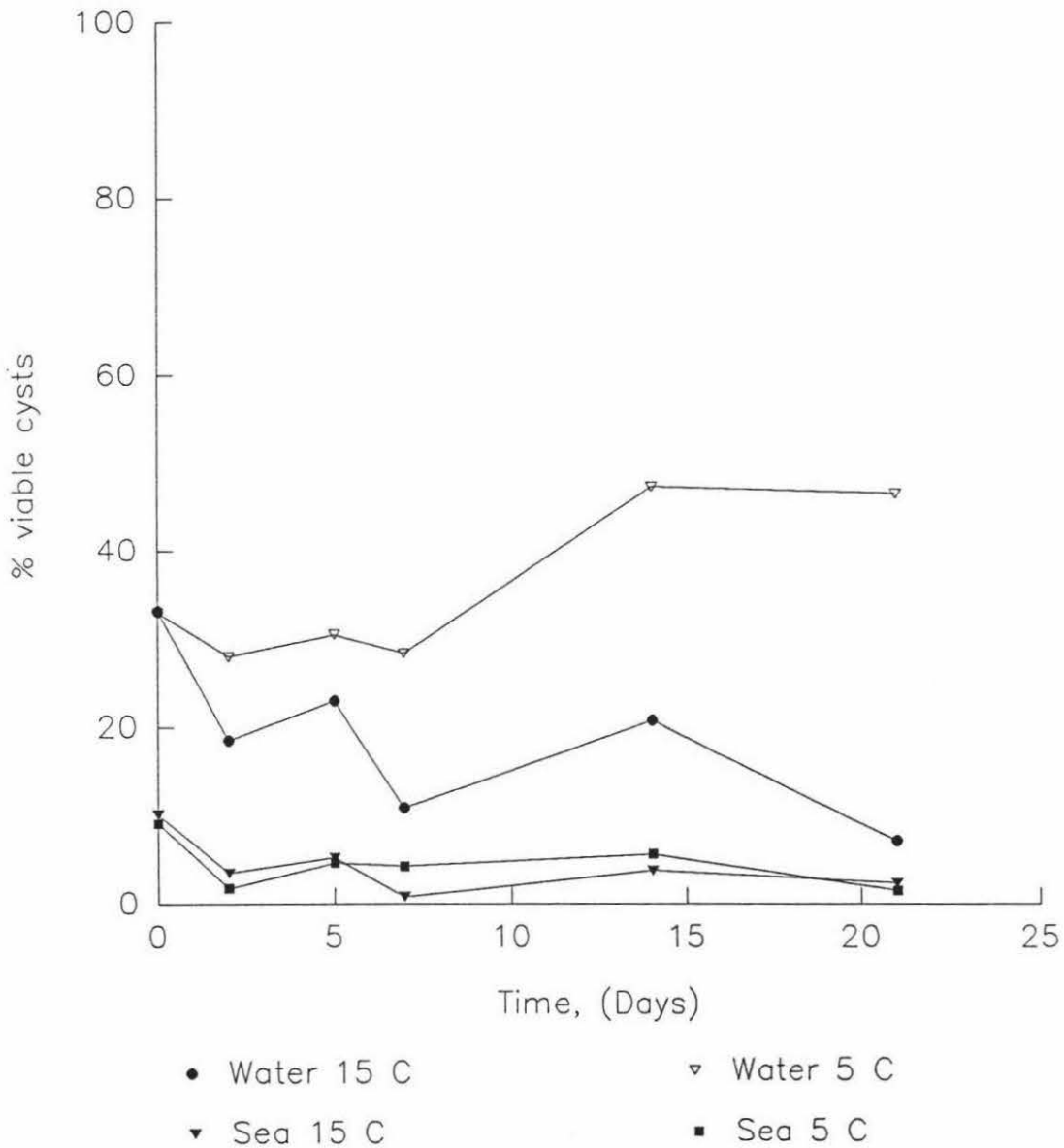


Figure 28 The percent viable cysts as determined by Excystation for G. intestinalis showing temperature and seawater effect.

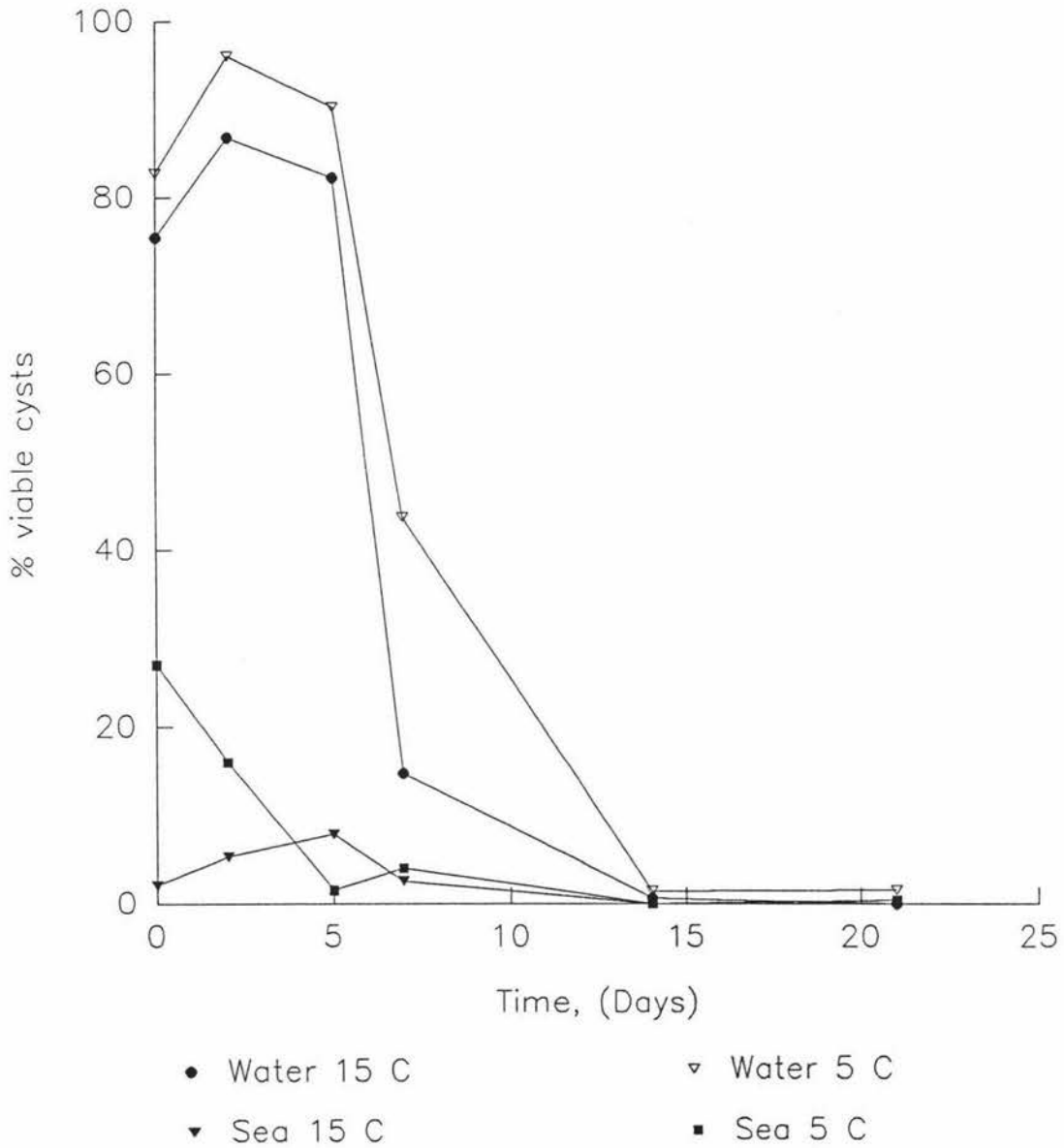


Figure 29 The percent viable cysts as determined by FDA for G. intestinalis showing temperature and seawater effect.

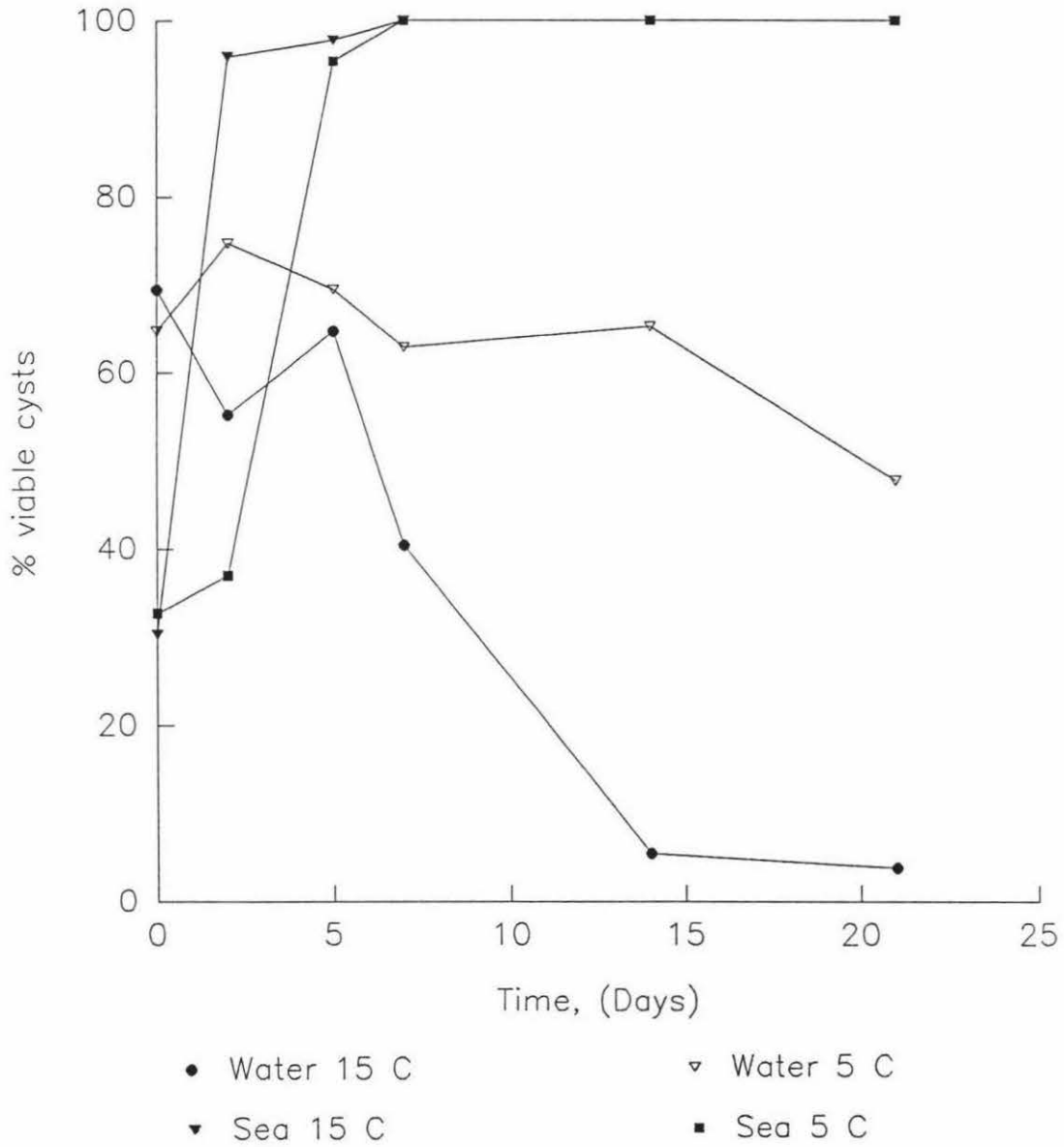


Figure 30 The percent nonviable cysts as determined by PI for G. intestinalis showing temperature and seawater effect.

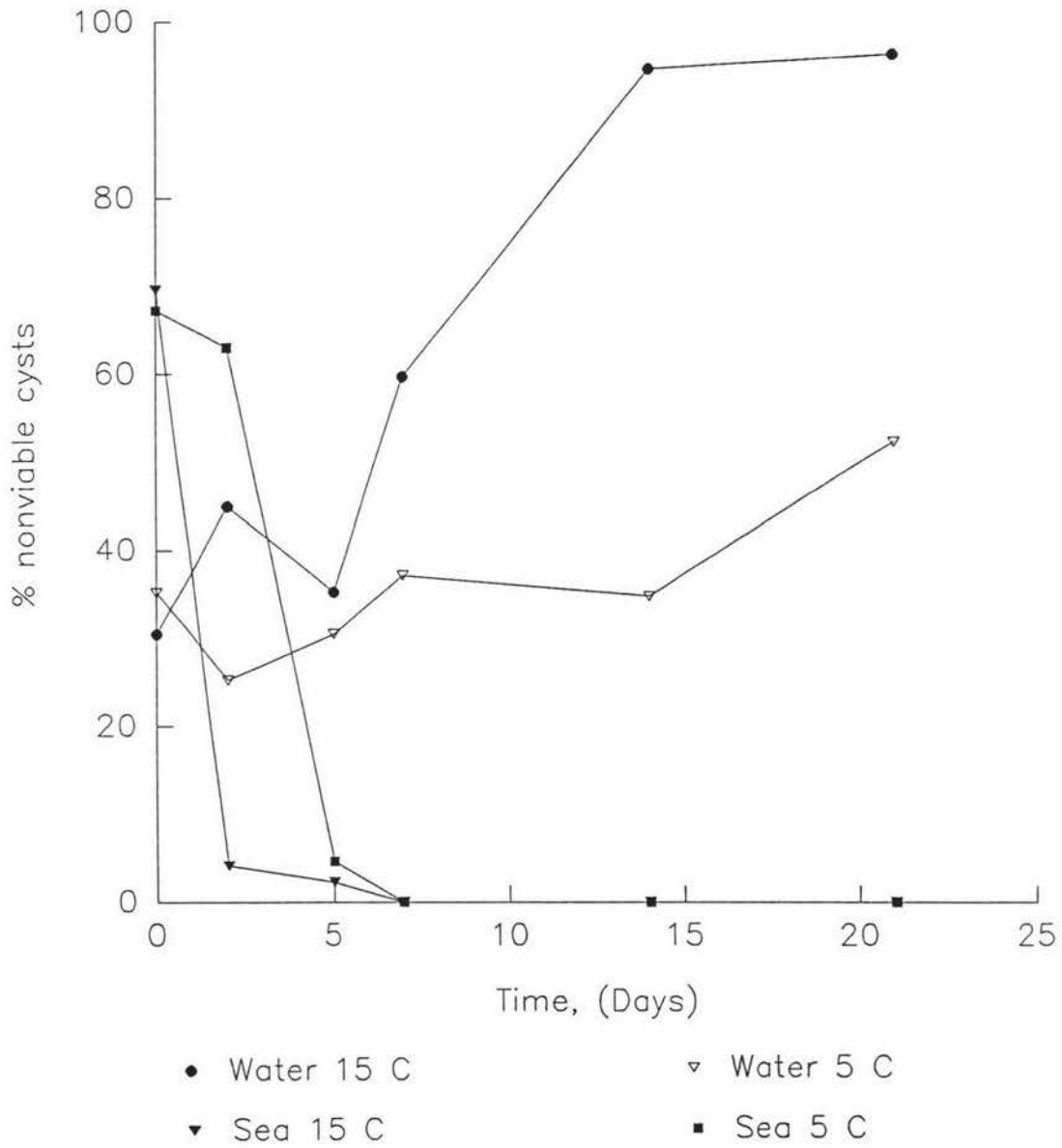


Table 3
Inactivation of *Giardia muris* using various chlorine treatments.

Temp°C	pH	FAC	Time min.	Inactivation efficiency.	Reference.
5	7.0	1	30	90%	Rice *et al.*, 1982
6	8.2	1.3	30	88.4%	Hoff *et al.*, 1985
5	7.0	1.5	30	97%	Sauch *et al.*, 1991
5	7.0	31.4	30	99%	Leahy *et al.*, 1987
5	6.0	0.95	62*	90%	This thesis.

*derived from log expressed data for excystation in Figure 34.

Figure 31 Chlorine inactivation of G. muris as determined by Nomarski DIC.

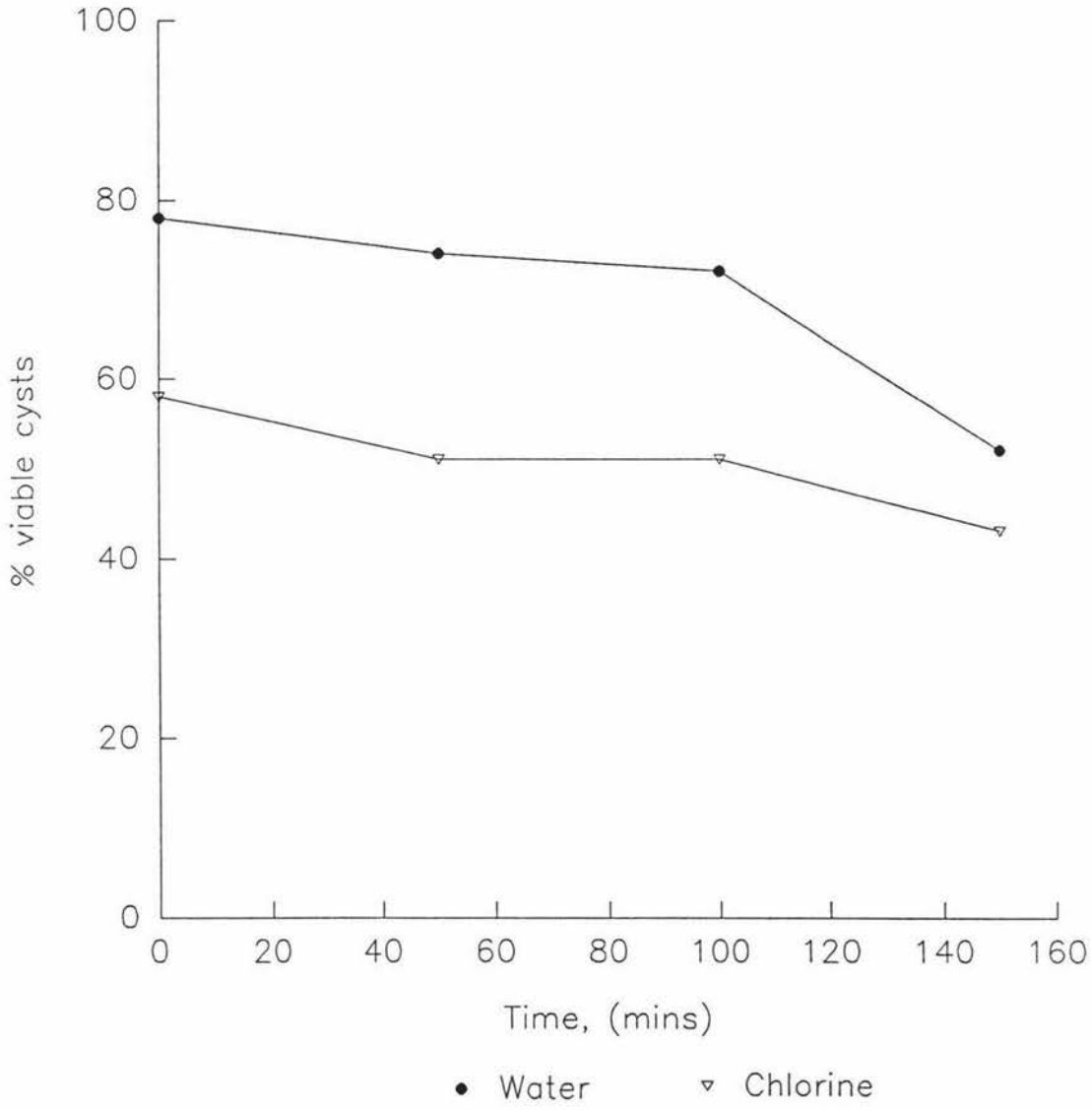


Figure 32 Chlorine inactivation of G. muris as determined by Excystation.

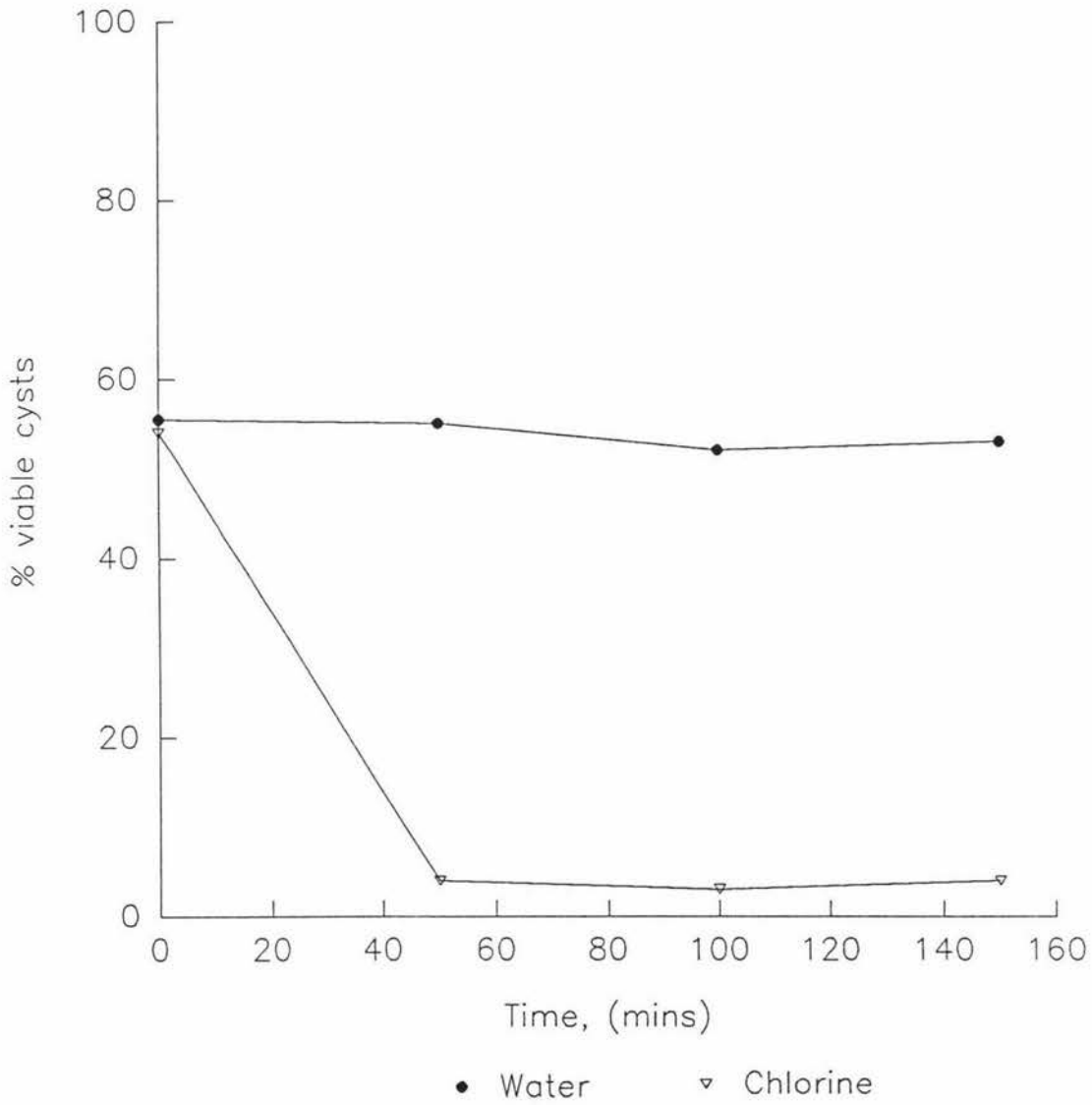


Figure 33 Chlorine inactivation of G. muris as determined by FDA/PI.

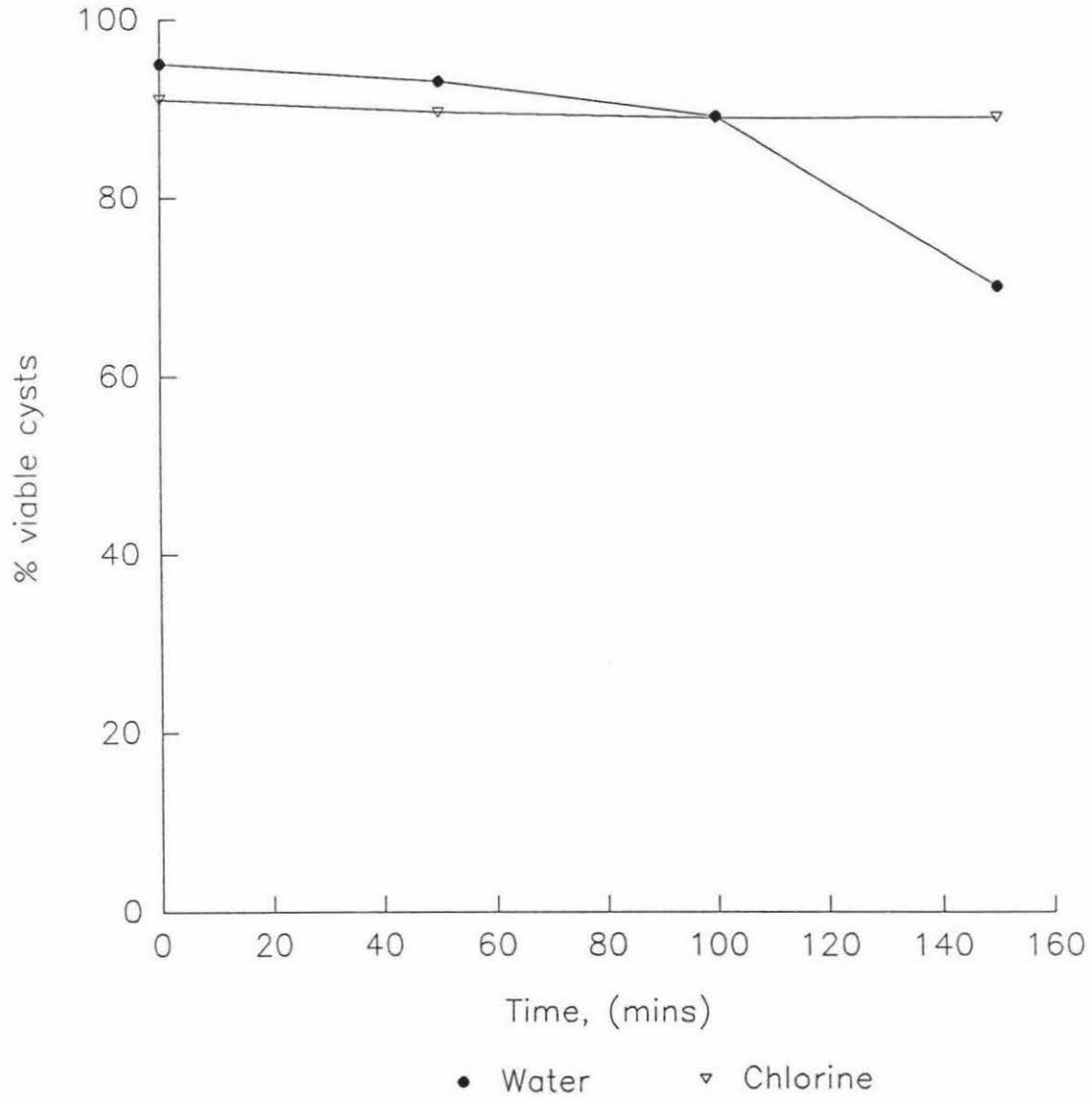
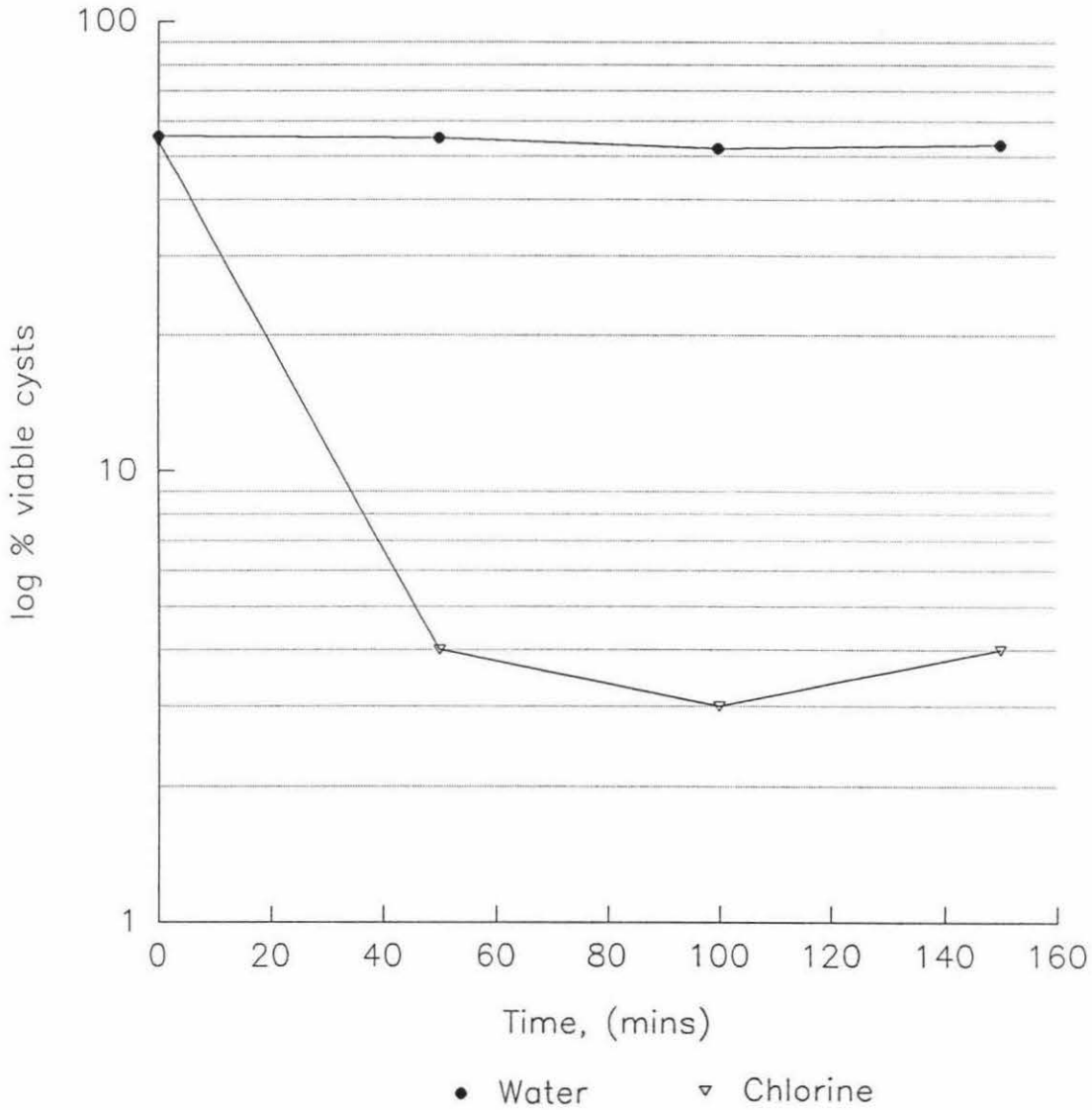


Figure 34 Chlorine inactivation of G. muris log data for Excystation.



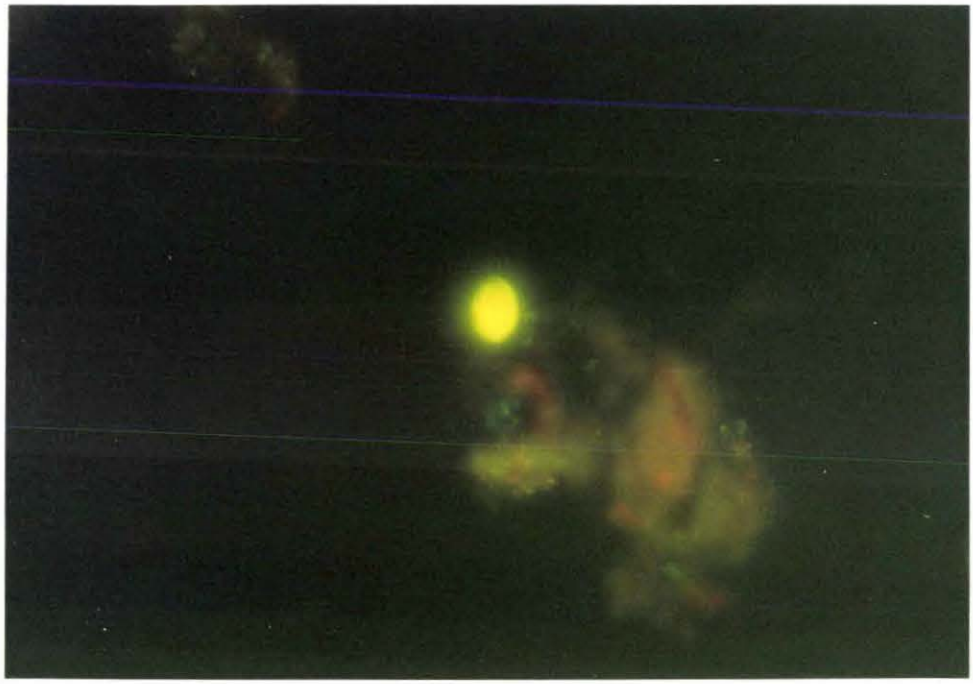


Plate 7

Fluorescein Diacetate stained *Giardia* cyst (x400).

Plate 8

Propidium Iodide stained *Giardia* cyst (x400).

Chapter Five Discussion.

Seawater Inactivation.

Very few areas of New Zealand are far from the sea. This enables many New Zealanders to enjoy the many recreational attractions that the sea offers, such as fishing, boating, swimming, surfing and seafood gathering.

Cities and towns produce large volumes of sewage. The disposal and treatment which, is expensive and sometimes controversial for many borough councils.

Historically proximity to the coast has meant local authorities opting for the infinitely cheaper option of disposal into the sea. In today's social climate this practice is rapidly becoming unacceptable and the move is towards 100% land based treatment. (eg. Wellington City proposed Milliscreening plant at Moa Point).

Seaborne waste disposal results in microorganisms such as viruses (eg. infectious hepatitis), bacteria (eg. *Escherichia coli*) and protozoa such as *Giardia spp.* being introduced into seawater. The length of time such micro-organisms remain viable in seawater has only received moderate attention. *E.coli* and virus survival in seawater (Akin *et al.*, 1975; Carlucci and Pramer 1959; Kornek 1926; Nusbaum and Garver 1955; Zobell 1936), and *Giardia* cyst survival in seawater (Fontaine *et al.*, 1984), are important considerations when evaluating the effects of a sewage outfall. Limited information concerning *Giardia* cyst viability in seawater prompted this study examining firstly whether *G. muris* cysts are able to be used as a model for *G. intestinalis* cyst inactivation and secondly whether seawater has any effect on the viability of cysts when compared to freshwater.

In vivo derived cysts were used for all experiments.

G. intestinalis cysts from human faecal samples excysted with a consistently high level of 90% in contrast to the findings of Bingham and Meyer (1990);

Buchel *et al.*, (1987); and Smith and Smith (1989) who showed rates of excystation from 15 - 60%.
The level obtained here was ideally suited to this study.
G. muris cysts as reported by Roberts-Thomsen *et al.*, (1976) showed excystation rates greater than 90% when cysts were freshly harvested from mouse faeces.

Experiments examining the seawater effect at 3 different temperatures (5, 10, and 15°C) with respect to a water control, using *G. intestinalis* and *G. muris* were conducted. Results at each temperature and for each species of *Giardia* reveals a significant difference (Analysis of Variance, ANOVA $p < 0.0001$) between viability of seawater and freshwater incubated cysts (Figs. 1-6).
G. muris follows a clear exponential decrease in viability with respect to time for each temperature, and *G. intestinalis* follows a similar pattern. These differences maybe species related.

The lower viability seen in seawater incubated cysts is probably due to some cysticidal factor in the seawater. Carlucci and Pramer (1959), stated "that on the basis of concentrations, inorganic salts are the most potentially toxic substance present in the sea". It is postulated that this will have an osmotic effect or perhaps exert a specific ion toxicity. Analysis of the composition of seawater suggests that seawater does indeed have the capacity to cause these effects.

Table 4 Top ten ion concentrations in seawater.

Rank	Element	[ion] mg/l
1	Chlorine	18800
2	Sodium	10700
3	Magnesium	1290
4	Sulpher	905
5	Calcium	412
6	Potassium	399
7	Bromine	67
8	Carbon	28
9	Strontium	7.9
10	Boron	4.5

([ion] based on 1 litre of 35% seawater = 1.028 kg)
(From Marine Pollution, Diagnosis and Therapy. Ed: A. Sebastion)

Results (Figs. 1-6) with both *G. muris* and *G. intestinalis* show a decrease in viability consistent with the statement of Carlucci and Pramer (1959).
At t=0, when cyst samples were in seawater and freshwater for less than one minute before viability determination, both species showed a marked difference in viability at all temperatures (Figs. 1-6; Table 1). (A difference being calculated as viability of cysts in freshwater at t=0 less viability of cysts in seawater at t=0) The differences shown in Table 1 illustrate what is thought to be the effect of osmotic shock. *Giardia spp* inoculated into seawater at a

sewage outfall would therefore show an almost instantaneous decrease in viability compared to the same population of cysts in freshwater. The magnitude of this viability loss in seawater is in the order of 33.5% for *G. muris* and 51% for *G. intestinalis* (with respect to water) such that seawater can be said to be cysticidal. The differences seen in Table 1 are illustrative of species differences between *G. muris* and *G. intestinalis*.

The argument that osmotic shock is the primary inactivation factor is strengthened by the photographic evidence in Plate 6. This Nomarski DIC photo shows a seawater inactivated cyst with morphology characteristic of a cell that has undergone plasmolysis in hypotonic solution. The cyst wall is intact and internal structures are concentrated in a central position in the cyst.

The term "specific ion toxicity" relates to the effect heavy metals such as Mercury, Lead, Arsenic, and Aluminium have. The seawater used in this study was collected from a remote coastal location on the West Coast of the North Island, New Zealand-Tangimoana Beach. The levels of other ions in seawater as illustrated previously may exert an effect other than osmosis on the cyst, contributing to the cysticidal effect of seawater ie. there may be unknown cysticidal radicals present.

Seawater pH is known to have an effect on the survival of *Escherichia coli* in seawater (Carlucci and Pramer 1959). Incubation of *E. coli* for 48 hrs in seawater at pH 5 yields a survival of 58%, compared with incubation in seawater at pH 8.0 (pH most commonly found in oceans, Walker 1975), yielding a survival of just 0.4%. Thus *Giardia* cysts may be similarly affected, and could contribute to the viability difference seen at $t=0$ (Table 1) and general decrease in viability seen with respect to time (Figs. 1-6). The high pH of seawater is due to the high concentration of salts present (Marine Pollution, Diagnosis and Therapy 1976) Tangimoana seawater has a pH of between 7.45 - 8.47, easily in the range to give a killing effect.

Log plots of Nt/No for *G. muris* illustrate a linear trend in the decrease of viability in freshwater and seawater (Figs. 7-10). This is most pronounced for *G. muris* at all temperatures, whereas *G. intestinalis* (Figs. 11-14) does not show the same degree of linearity. This is probably species related.

An average of data combining the rates of inactivation for the three temperatures shows *G. muris* is inactivated at a slower rate in seawater than fresh water (Table 2). *G. intestinalis* does not show this same linear trend and we can say that there is a species difference in the pattern of inactivation in seawater and freshwater. This difference is significant (ANOVA $p < 0.0001$). By comparing the inactivation trends of *G. muris* and *G. intestinalis* we can see that the animal strain has a greater resistance to inactivation by seawater.

This effect may be compared to the observations of Jaroll (1988) for chlorine inactivation of *G. muris*. They observed that *G. muris* cysts have a greater resistance to chlorine inactivation. This study agrees with these observations with respect to seawater such that one can suggest that *G. muris* is a more hardy species of cyst. This is maybe due to structural differences in the cyst giving it greater resistance.

In these comparisons the temperature effect stated in the literature (Bingham *et al.*, 1979), does not seem to be evident, ie. one would have expected to see an increase in inactivation rate with an increase in temperature. With both *G. muris* and *G. intestinalis* the relationship between temperature and increased inactivation rate is unusual with greater inactivation being shown at 5 and 15°C than at 10°C. There is no obvious explanation for this but it could be that the cysts are being influenced by a temperature dependent enzyme effect ie. enzymes concerned with the viability of the resting cyst and the cysts ability to undergo excystation may have optima which support these observations.

Assessment of Methods of Determining Viability of *Giardia* cysts.

1. Seawater inactivation.

Investigations into the methods for determining *Giardia spp.* cyst viability have been in progress for some time now (Belosevic *et al.*, 1983; Bingham *et al.*, 1979; Feely 1986; Feely *et al.*, 1991; Filice 1952; Hoff *et al.*, 1984; Hoff *et al.*, 1985; Labutuik *et al.*, 1991; Leahy *et al.*, 1987; Rendtorff 1879; Rice and Schaefer 1981; Roberts-Thomsen *et al.*, 1976; Sauch *et al.*, 1991; Schaefer *et al.*, 1991; Schupp and Erlandsen 1987; Schupp *et al.*, 1988). The accepted methods used for viability testing are few at present and consist mainly of excystation, fluorogenic dye staining, infectivity, and morphological determination using Nomarski Differential Interference Contrast Microscopy (Nomarski DIC), (Belosevic *et al.*, 1983; Bingham *et al.*, 1979; Feely 1986; Feely *et al.*, 1991; Filice 1952; Hoff *et al.*, 1984; Hoff *et al.*, 1985; Labutuik *et al.*, 1991; Leahy *et al.*, 1987; Rendtorff 1879; Rice and Schaefer 1981; Roberts-Thomsen *et al.*, 1976; Sauch *et al.*, 1991; Schaefer *et al.*, 1991; Schupp and Erlandsen 1987; Schupp *et al.*, 1988).

Excystation is considered the "benchmark" for viability by many researchers. But complex media, length of time needed to conduct this assay and the need for $> 10^5$ cysts per ml to test precludes its use in water testing, where frequently < 10 cysts are found. Therefore the alternatives are investigated in this study.

The effective merits of these methods have been examined in what is mainly "time independent" studies using single samples and single field of view experiments (Schupp and Erlandsen 1987; Smith and Smith 1989). Investigators using time dependent studies (Sauch *et al.*, 1991; Labutuik 1991), use cyst inactivations that are not capable of showing trends over a time period because serial sampling and testing was not practised. They involve a single set exposure time. This study differs majorly from previous work as follows : the methodology involved taking samples at time intervals over a period of 21

days for seawater inactivation and 150 minutes for chlorine inactivation, then analyzing these samples using

a) Excystation,
b) Nomarski DIC,
c) Fluorogenic Dyes.

Infectivity (Roberts-Thomsen *et al.*, 1976), was not used as a measure of viability because of major problems obtaining specific pathogen free mice.

Inactivation using seawater and chlorine was trialled because of previous reports that fluorogenic dye staining was thought not to be effective in a chlorine inactivation system, and that dyes would be better suited to an environmental inactivation system (Sauch *et al.*, 1991).
Bearing these points in mind an environmental system using seawater as the inactivating agent was used. This was shown earlier in this study to be an effective inactivation agent for both *G. muris* and *G. intestinalis*.
Verification of the observations of Sauch *et al.*, (1991) with chlorine inactivation was conducted by performing a similar experiment. The experiment was conducted using *G. muris* as at the time *in vivo* *G. intestinalis* were difficult to source.
Chlorine levels for the inactivation were determined by the N.Z. Health Department as being suitable for 99.9% inactivation of *G. intestinalis* cysts. The experiment entailed; 1 ppm Free Available Chlorine, 149 minutes, pH 6-7, and 5°C (Ampofo *et al.*, 1991).

'Black cysts' as seen by (Sauch *et al.*, 1991; and Shupp and Erlandsen 1987), were not observed in any of the samples tested using the fluorogenic dyes, and were therefore not considered in the conclusions stated here.

Examination of figures for *G. muris* incubated in freshwater at 5 and 15°C (Figs. 15-16), shows Fluorescein Diacetate (FDA) and Nomarski DIC account for similar levels of viability with FDA recording a slightly higher proportion at each sampling time. The level of viability determined by the 'benchmark' excystation is considerably greater (at all data points), and thus Nomarski DIC and FDA although very similar to each other both underestimate viability with regard to excystation. Propidium Iodide (PI) staining represents the proportion of non-viable cysts and was calculated from the same data as that for FDA viability. ie. Total number in the sample = FDA positive scored cysts + PI positive scored cysts. This explains the mirrored pattern seen in these experiments with respect to the fluorogenic dyes. PI under estimates the level of viability.

In seawater at 5 and 15 °C (Figs. 17-18), *G. muris* cyst staining initially (t = 0) showed indications of following the same trends as in water. But after 1 - 2 days results were very unusual. ie. FDA displays 100% viability and PI 0% viability for each temperature. This effect was not seen in freshwater and is also observed for the same experiment conducted with *G. intestinalis* (Figs. 25-26). This effect was probably due to a) the cysticidal effect of the seawater or b) the seawater possible osmotic effect on the action of the stains. Plate 6 shows the apparent effect seawater has on *Giardia* cysts, in that it seems the primary cause of death is plasmolysis.

FDA action as a vital stain means that only viable actively metabolising cysts are able to fluoresce green at an excitation wavelength 490nm. PI relies on diffusion into the cyst where it intercalates nucleic acids yielding a red/orange fluorescing cyst at excitation wavelength 490nm (Schupp and Erlandsen 1987). Thus if cysts have been metabolically altered by the action of the seawater and show characteristics such as those in this experiment then the reliability of the use of these fluorogenic dyes for estimating viability of seawater inactivated cysts must be questioned.

The response the stains give in freshwater, although underestimating the viability with respect to excystation seem to behave 'normally'. Their use in classifying cysts isolated from freshwater samples as either viable or non-viable may be possible.

Commercially available and highly specific immunofluorescent stains (using Fluorescein label), have been shown to be compatible with Propidium Iodide (Sauch *et al.*, 1991) and they could therefore be used together to detect and determine viability of *Giardia* cysts isolated from freshwater.

The viability as assessed by excystation and Nomarski DIC in seawater, is much lower than that seen in freshwater for the same population of cysts, this is due to the seawater as stated earlier (osmotic and other unknown effects). Nomarski DIC consistently under estimated viability when compared to the 'benchmark' excystation.

A similar experiment conducted using *G. intestinalis* varies from those given by *G. muris* (Figs. 23-24). Incubation in water and for both temperature cases (5, 15°C) shows Nomarski DIC under estimates viability when compared with FDA. This agrees with the pattern for *G. muris*. PI staining under estimates the viable proportion of cysts compared to Nomarski DIC. Excystation shows a different pattern to that for *G. muris* decreasing at a faster rate and suggesting a species difference.

In seawater at 5 and 15°C the pattern of inactivation for *G. intestinalis* (Figs. 25-26), as shown by FDA and PI, is identical to that shown by *G. muris* (Figs. 17-18). Excystation and Nomarski DIC do give a reading but this does not rise above 10%, suggesting that *G. intestinalis* is a more susceptible species to inactivation by seawater. This contrasts with *G. muris* where viability by these methods was measured initially at above 20% in each case, and drops in the same exponential fashion seen in Figs. 1-3.

The unusual results for FDA and PI may be hypothesized as being due to the same effects discussed for *G. muris* (Plasmolysis and seawater having an effect on their ability to stain the cyst).

A re-emphasis of the data allows an examination of each method of determining viability, both with respect to temperature (5, 15°C) and treatment (freshwater/seawater). Viability as determined by excystation in Figure 20 for *G. muris* and Figure 28 for *G. intestinalis* show a clear difference between viability in freshwater and seawater for each species. The lower temperature of 5°C appears to "protect" the cysts in each treatment yielding a higher viability over the time period. This effect is less evident in previous seawater inactivations (this study). The lethal temperature dependent killing effect seen in freshwater is documented (Bingham *et al.*, 1979), and seems to apply to seawater.

Viability as determined by Nomarski DIC (Figs. 19 and 27) shows clear differences when compared to excystation, ie. at $t=0$, the viability being approximately 50% lower. The difference between freshwater and seawater treatments is also extremely clear. Temperature as seen for excystation, 'protects' cysts at the lower temperature of 5°, with 15°C. showing a faster rate of inactivation.

2. Chlorine Inactivation.

In the supply of freshwater to townships and cities, effective disinfection is critical for the safety and well being of the inhabitants. Filtration, coagulation, and sedimentation although necessary are not always 100% effective and a subsequent disinfection programme must be effective. The New Zealand Health Department has set guidelines for disinfection of domestic supply water. A chlorine concentration of 1 mg/l Free Available Chlorine for 149 minutes, at pH 6-7, and 6°C, is deemed to give a 99.9% inactivation of *G. intestinalis* (Ampofo *et al.*, 1991).

Detection of *Giardia* cysts either pre or post treatment is indicative of a potential problem. ie. *Giardia* cysts in the water supply. Unfortunately the potential infectivity cysts is not presently assessable. Many researchers have addressed the problem of viability post disinfection (Leahy *et al.*, 1987; Neuwirth *et al.*, 1988; Hoff, Rice and Schaefer 1985; Rice *et al.*, 1982) using excystation as their measure of viability.

Water testing for *Giardia* cysts is laborious and time consuming. The methodology involved (Standard Methods), results in approximately 50% of the cysts present being recovered (Jakubowski 1987). The laboriousness of the testing combined with the complex materials and methodology needed for excystation, makes Nomarski DIC, and Fluorogenic Dye Staining more appealing as an alternative for determining viability. Cysts isolated are able to be kept on slides for detection, then perhaps viewed with Nomarski DIC or stained with a vital stain.

With the testing in mind methods for determining viability such as Fluorogenic Dye Staining and Nomarski DIC are less complex both in materials used and the methodology followed when compared to excystation, and also take considerably less time to complete.

The experiments show both Nomarski DIC and FDA/PI over estimate viability when compared to the 'benchmark' for viability excystation. This occurs in freshwater and to a greater extent in chlorine treated water.
The actual response to chlorine is minimal when compared to excystation which shows a large decrease. This is the 'benchmark' for viability and thus in interpreting these results we would be more inclined to believe excystation. Comparison of the data here with the observations made by other researchers in Table 3, shows viability as assessed by excystation here follows a similar trend to their experiments (References in Table 3). These researchers achieved a 90% inactivation under conditions similar to those used here.

The response shown by Nomarski DIC and FDA/PI does show signs of decreasing, and it may be that given more time these methods will show an appreciable decrease in viability. Unfortunately in the disinfection of large volumes of water with high flow rates, this doesn't allow a lengthening of retention time to enable the viability of cysts detected to be determined.

The use of Nomarski DIC and FDA/PI in determining viability of pretreated water would give an exaggerated estimate of the viability of the cysts tested. This in itself would not be an unwarranted use of Nomarski DIC or the stains as any cyst detected should prompt a thorough examination of water treatment practises and make staff wary of the risk that *Giardia* poses. The same theory can be applied post-chlorine treatment.

Chapter Six Conclusions.

In Figs. 19-22 & 27-30, the effect of temperature is evident more in some cases than others. *G. muris* and *G. intestinalis* both exhibit considerably faster rates of inactivation in seawater as compared to freshwater. The pattern of inactivation (Figs. 1-3) for *G. muris* is different to that for *G. intestinalis* (Figs. 4-6) and therefore does not form a good model for *G. intestinalis*.

The cysticidal effect that seawater has on cysts has been postulated as being due to the salt in seawater and the alkaline pH. Plate 6 shows a seawater inactivated cyst which has the morphological character of a plasmolysed cell. Thus the seawater exerts an osmotic effect on the cysts and in all cases this effect yields an almost instantaneous drop in viability.

In conjunction with other factors determining the fate of sewage pumped into the sea, dilution. This is a variable factor and is dependent on tidal patterns and the direction and rate of currents (Hyashi and Ito 1975). It can be assumed that in the case of outfalls pumped directly into the sea as opposed to harbours or estuaries that the effect of dilution would be massive. Further, sedimentation of *Giardia* cysts physically associated with relatively large particulate matter and with the flocculating effect of seawater (Mitchell and Chamberlain 1975), absorption to marine sediments stimulates effective cyst reduction. Thus we can say the risk of contracting a giardiasis infection from contaminated sea would be much reduced and significantly less than fresh water.

The use of Fluorogenic Dyes as an alternative methods of determining viability in seawater is not possible because of the unusual and as yet unexplained results in Figs. 17-18, 21-22, 25-26, 29-30. Nomarski DIC is able to be used in a limited capacity to determine viability of cysts isolated from seawater, but one must remember that this method under estimates viability as compared to

the benchmark method excystation. In freshwater the effective merits of the Fluorogenic Dyes are more useful with FDA/PI only slightly under estimating viability as compared to Nomarski DIC, but under estimating viability to a larger extent when compared to excystation.

In chlorine inactivation the use of Nomarski DIC and Fluorogenic Dyes to determine viability of cysts isolated from water samples would seem feasible at first. The relative ease with which these methods can be conducted when compared to excystation means that little training would be required to enable their use in treatment plants. The cost of the microscopes needed is unfortunately quite inhibitory to their set up.

An over estimation of viability when determined by Nomarski DIC and Fluorogenic Dyes means that a greater proportion of the cysts detected would be classed as viable. This could lead to heightened awareness of the risk that *Giardia* poses to the wider community.

Anomalies in the results concerning an underestimation of viability by Nomarski DIC when compared to excystation in seawater and an over estimation when inactivated by chlorine is probably due in part to the highly osmotic nature of seawater. The much greater chlorine ion concentration in seawater in conjunction with the many other ions present produces more non-viable Nomarski DIC cyst morphology's and therefore greater inactivation. When acted on solely by chlorine, it maybe that this does not provide an effect great enough to change the morphology as seen by Nomarski DIC. Thus giving an over estimation of viability.

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