

OCCURRENCE AND SOURCE OF CRYPTOSPORIDIUM AND GIARDIA IN CATCHMENT AREAS AND WASTEWATER WORKS

Report to the Water Research Commission

by

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EXECUTIVE SUMMARY

Background and motivation

A Water Research Commission sponsored workshop was held at Umgeni Water, Pietermaritzburg in February 1996 on "The occurrence and control of Cryptosporidium in the Umgeni catchment." The major stakeholders were present and discussed the present and future problems, research needs, collaborative partners and responsible researchers and laid out the aims of a research project. A collaborative effort was required to solve the problem of the source of the parasitic protozoa in the environment and assess the risks to health to both the rural communities who may use raw water as their only source and also the millions who are supplied with potable water directly, which could still be contaminated. The three organizations that were working on the parasitic protozoans in closely related areas, Umgeni Water, CSIR and University of Pretoria who had applied for funding were asked to combine projects and expertise.

A number of related WRC funded research projects have been undertaken at Umgeni Water. One is the investigation of the production of a novel Slide Immuno-Assay field test kit (report no. 825/1/03), for the protozoan parasites that could be tested in the high-risk areas pinpointed by this study. Another is Alternative Disinfection for the treatment of final wastewater effluents (report no. 1030/1/02); pilot studies have shown that large quantities of the parasites were present on occasions in some sewage effluents. Another project seeks to explore the relationship between the provision of rural water supply and community health, (Report no. 925/1/03).

Although there are many causes of diarrhoea, the enteric protozoons *Cryptosporidium parvum* and *Giardia lamblia* have been recognised as important causes of both outbreak-related and sporadic diarrhoea in humans (Casemore, 1990). Contamination of a water source may arise as a result of run-off, accidental spillage of farm slurry from agricultural land or from sewage effluent particularly from abattoirs or when infection exists in the community (Anon., 1990). In addition, the incidence of cryptosporidiosis and giardiasis is dependent on the lifestyle, socio-economic level and general health of the community (Fayer and Ungar, 1986; Feachem *et al.*, 1983). The largest waterborne outbreak in US history occurred in Milwaukee during spring 1993. Considerable epidemiological and environmental evidence indicates that the outbreak was caused by a large amount of *Cryptosporidium* passing through one of the drinking water treatment facilities of the Milwaukee Water Works. This waterborne disease outbreak caused illness in more than 400,000 people and resulted in several deaths (Solo-Gabriele and Neumeister, 1996).

From all assembled evidence it appears that occasional waterborne transmission of Cryptosporidium is likely. However, due to numbers likely to be present in raw waters and to probable immunity in the population, incidence is likely to be low. It is clear that there can never be absolute certainty that viable oocysts will not penetrate water treatment and so pass into supply. The problem therefore becomes one of risk assessment and minimisation by stricter regulations and guidelines to protect public health (Haas et al., 1996). One of the requirements, therefore, is for good evidence on the occurrence of Cryptosporidium or Giardia in raw water sources. Using untreated surface water as a source of drinking water exposes a great number of the South African population to higher numbers of these pathogens and increases the risk of infection. For the accurate enumeration of oocysts in samples, microscopy is still the method of choice. Under certain conditions the confirmation of microscopic results might be important, especially if oocysts are detected in treated drinking water. PCR (polymerase chain-reaction) is one of the techniques that could be used for this purpose.

Aims and objectives as specified in contract

The objectives of the project were:

- To establish a PCR method for confirming the presence of Cryptosporidium and Giardia in environmental water samples.
- (2) To compare and contrast the PCR method of detection with immunofluorescence microscopy (CSIR; Umgeni Water) and flow cytometry (Rand Water) following concentration by cartridge filtration (CSIR), flocculation (Umgeni Water; Rand Water) or immuno-magnetic separation (Umgeni Water).
- (3) To investigate the occurrence of parasitic protozoa in a catchment area.
- (4) To establish to what extent diarrhoeal disease within a community is caused by Cryptosporidium and Giardia.
- (5) To establish the presence of viable Cryptosporidium oocysts and Giardia cysts in the surface water of a high-risk community and any correlation with the occurrence of diarrhoea.
- (6) To investigate the presence, viability and removal of parasitic protists during wastewater treatment processes and to evaluate the contribution of this source to their occurrence in the aquatic environment.
- (7) Once sources of contamination have been identified, the necessary health education and guidelines can be implemented according to the Risk Assessment to minimise the health risk to communities and to potable water supplies.
- (8) To train technicians from previously disadvantaged backgrounds in parasitological techniques.

Study design

One main objective of this study was to investigate the use of PCR based methods for the confirmation of results and to evaluate it's compatibility with the commonly used concentration, purification and detection methods. It was decided that the research would focus on Cryptosporidium parvum in the light of the difficulties experienced during detection. The findings of this study could thereafter be applied to the detection of Giardia if shown to be a viable option.

The study consisted of two parts, first, PCR reactions detecting all species of *Cryptosporidium* or only *Cryptosporidium* parvum had to be established in the lab. It was decided to use the primers described by Rochelle et al. (1997) because these primer sets can not only be used for the standard detection of oocysts but also for RT-PRC to test for viability. They were also compatible and could be used as part of a multiplex PCR for the simultaneous detection of *Cryptosporidium* and *Giardia*. The second part of the project was to evaluate whether this method was compatible to be used in conjunction with other methods used for the concentration and separation of *Cryptosporidium* oocysts in source and treated water samples.

The Pietermaritzburg catchment in KwaZulu-Natal was chosen for investigation of protozoon incidence in its rivers as the area is impacted by rural, peri-urban and urban settlements. Water sampling sites were chosen along the uMsunduze River and its tributaries as they make their way through the rural and peri-urban areas of Vulindlela and through the city of Pietermaritzburg. In addition, influent, effluent and sludge samples were collected from Darvill wastewater works and the viability status of (oo)cysts determined. As sludge samples are disposed of onto land, the effect of desiccation on the viability of (oo)cysts present in these samples was evaluated. The Regional Veterinary laboratories and local veterinarians were contacted to obtain samples of dung and details of any Cryptosporidium detected.

This study tried to determine whether the cause of diarrhoea, suffered by some patients in the Pietermaritzburg catchment and the Vulindlela area in KwaZulu-Natal, was indeed caused by Giardia and Cryptosporidium. Stools were examined for cysts and oocysts and the extent of diarrhoeal diseases, in the Vulindlela area, was established using information obtained from five clinics. An attempt was made to correlate the water source types used for domestic purposes in the Vulindlela area with the occurrence of diarrhoea in the community. The risk of Giardia and Cryptosporidium infections was quantified based on results obtained for different sampling sites on the uMsunduze River and its tributaries and Darvill sewage treatment works' final effluent. The Risk Assessment aimed to provide a quantitative estimate of the probability of illness associated with environmental exposures and focused on human health risk assessment from Giardia cysts and Cryptosporidium oocysts present in river water.

Brief summary of results and conclusions

- PCR-based methods have the potential to be used to confirm the presence of protozoan cysts and oocysts in water samples but owing to sensitivity problems it would be difficult to implement this technology on a routine basis in the near future.
- Occurrence in rivers was sporadic with Giardia cysts detected in 8% of samples and in higher numbers than Cryptosporidium oocysts, detected in 2% of samples; mainly during the wet summer months (although this may have been due to sewer breaks).
- As Cryptosporidium and Giardia were detected widely in river and wastewater samples, they are
 probably ubiquitous in the Pietermaritzburg area community and possibly livestock.
- Darvill Wastewater Works was found to remove up to 99.9% of Giardia cysts, after the activated sludge process, chlorination and maturation, in the final effluent.

- Some 70% of Darvill effluent samples were positive for the protozoa, however, containing up to 520 Giardia cysts 10 ℓ⁻¹ and 110 10 ℓ⁻¹ Cryptosporidium oocysts with up to 200 cysts 10 ℓ⁻¹ in the river downstream.
- The potential Darvill effluent loading could be some 2.5 Billion cysts and 0.5 Billion oocysts per day into the uMsunduze River (at a dry weather flow of some 50 Mℓ /day), which constitues half the dry weather (winter) flow of the river.
- Fresh sludge disposed onto land was found to contain up to 3000 oocysts ℓ⁻¹ and 700 000 cysts ℓ⁻¹ and needs to be monitored if used in sensitive locations; desiccation experiments proved that viability could be reduced significantly.
- Neither the activated sludge process, nor the anaerobic digestion of sludge, appeared to significantly affect the viability status of the Cryptosporidium or Giardia.
- Treated effluents need to be monitored prior to discharge into rivers, which could be a source of water for both humans and animals and possibly treated further to remove them.
- The potential risk of infection for Giardia and Cryptosporidium posed by the river water, downstream of sewage effluents would appear to be high, if used for drinking or recreation.
- The occurrence of Giardia cysts throughout the year in the effluent samples indicates that giardiasis is probably endemic in the Pietermaritzburg population, while cryptosporidiosis infections may be sporadic and quite possibly asymtomatic.
- Giardia cysts were present in (black) schoolchildren's stool samples analysed, with 9.5% prevalence from the Kranskop area and 5.5% from semi-rural Vulindlela; Cryptosporidium oocysts were not detected, however.
- Reported incidence of diarrhoea was high in the Vulindlela area, with 39% overall and 49% in children under 5.
- A total of 20 household and source water samples were analysed from families who reported diarrhoea, but all were negative for Giardia and Cryptosporidium
- The actual cause of diarrhoea could generally not be established from local clinic, hospital or laboratory records; very few stools tested were positive for Giardia but approximately 3% were Cryptosporidium (mostly AIDS related therefore probably not of significance for water use).
- It would appear that diseases caused by these pathogens are not prevalent in livestock in this
 area or in South Africa in general, despite international reports they are the most common
 cause of diarrhoea in calves, foals and lambs worldwide.

Extent to which objectives were reached and actions to be taken as a result of the findings

The above objectives were generally achieved as described above, except for a part of number 7. The sources of contamination were identified and some preliminary guidelines drawn up for the community, veterinarians and for safe-guarding potable water supplies, but the implementation of these is the responsibly of the relevant authorities.

Health education and guidelines (for Schools, Depts. Educations and Health, Environmental Health Officers, Veterinarians, DWAF, farmers etc)

- The potential risk of infection downstream of wastewater effluents, for human consumption and recreation (swimming), would appear to be high. Therefore the community, especially children must be made aware of this and steps taken to restrict access.
- The effluents themselves and maturation ponds etc are often used, as they appear clean, for portable water, swimming and fishing. Access control for these areas should be considered, along with warning signs and pictures in various languages.
- Livestock is also at risk from the above
- Health education at schools should include the above and that even chlorination will not remove these protozoa.
- Direct faecal-oral transmission amongst children especially at crèches would seem to be important, therefore personal hygiene should also be emphasised at school.
- Screening of high-risk school children would enable carriers of intestinal parasites to be identified, as these infections can seriously affect cognitive ability.
- Access by livestock to areas of fresh sewage sludge should also be restricted and the sludge should not be used to fertilise crops that are eaten raw.
- If used as soil conditioner etc the sludge should be dried first and monitored for the protists before being transported off-site.
- Veterinarians should be encouraged to send dung samples to Veterinary laboratories for identification purposes.

General guidelines (for Municipal Managers, Water Boards, DWAF, farmers etc)

Tertiary treatment should be considered at wastewater works or alternative disinfectants eg UV, which would inactivate (oo)cysts.

- Computerisation of hospital, clinic and laboratory records to aid prevalence studies.
- Catchment management to ensure recreational and abstraction points are not close downstream to wastewater works
- Catchment surveys to identify water treatment plant abstraction points at risk from broken sewers or septic tank effluents; these should be monitored regularly.

Recommendations for further research and technology transfer

- Intensive monitoring of specific areas is the only way to get an accurate picture of the presence
 of parasites, as these are likely to be sporadic, reflecting infection in the community and rainfall
 events.
- The data acquired from such monitoring is invaluable to indicate which sources of contamination need to be addressed as a priority as well as providing data about which types of future development are likely to be potentially the most dangerous.
- Risk assessment of each Water Treatment Plant is necessary to prioritise resources for monitoring and should comprise both process and catchment assessments.
- 4. The role of livestock (and possibly wild animals), as the source of the protozoan parasites in rivers has not been properly investigated, although indications are that this is relatively unimportant and surveys of feedlots and farms needs to be undertaken in this regard.

- Typing of the different strains is now possible and early data suggests that there are different types for animals and human sources.
- Investigation into resistance patterns in humans, to the parasitic protozoa could establish how widespread these infections are.

Technology transfer

The following workshops were attended/given to publicise the research work:

- A KZN Parasite Control Programme workshop, hosted by the Provincial Environmental Health department, was attended by Mr Bailey and Ms Jarmey-Swan, who presented an overview of the occurrence of Cryptosporidium and Giardia in faecal and water samples with particular reference to the Pietermaritzburg area.
- A KZN Regional Communicable Disease Control meeting was also attended at which a similar presentation was made to highlight the occurrence of these protists in KwaZulu-Natal.
- The Allerton Regional Veterinary laboratories and local veterinarians were visited and the
 project described to them and the significance of the protozoan parasites was explained.
 Training was given on laboratory procedures especially the immuno-fluorescence method
 for their detection.

Publications emanating from this project

C Jarmey-Swan, IW Bailey and AR Howgrave-Graham. 1997. Detection, occurrence and epidemiology of Cryptosporidium in KwaZulu-Natal, South Africa. In 1997 International Symposium on Waterborne Cryptosporidium Proc., Editors: CR Fricker, JL Clancy and PA Rochelle, 159-170.

C Jarmey-Swan, RA Gibbs, GE Ho, IW Bailey, and AR Howgrave-Graham, 2000. A novel method for detection of viable Giardia cysts in water samples. Wat. Res. 34 (6), 1948-1951.

C Jarmey-Swan, IW Bailey and AR Howgrave-Graham. 2000. Ubiquity of the waterborne pathogens Cryptosporidium and Giardia in KwaZulu-Natal populations. Water SA January (1) 2000.

C Jarmey-Swan, IW Bailey and C Johnson 2000. Occurrence and source of Cryptosporidium and Giardia in catchment areas and wastewater works in KwaZulu-Natal. In The Water Institute of Southern Africa (WISA) biennial conference and exhibition: Health Impacts Proc.

Archiving

The raw data for the relevant sections is archived as follows:

Umgeni Water: Occurrence of parasitic protozoa in a catchment area.

CSIR: Occurrence of Cryptosporidium and Giardia in a community

University of Pretoria: Development of a PCR technique for Cryptosporidium and Giardia

Capacity building

Six previously disadvantaged individuals were employed during the duration of this project namely Ms Lungile Mthembu, Ms Zola Msiska, Ms Vashnee Chinnah, Ms Nosipho Gulwa, Ms Shantel Pecku Ms Caron Johnson and Ms Tracy Schmidt They were trained in concentration, detection and viability staining of protists in water, wastewater and sludge samples. Ms Johnson was trained in techniques for identifying *Cryptosporidium* and *Giardia* in stool samples under the auspices of the Medical Research Council, Durban. Ms Tracy Schmidt was trained in *Cryptosporidium* and *Giardia* concentration and detection techniques

Ms Mthembu has subsequently been employed as a permanent technician with the Microbiology and Public Health section of Analytical Services, Umgeni Water. Ms Msiska is employed as a Plant Pathologist with the Agricultural Research Council while Ms Chinnah declined a position as Senior Technologist with the Medical Research Council. Ms Gulwa is now employed as a microbiologist with Amatola Water in the Eastern Cape. Ms Johnson obtained a post with the Dept Health in Malaria Research, while Ms Schmidt was subsequently employed by the Allerton State Veterinary Laboratories.

Ms Zola Msiska obtained a BSc Hons Ms Shantel Pecku obtained a BSc Hons Ms Claire Jarmey-Swan obtained an MSc

User groups

Water Boards, local and national Departments of Health, Environmental Health Officers and other medical personnel interested in epidemiology within communities, Department of Water Affairs and Forestry, Municipal Managers, farmers and veterinarians.

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ABBREVIATIONS

AIDS Acquired Immunodeficiency Syndrome

Ca2+ Calcium ions CaCl₂ Calcium chloride

CDC Center for Disease Control

DNA Deoxyribonucleic acid

DOH Department of Health

EHO Environmental Health officer

EPA Environmental Protection Agency

FDA Fluorescein diacetate

FITC Fluorescein isothiocyanate

HCI Hydrochloric acid hsp Heat shock protein

IMS Immunomagnetic separation

MgCl₂ Magnesium ions MgCl₂ Magnesium chloride

mRNA Messenger ribonucleic acid

NaCl Sodium chloride NaHCO₃ Sodium bicarbonate NaOH Sodium hydroxide

PBS Phosphate buffered saline PCR Polymerase chain reaction

rRNA Ribosomal ribonucleic acid RT-PCR Reverse transcriptase PCR

TMR Tetramethyl rhodamine

US-EPA United States Environmental Protection Agency

USA United States of America

UW Umgeni Water

WRC Water Research Commission

1 INTRODUCTION

Although there are many causes of diarrhoea, the enteric protozoans *Cryptosporidium* parvum and *Giardia lamblia* have been recognised as important causes of both outbreak-related and sporadic diarrhoea in humans (Casemore, 1990). These parasites are transmitted by ingestion of (oo)cysts excreted in the faeces of infected humans or animals. The infection can therefore be transmitted from person to person, through ingestion of contaminated water (drinking water and water used for recreational purposes) or food, from animal to person, or by contact with faecal contaminated environmental surfaces (Juranek, 1995).

Contamination of a water source may arise as a result of run-off, accidental spillage of farm slurry from agricultural land or from sewage effluent particularly from abattoirs or when infection exists in the community (Anon., 1990). In addition, the incidence of cryptosporidiosis and giardiasis is dependent on the lifestyle, socio-economic level and general health of the community (Fayer and Ungar, 1986; Feachem et al., 1983).

Six well-documented outbreaks of Cryptosporidiosis attributed to drinking water have been recognised in the United States (Herwaldt et al., 1991; Herwaldt et al., 1993). The source of drinking water used by utilities in these outbreaks included surface water (lakes, rivers, streams), well water, and spring water. Several outbreaks have also been associated with swimming pools and amusement park wave pools or water slides (Sorvillo et al., 1992; Bell et al., 1993). The largest waterborne outbreak in US history occurred in Milwaukee during spring 1993. Considerable epidemiological and environmental evidence indicates that the outbreak was caused by a large amount of *Cryptosporidium* passing through one of the drinking water treatment facilities of the Milwaukee Water Works. This waterborne disease outbreak caused the illness in more than 400,000 people and resulted in several deaths (Solo-Gabriele and Neumeister, 1996).

In all outbreaks it has been impossible to establish a casual relationship absolutely, largely because the investigations have proceeded from the detection of increased incidence of cryptosporidiosis in a community, followed by identification of water supply as a common link in the occurrence of primary cases. In some cases occysts were detected in source and/ or treated water but because of the time delay between the onset of illness and suspecting water supplies, it is likely that the peak values were not recorded (Carrington and Miller, 1993).

From all assembled evidence it appears that occasional waterborne transmission of Cryptosporidium is likely. However, due to numbers likely to be present in raw waters, and to probable immunity in the population, incidence is likely to be low. It is clear that there can never be absolute certainty that viable oocysts will not penetrate water treatment and so pass into supply. The problem therefore becomes one of risk assessment and minimisation by stricter regulations and guidelines to protect public health (Haas et al., 1996). One of the requirements, therefore, is for good evidence on the occurrence of Cryptosporidium or Giardia in raw water sources. Both Cryptosporidium and Giardia are currently considered of major importance for safety of water used for drinking. The reasons are their high infectivity and their resistance to chemical disinfection. This has been demonstrated by waterborne outbreaks caused by Cryptosporidium where concentrations were at levels below the detection limit of the detection method (Goldstein et al., 1996). Prevalence rates in the developed world have ranged from 0.6% to 20.0%, while prevalence rates in developing countries have ranged from three percent to 30.0% (Casemore, 1990; Ungar, 1990). Although surveys have noted the presence of Giardia cysts and Cryptosporidium oocysts in surface and drinking water the extent of diarrhoea caused by these parasites is not known (du Preez and Gericke., 1999). Using untreated surface water as a source of drinking water exposes a great number of the South African population to higher numbers of these pathogens and increases the risk of infection.

The number of Cryptosporidium oocysts needed to initiate a gastro-intestinal infection in healthy human volunteers have proved to be extremely low (Dupont et al., 1995). This necessitates the examination of large volumes of water when monitoring the quality of drinking and environmental water samples. The methods for the detection of Cryptosporidium and Giardia in water can mainly be divided into three stages: concentration, purification and detection of (oo)cysts.

In South Africa, commonly used procedures for concentrating protists from water samples include the filtration of large volumes of water (20 − 1000 ℓ) through a cartridge filter or Calcium carbonate flocculation (Vesey et al., 1993) of smaller volumes (10 − 20ℓ). Recoveries of oocysts from seeded tap water samples using the former technique ranged from 0.4 to 129.0% (Gilmour et al., 1991; Whitmore and Carrington, 1993; LeChevallier et al., 1995; Nieminski et al., 1995) while cyst recoveries from seeded tap water samples ranged from 9.8 - 68% (Gilmour et al., 1991). Recovery of oocysts and cysts from seeded turbid water samples ranged from 3.9 − 14.2% and 9.8 − 20.4% respectively (Shepherd and Wyn-Jones 1996).

Calcium carbonate flocculation is less labour intensive and has been shown to have consistently high recoveries (71.3 - 76.0%) in deionised, tap and river water (Vesey et al., 1993; Shepherd and Wyn-Jones, 1996). A number of *Cryptosporidium* and *Giardia* concentration procedures include a density gradient to separate oocysts or cysts from debris in the concentrated sample. Researchers have found low recoveries of oocysts when sucrose flotation techniques were used with compared to higher recoveries without the use of the density gradient (Shepherd and Wyn-Jones, 1995). Although sucrose flotation removes interfering debris, which may make microscopy easier, the discrepancy in recoveries highlights the need to evaluate the use of this technique with various concentration procedures.

The standard method for the purification of (oo)cysts from the rest of the concentrate is floatation on sucrose or percol, but the recovery is usually poor (seeded tap water: 0.7 - 16.0% and river water samples: 0.4 - 11.2%). Presently, two of the South African groups involved in the routine monitoring of *Cryptosporidium* and *Giardia* utilise flow cytometry to separate oocysts from normal background debris. The sorted material is collected and

viewed on a microscope slide or membrane. Immuno-magnetic separation is another technique that has shown great promise for the separation of the oocysts. Immuno-magnetic separation allowed high recoveries for *Cryptosporidium*: 49 to 67% from raw water and 87 to 97.4% from turbid waters (Stanfield et al., 1999). As the detection limit is 1 (oo) cyst in the sampled volume, IMS is today becoming the method of choice for purifying concentrates in routine analysis.

For the accurate enumeration of oocysts in samples, microscopy is still the method of choice. Under certain conditions the confirmation of microscopic results might be important, especially if oocysts are detected in treated drinking water. PCR is one of the techniques that could be used for this purpose.

1.1 Project objectives

The objectives of the project as stated in the original proposal were:

- To establish a PCR method for confirming the presence of Cryptosporidium and Giardia in environmental water samples.
- To compare and contrast the PCR method of detection with immunofluorescence microscopy (CSIR; Umgeni Water) and flow cytometry (Rand Water) following concentration by cartridge filtration (CSIR), flocculation (Umgeni Water; Rand Water) or antibody-magnetite (Umgeni Water).
- To investigate the occurrence of parasitic protozoa in a catchment area.
- To establish to what extent diarrhoeal disease within a community is caused by Cryptosporidium and Giardia.
- To establish the presence of viable Cryptosporidium oocysts and Giardia cysts in the surface water of a high-risk community and any correlation with the occurrence of diarrhoea.
- To investigate the presence, viability and removal of parasitic protists during wastewater treatment processes and to evaluate the contribution of this source to their occurrence in the aquatic environment.
- Once sources of contamination have been identified, the necessary health education and guidelines can be implemented according to the risk assessment to minimise the health risk to communities and to potable water supplies.
- To train technicians from previously disadvantaged backgrounds in parasitological techniques.

2 LITERATURE REVIEW

2.1 Detection and confirmation of Cryptosporidium parvum and Giardia duodenalis

The number of protozoan parasites cysts and oocysts needed to initiate a gastrointestinal infection in healthy human volunteers has proved to be extremely low (Dupont et al., 1995; Faubert, 1996). This necessitates the examination of large volumes of water when monitoring the quality of drinking and environmental water samples. The main focus at present is on the detection of *Giardia* and *Cryptosporidium*. The detection methods can mainly be divided into three stages: concentration, purification and detection of cysts and oocysts.

In South Africa, water samples have been concentrated using either membrane filters, various cartridge filters or by means of calcium carbonate flocculation. Yarn wound filters were originally used to concentrate large volumes of water ($100 - 1000 \ell$), but low recovery efficiencies have been reported. The recovery efficiencies for the flocculation method were reported to be significantly higher but is only suitable for volumes of less than 20ℓ of water. The new generation of pleated or foam filters have much higher recoveries, but are relatively expensive.

Methods for the purification of cysts and oocysts from the rest of the concentrate were by means of sucrose, percol or salt floatation, but the recovery was usually poor. Flow cytometry can be used to separate cysts and oocysts from normal background debris based on their size, fluorescence and light scatter after staining with fluorescent antibodies, but is usually prohibitively expensive. The sorted material can than be collected and viewed on a microscope slide for confirmation. Immuno-magnetic separation (IMS) is another technique gaining popularity that has shown great promise for the separation of the cysts and oocysts. Recoveries are usually high but they could be affected by the turbidity of the samples (Gasser and O'Donoghue, 1999); but this is also very expensive.

Cryptosporidium oocysts and Giardia cysts are finally detected in samples by means of epifluorescent microscopy after staining with monoclonal antibodies. Owing to their size, Giardia cysts are easier to detect and identify, whilst most problems are experienced with the detection of Cryptosporidium. The commercially available antibodies react with all members of the genus and cannot be used to detect the human pathogen Cryptosporidium parvum alone. Due to their specific size, it is also difficult to differentiate the oocysts from auto-fluorescing algae that are commonly found in these samples. Under certain conditions the confirmation of microscopic results may be of great importance, especially in the case where oocysts are detected in treated drinking water. Reports indicate that polymerase chain reaction (PCR) based methods hold potential to be used for this purpose (Toze, 1999).

2.2 PCR detection of Giardia

A few PCR methods have been described for the detection of Giardia. Mahbubani et al. (1991; 1992) described two approaches, both targeted at a 171 bp region of the giardin

coding gene. Using normal PCR combined with a gene probe they could discriminate between G. duodenalis and other Giardia spp., which are not human pathogens. Using RT-PCR they could also distinguish live from dead cysts. Abbaszadegan et al. (1997) used a similar approach by amplification of heat-shock induced mRNA.

2.3 PCR detection of Cryptosporidium

In the light of the many problems associated with the correct identification and speciation of Cryptosporidium, a number of PCR-based detection methods have been described. The methods are for the detection of both Cryptosporidium spp. and Cryptosporidium parvum and targeting a variety of genes. Different lysis and DNA extraction protocols were used and the reported sensitivity for some of the methods ranged from 1 –100 oocysts (Rochelle et al., 1997a)

Laxter et al. (1991) targeted a 400-bp region of the *Cryptosporidium* genomic DNA as an amplification target. Webster et al. (1993) also selected a 329-bp fragment from an undefined region of the DNA sequence. The specific regions were selected because they contained a unique restriction endonuclease site (Sty 1), which could serve as a useful marker.

In another study (Awak-El-Kariem et al., 1994), the polymerase chain reaction was used to amplify a 556-bp nucleotide product, by employing primers based on the published sequence of the 18S rRNA genes in *Cryptosporidium parvum* and C. muris. The product contained three Mae 1 endonuclease restriction sites, one of which was only present in C. parvum. In the above-mentioned PCR methods, restriction enzyme digestion was used to confirm the PCR product sequence and to distinguish between C.parvum and C.muris. This approach obviates the need to use lengthy and complicated hybridisation protocols to verify the origin of the amplified products.

Johnson et al. (1995) developed a reliable PCR method for the detection of Cryptosporidium occysts in environmental samples. The area targeted for amplification was a portion of the sequence encoding the small 18S rRNA sub-unit and a 435-bp product was produced. The assay routinely detected 1 to 10 occysts in a purified occyst preparation, but a 1 000-fold lower sensitivity was reported for seeded environmental water samples.

Mayer and Palmer (1996) developed a nested PCR to detect *Cryptosporidium* oocysts in water samples. The assay was targeted at a gene coding for an oocyst cell wall protein and a 753-bp product was amplified during the first round PCR. Thereafter, the nested primers amplified a 283-bp product within the first amplicon. It was reported that the nested PCR could detect as few as 10² oocysts per litre.

One of the major disadvantages of normal PCR is the fact that intact DNA will be detected even if the cells present are dead. To overcome this problem Stinear et al. (1996) used Reverse Transcriptase PCR (RT-PCR). Viable oocysts were detected by RT-PCR targeting the mRNA of a heat-shock protein (hsp) gene. Before mRNA extraction, oocysts were exposed to 45 °C for 20' to maximize heat-shock protein mRNA production.

The one question that still remained was whether the viable oocysts detected were also capable of causing infections. An assay combining cell culture with RT-PCR described by Rochelle et al (1997b) was developed to provide such information. Oocysts were inoculated onto monolayers of Caco-2 cells and grown on a microscopic slide, thereafter RT-PCR targeting the hsp 70 gene mRNA was performed. It was reported that a single infectious oocyst could be successfully detected using this technique.

PCR has provided the basis for the development of a number of new approaches for the detection of *Cryptosporidium* in clinical and environmental samples. While this method holds great promise, inhibition and compatibility with concentration and purification methods used has yet to be fully resolved (Gasser and O'Donoghue, 1999).

2.4 Sources of Cryptosporidium and Giardia contamination of water

Sources of contamination of watercourses by *Cryptosporidium* and *Giardia* have included run-off from farmlands and informal settlements, treated wastewaters and broken or blocked sewers (Solo-Gabriele and Neumeister, 1996). Electrophoretic characterisation of *Giardia* isolates from humans, cattle, sheep and dogs have shown similarity (Stranden et al., 1990) confirming that vertebrate species do not seem to possess their own variant (Meloni et al., 1988). This data suggests that domestic animals in certain geographic areas, may serve as a reservoir of human *Giardia* infections and that cross-transmission between humans and animals is likely to occur. All species are transmitted by means of cysts passed out in faeces and G. canis and G. lamblia are known to be pathogenic, the latter inhabiting the small intestine of man, monkeys and pigs worldwide; beavers may also serve as a reservoir for human infection (Kreier and Baker, 1987).

Recently the Center for Disease Control (CDC), USA, identified two new *Cryptosporidium* genotypes, which are able to infect humans (Pieniazek et al., 1999). This may suggest a unique susceptibility to infections of animal or livestock origins as these genotypes were previously known to only infect cats or dogs respectively. Faecal specimens from patients following a waterborne outbreak of cryptosporidiosis in the United Kingdom were identified as *Cryptosporidium parvum* genotype 2, the zoonotic strain (Hunter, 1999).

The incidence of cryptosporidiosis and giardiasis is dependent on the lifestyle, socioeconomic level and general health of the community (Feachem et al., 1983; Fayer and
Ungar, 1986). Madore et al. (1987) found that the two parasites often co-exist in polluted
waters and the frequency of oocysts/cyst detection has been shown to be directly related to
the degree of human activity in the watershed. As urban and rural settlements along South
Africa's rivers and dams have increased in number and these settlements are often informal
possessing no infrastructure, they are at risk to both human and animal faecal
contamination of water sources. The outbreak in the USA in 1984 involved water supply
from a well in Texas where the treatment was chlorination and evidence pointed to sewage
contamination (Carrington and Miller, 1993).

An outbreak in the United Kingdom occurred in Ayrshire in Scotland in 1988 was almost certainly due to post-treatment contamination resulting from the ingress of drainage water

from an area frequented by cattle. Another outbreak occurred in 1989 in Swindon and parts of Oxfordshire in England, where the source of the oocysts was not positively identified, but investigators from the water supply company believed there was evidence of severe diarrhoea in animals at a farm located close to a tributary of the River Thames from which water was abstracted for the plant via a pumped storage reservoir (Carrington and Miller, 1993).

The largest waterborne outbreak in US history occurred in Milwaukee during spring 1993. Considerable epidemiological and environmental evidence indicates that the outbreak was caused by a large amount of *Cryptosporidium* passing through one of the drinking water treatment facilities of the Milwaukee Water Works. This waterborne disease outbreak caused the illness in more than 400,000 people and resulted in several deaths (Solo-Gabriele and Neumeister, 1996).

Giardia is the most common identifiable etiological agent of all waterborne outbreaks. It has been estimated that 60% of all Giardia infections are acquired through contaminated water (Bennet et al., 1987). Giardia cysts may be found in water as a result of the deposition of faecal material from both man and animals. Surveys of Giardia cyst levels in various waters indicate that 26% - 43% of surface waters were contaminated with Giardia cysts ranging in concentrations from 0.3 to 100 cysts per 100ℓ (Hibler, 1988).

Five outbreaks of giardiasis, which affected an estimated 385 people, were associated with community water systems, three with surface water and two with well water. High concentrations of *Giardia* cysts were also found in tap water. Of 4244 stool specimens from people in the test group, 110 were positive for *Giardia* and 42 others for Entamoeba histolytica (MacKenzie et al., 1994).

In South Africa, Harding and Genthe (1998) reported that approximately 52.9% (9/17) of raw waters in the Western Cape were positive for *Giardia* ranging from 0-1.8 cysts ℓ^{-1} (average 1.04 cysts ℓ^{-1}). Approximately 11.8% (2/17) of the same raw waters sampled were positive for *Cryptosporidium* and were found to have up to 1.2 oocysts ℓ^{-1} (average 0.8 oocysts ℓ^{-1}). One river in the Western Cape, which was sampled four times, was reported by the authors to have 0.25 - 1.6 cysts ℓ^{-1} while oocysts were only detected once (0.15 oocysts). Nine recreational sites were sampled over a three-month period and cyst numbers ranged from 0-1.9 ℓ^{-1} while oocysts were not detected.

Du Preez and Gericke (1998) sampled three groups of water: 1) raw and unprotected; 2) protected but receiving no treatment; 3) water receiving treatment. Seventeen percent (22/131) of samples analysed for the presence of *Giardia* cysts were positive with cysts ranging from $0 - 20 \ell^{-1}$. Cryptosporidium oocysts were detected at seven of the twelve sampling sites, which included two protected boreholes, four raw unprotected waters and one treated water. Cryptosporidium counts ranged between zero and $10.2 \ell^{-1}$. Eight percent (11/132) of water samples were found to be positive for oocysts.

A higher number of samples from rural areas (19%) were positive for *Giardia* than those from semi-urban areas (14%). Fewer samples (6%) from rural areas were positive for *Cryptosporidium* oocysts than semi-urban areas where 10% of samples contained *Cryptosporidium*. Increases in occurrence was seen in summer months although an analysis of variance for seasonal differences was not found to be significant (p=0.9) for the occurrence of *Giardia* cysts while *Cryptosporidium* was found to be statistically significant (p=0.01)(Du Preez and Gericke, 1998).

Gericke et al (1996) evaluated one hundred and fourteen raw water samples over a twelve-month period. Giardia cysts were detected in 75 and Cryptosporidium oocysts in 59 of the samples. The average number of oocysts and cysts detected did not exceed 2 (oo) cysts ℓ^{-1} . To evaluate seasonal variation of parasite levels in the raw water, results were sorted according to season. Six of the eight plants showed higher average counts for Giardia during summer while Cryptosporidium oocysts showed higher counts during summer for 5 of the 8 plants monitored. Similar results were recorded by Rose (1995) who found that the average concentration of oocysts and cysts were higher during summer and autumn. Gericke et al. (1996) also found that Giardia cysts were detected in a higher number of samples than were Cryptosporidium oocysts as did Harding and Genthe (1998), Du Preez and Gericke (1998) and Kfir et al. (1995). Kfir et al. (1995) analysed 650 surface water samples, which contained up to 46 oocysts ℓ^{-1} (average 3 oocysts ℓ^{-1}) and 25 cysts ℓ^{-1} (average 0.6 cysts ℓ^{-1}).

Midmar Dam, a source of potable water for the Pietermaritzburg area, generally contained low concentrations of oocysts and cysts and often no protists were detected. It is possible that any protists present could sediment out in the dam if attached to solid particles (Anon. 1990). The highest recorded numbers occurred after rainfall perhaps due to run-off from the surrounding agricultural land or adjacent wastewater works. (Jarmey-Swan and Bailey, 1996).

Although researchers are able to recover small numbers of oocysts from treated drinking water, current laboratory methods do not enable them to determine if these oocysts are viable or infectious. Moreover it is not known if the number of oocysts present in drinking water constitutes a sufficient dose to cause illness in humans, whether immuno-suppressed persons are more susceptible to lower doses of oocysts than are immuno-competent persons, or if there are strains of C. parvum that vary in infectious dose and virulence (Du Pont et al., 1995).

A summary of the most common sources of faecal contamination of waters which have resulted in the detection of *Cryptosporidium* and *Giardia* infections is presented in Table 2.1.

Table 2.1 Suspected sources of contamination of water sources

Raw water source	Source of contamination				
Well	Raw sewage Septic tank effluent into nearby creek Infiltration of runoff from cattle or sheep grazing areas Treated wastewater				
Lake / Dam	Cattle wastes, slaughter-house wastes, sewage carried by tributary rivers Backflow of sewage or septic tank effluent into distribution, raw water inlet lines, or both Treated wastewater, sewage from boats				
River	Raw sewage and run-off from cattle grazing areas Surface water, treated wastewater, run-off from agricultural areas				
Surface water Surface run-off from livestock grazing areas					

2.5 Occurrence of Cryptosporidium and Giardia in wastewater works

Whitmore and Robertson (1995) found the ability of primary sewage sedimentation to remove *Cryptosporidium* oocysts poor. Aerobic digestion and sludge pasteurisation at 55°C was found to be an effective treatment to inactivate oocysts and only 10% of the oocyst population was viable. A laboratory simulation of the same process demonstrated an 80-84% reduction in oocyst numbers although those remaining in the effluent were capable of causing infection in mice (Villacorta-Martinez de Maturana et al., 1992).

Grimason et al. (1993) determined the occurrence and removal of *Cryptosporidium* spp. and *Giardia* spp. in municipal wastewater by waste stabilisation ponds in Kenya. No oocysts were detected in the final effluent of all pond systems (11/11) while no cysts were detected in the final effluent of 10 of the 11 waste stabilisation pond systems. One pond discharged 40-50 cysts ℓ^{-1} in the final effluent.

Almost 50% of raw sewage samples studied by Kfir et al. (1995) contained *Giardia* cysts and 30% contained both *Giardia* and *Cryptosporidium* oocysts. The relatively high incidence of cysts in the raw sewage may indicate a high prevalence of giardiasis in the South African population with the possibility of a high carrier rate. Thirty percent of treated effluents were positive for *Giardia* (Average 12 ℓ⁻¹) and 25% had both cysts and oocysts)(Kfir et al., 1995).

Pre-thickener sludge from Darvill wastewater works contained up to 70 x 10^4 oocysts ℓ^1 and 280 x 10^4 cysts ℓ^1 from monthly samples taken whilst the post-thickener sludge from Darvill contained 0 - 25 x 10^4 oocysts ℓ^1 and 0 - 140 x 10^4 cysts ℓ^1 . The highest number of cysts recorded followed the 1995 December floods in Pietermaritzburg, although oocysts

were not detected on this occasion. Darvill treated effluent, which enters the uMsunduze River, contained up to 150 occysts ℓ^{-1} and 40 cysts ℓ^{-1} (Jarmey-Swan and Bailey, 1996).

2.6 Significance of Cryptosporidium and Giardia in human populations

It is difficult to determine incidence rates or prevalence relating to *Cryptosporidium* or *Giardia* as population surveys are often based on diagnostic specimens received by laboratories with which age and gender data are often not recorded (Casemore et al., 1997). In addition, few surveys are adequately controlled and the resulting data may be biased towards children, as medical intervention is more likely to result from gastrointestinal symptoms appearing in younger patients (Casemore, 1990).

Prevalence rates in the developed world have ranged from 0.6% to 20.0%, while prevalence rates in developing countries have ranged from three percent to 30.0% (Casemore, 1990; Ungar, 1990). Few studies have been undertaken in South Africa to determine the incidence or prevalence of cryptosporidiosis or giardiasis in the population. Published reports have, however, highlighted the risk to young children (Schutte et al., 1981; Berkowitz et al., 1988; Walters et al., 1988; Moodley et al., 1991a). Seasonal and temporal trends of human infections have been observed in a number of investigations. Those trends often coincide with periods of maximum rainfall, which may reflect seasonal agricultural practices and environmental and zoonotic transmission (Moodley et al., 1991a).

Pegram et al. (1998) noted that more than 12 million people were without adequate levels of water supply and sanitation. They also estimated that some R3.4 billion might be directly attributed to diarrhoeal diseases in South Africa. Microbial contamination of water can result in increased morbidity and mortality rates. This increases medical treatment costs and decreases productivity. Severe flooding like that experienced during the last summer of 2000 throughout the South African subcontinent can increase the potential health risk associated with waterborne diseases like giardiasis and cryptosporidiosis. Both sewage treatment and water supply systems can be severely compromised, forcing people to use contaminated waters for domestic purposes. Although surveys have noted the presence of Giardia cysts and Cryptosporidium oocysts in surface and drinking water the extent of diarrhoea caused by these parasites is not known (du Preez and Gericke., 1999)

In KwaZulu-Natal, Cryptosporidium and Giardia were found to be endemic in KwaZulu-Natal, with laboratory-confirmed incidences ranging from 2.9 - 3.7% and 2.9 - 3.0%, of cases submitted for analysis, respectively However, the incidence of Cryptosporidium and Giardia did not appear to correlate (Pearson's correlation test) with climatic factors such as rainfall, season or year, possibly indicating that water-borne transmission is not the predominant route and other factors such as personal hygiene, potable water supply, sanitation and education probably have a more significant impact (Jarmey-Swan et al., 2000).

3 DEVELOPMENT OF A PCR TECHNIQUE FOR CRYPTOSPORIDIUM AND GIARDIA

All the methods used routinely for the detection of *Cryptosporidium* oocysts and *Giardia* cysts employ epi-fluorescent microscopy after staining the sample with monoclonal antibodies. Due to their size, *Giardia* cysts are easier to detect and identify. The detection of *Cryptosporidium* is more problematic. The commercially available antibodies react with all members of the genus and cannot be used to detect the human pathogen *Cryptosporidium* parvum alone. It is also difficult to differentiate the oocysts from auto-fluorescing algae, which are commonly found in these samples due to the similarity in size. A need therefore exists to confirm the presence of *Cryptosporidium* spp. or *Cryptosporidium* parvum in particular, once detected. Based on the reports sited in the literature survey it was decided that PCR based methods hold potential to be used for the confirmation of microscopic results.

The main objective of this part of the study was to investigate the use of PCR based methods for the confirmation of results and to evaluate it compatibility with the commonly used concentration, purification and detection methods. It was decided that the research would focus on *Cryptosporidium parvum* in the light of the difficulties experienced during detection. The findings of this study could thereafter be applied to the detection of *Giardia* if it is shown to be a viable option.

The study consisted of two parts, first, PCR reactions detecting all species of Cryptosporidium or only Cryptosporidium parvum had to be established in the lab. It was decided to use the primers described by Rochelle et al. (1997a) because these primer sets can not only be used for the standard detection of oocysts but also for RT-PRC to test for viability. They were also compatible and could be used as part of a multiplex PCR for the simultaneous detection of Cryptosporidium and Giardia. The second part of the project was to evaluate whether this method was compatible to be used in conjunction with other methods used for the concentration and separation of Cryptosporidium oocysts in source and treated water samples.

3.1 Methodology

3.1.1 Protozoon cultures

A highly purified preparation of *Cryptosporidium parvum* oocysts (Iowa strain, bovine genotype) was obtained from the Department of Veterinary Science and Microbiology, University of Arizona. The oocysts were supplied at a concentration of 2 × 10⁸ oocysts me⁻¹, and stored at 4 °C. These oocysts were used to produce the seeded samples used during the experiments. Human faecal samples containing *Cryptosporidium* were obtained from the Department of Medical Virology, University of Pretoria.

3.1.2 DNA Extraction

Bacterial Mini-prep extraction

Total oocyst DNA was extracted from the concentrated oocyst samples (3 $\mu\ell$) by resuspending it in lysis buffer (50 mM Tris-HCI [tris-(hydroxymethyl)amino methane) - 20 mM EDTA (ethylenediaminetetra-acetic acid), pH 8.0], containing 2 mg of proteinase K (Boehringer Mannheim) per $m\ell$ and 0.5 % Sarkosyl. After the addition of the reagents the samples were incubated at 37 °C for 1 h. Thereafter 5 M NaCl was added to give a final concentration of 1 M, and CTAB (Merck) was added to a concentration of 1 %. Following incubation at 65 °C for 10 min the oocysts were exposed to one freeze-thaw cycle consisting of an freezing for 1 min. at -70 °C and heating for 1 min. at 96 °C thereafter. After the freeze-thaw cycle DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and precipitated by the addition of 0.6 volumes of isopropanol. The DNA pellets were then washed with 70 % ethanol and after desiccation resuspended in 100 $\mu\ell$ of low-salt TE buffer (1 mM Tris; 0.1 mM EDTA, pH 7.6) and stored at -20 °C, ready for DNA amplification.

QIAamp Purification Kit

A commercial DNA purification kit available from Qiagen was also evaluated for DNA extraction. Oocysts were lysed by incubating them for 3 hours at 55 °C in the presence of Proteinase K. After the release of the DNA, the kit is used for purification. The basic principle of the kit is that DNA in the sample is absorbed on the QIAamp silica membrane during a brief centrifugation step. Salt and pH conditions of the buffers used, ensure that the proteins and other contaminants, which may inhibit PCR and other enzymatic reactions, are not retained on the membrane. After all the contaminants have been removed by a washing procedure, the DNA is eluted by the addition of the supplied elution buffer, and stored at – 20 °C.

3.1.3 Oligonucleotide Primers

The amplification reaction included forward and reverse oligonucleotide primers, which correspond with unique sequences within the *Cryptosporidium* heat-shock Protein 70 (hsp 70) gene. Two sets of PCR primers were synthesised by MWG-Biotech (Germany) based on sequences published by Rochelle et al. (1997b)(Figure 2). The CPHSP 1 set consists of two 21bp primers and has a broad specificity to recognise all species of *Cryptosporidium*:

cphsp 2386F (5'-CTG TTG CTT ATG GTG CTG CTG-3') and cphsp 2672R (5'-CCT CTT GGT GCT GGT GGA ATA-3').

The other primer set, CPHSP2 was designed to be specific for Cryptosporidium parvum:

cphsp 2423F (5'-AAA TGG TGA GCA ATC CTC TGC CG-3') and cphsp 2746R (5'-CTT GCT GCT CTT ACC AGT AC-3').

2304 acacgtatic caaaggitca ggccttgati caggaatict titaacggtaa agagccatgc
2364 aaagcaatca atccagacga ag ctgttgct tatggtgctg ctg tacaagc tgctatctt a
2424 aatggtgagc aatcctctgc cg tacaggat ctcttattat tggatgttgc tccattatca
2484 ctcggtttag aaactgctgg tggtgttatg accaagctta ttgaacgtaa tacaactatc
2544 ccagcaaaga agacacaagt cttcactact tatgctgata accagagtgg tgtcttgatc
2604 caagtttatg agggtgagag agccatgact aaggataacc atctcctcgg aaagttccat
2664 cttgatgg ta ttccaccagc accaagagg t gtaccacaaa ttgaagtcac ctttgatatt
2724 gatgctaatg gtatcttgaa tgtgtctgct gttgataaga gtactggtaa gagcagcaag
2784 atcactatta ctaacgataa gggtagatta tcaaaggacg atattgaacg tatggttaat

Figure 3.1 Partial sequence (base pair 2304 - 2834) of the Cryptosporidium parvum Heat Shock Protein 70 (Genbank Accession number U69698), indicating primer locations. (Primer set CPHSP 1 is underlined and primer set CPHSP 2 is indicated using italics)

Amplification of DNA by using the above primers, cphsp2386 with cphsp2672 and cphsp2423 with cphsp2746, resulted in the production of a 307 and 361 bp product respectively. Through empirical evaluation of the primers, the optimum annealing temperatures were determined to be 53 and 56 °C and the MgCl₂ concentrations to be 2.5 and 1.5 mM for CPHSP 1 and CPHSP 2 respectively.

3.1.4 DNA amplification

The PCR reaction mixture contained the following components: 2.5 U Taq polymerase (Promega), 1 X Reaction Buffer (50 mM Tris-HCl pH 8.0, 100mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 50 % glycerol and 1 % Triton X-100), 0.25 μ M of each of the forward and reverse primers, 200 μ M of each of the dNTPs, and MgCl₂ at a concentration of 2.5 and 1.5 mM for CPHSP 1 and CPHSP 2, respectively. Subsequently 10 μ l of the DNA template was added to the reaction mixture and the volume made-up with double-distilled water to a final volume of 100 μ l. Sterile distilled water was used as a negative control in the reactions.

PCR reactions were performed in a GeneAmp 2400 DNA thermal cycler (Perkin Elmer), with the following temperature cycle: 94 °C for 2 min for the initial denaturation, followed by 30 cycles. For the CPHSP1 set the cycle was: 30' at 94 °C (denaturation), 30' at 53 °C (annealing) and 30' at 72 °C (extension).

For the CHSP 2 set of primers the cycle was: 30' at 94 °C (denaturation), 30' at 56 °C (annealing) and 30' at 72 °C (extension). For both programmes, final extension was done for 5 min at of 72 °C.

3.1.5 Product Analysis

Following DNA amplification, 18 $\mu\ell$ aliquots of reactions were separated on a 1 % agarose gel made up with TAE buffer (40 mM Tris-acetate, 1mM EDTA disodium salt). The agarose gels were stained with 2 $\mu\ell$ ethidium bromide (10mg/m ℓ). To detect the fluorescent bands, the gels were visualised by an ultraviolet transilluminator. Molecular size markers included in gels were either the 100 bp DNA ladder (Promega) or the molecular DNA size marker VI (Roche).

3.1.6 Concentration Methods

Seeded samples

For this set of experiments samples consisting of 10 litres of drinking water were each seeded with at least 1000 oocysts.

Filtration

The seeded samples were concentrated using 1.2 μm nitrocellulose flatbed membrane filters. After filtration membranes were rinsed using a rubber squeegee. The oocysts in the eluate were collected by centrifugation. The pellets were resuspended in distilled water and used for the preparation of DNA (Falk et al., 1998).

Flocculation Method

Concentration of the seeded samples using flocculation was performed according to the method described by Vesey et al., 1993. Ten litre samples were used and 100 mℓ of a 1 mol calcium chloride (CaCl₂) and 100 ml of a 1-molar sodium bicarbonate (NaHCO₃) solution were added. After mixing, the pH of the sample was adjusted to pH10 by the addition of 1 mol sodium hydroxide (NaOH). After the supernatant fluid was discarded and the formed calcium carbonate precipitate was collected and dissolved in 200 mℓ 10 % v/v sulphamic acid. The dissolved residue was then collected by centrifugation. After washing the pellets they were resuspended in PBS Buffer pH 8.0. DNA was extracted from these concentrates by using the QIAamp Purification Kit as described above.

Immuno-magnetic separation

Cryptosporidium oocysts were separated from faecal material, or seeded water samples (after concentrated by flocculation), using para-magnetic beads coated with anti-Cryptosporidium antibodies (Dynal) according to the manufacturer's instructions. After collection of the oocysts, the beads were removed and the DNA was extracted using the QIAamp Purification Kit as described above.

3.2 Results and discussion

3.2.1 DNA extraction methods

Two methods were evaluated for the extraction of DNA from *Cryptosporidium* oocysts. Although the CTAB DNA extraction method was a very efficient way to extract oocysts DNA, losses still occurred during the phenol/chloroform and precipitation steps. The use of the QIAamp purification Kit was therefore evaluated because it does not involve a phenol/chloroform extraction step. Under specific pH and salt conditions, DNA will be retained on the silica in the spin column, whereas proteins and other contaminants will be passing through. After washing the column the DNA can be eluted by changing the conditions. The QIAamp purification Kit was found to produce DNA of sufficient quantity and quality. It should also be noted that a number of other groups were also using this method to obtain DNA from *Cryptosporidium* oocysts (Chung et al., 1998; Gibbons et al., 1998)

3.2.2 DNA amplification

Amplification of DNA extracted from the *Cryptosporidium parvum* reference strain was performed. Using the CPHSP1 and CPHSP 2 primer sets both the expected 307 bp and 367 bp products were successfully amplified. After the initial results were obtained using a 40 cycle PRC reaction problems were experienced with the development of smears in all reactions, including the negative controls. A variety of experiments were performed to investigate and eliminate this phenomenon. It was also noted for other PCR reactions used to amplify other non-related *Cryptosporidium* genes. It was eventually established that only a reduction in the number of cycles could eliminate the formation of these smears. Unfortunately this led to a decrease in sensitivity of the PCR-based method.

3.2.3 Sensitivity of PCR reactions

The sensitivity of both primer pairs, CPHSP 1 and CPHSP 2, were determined by performing PCR reactions on samples seeded with a specified number of oocysts. After the number of cycles had to be reduced to 30, the sensitivity for both reactions was in the order of more than 10³ oocysts.

3.2.4 Combination of PCR and oocyst concentration methods

After concentrating seeded samples using flatbed membrane filters, DNA was extracted and the PCR reaction was performed. This resulted in the amplification of the expected product, indicating that the PCR-based method was compatible with membrane filtration.

To evaluate the effect of calcium carbonate flocculation on PCR reactions, a 10ℓ treated effluent sample was concentrated to a volume to $3 \text{ m}\ell$. Half of the concentrate was seeded with oocysts and the DNA was extracted and amplified. No product was detected for both of the primer pairs. These experiments were repeated but amplification was also not noted in any of these experiments.

In another experiment, DNA was extracted normally after flocculation. The extracted DNA was then used to set the PCR reaction mixtures. Concentrated *Cryptosporidium* DNA was also added to these reactions. The PCR assay showed total inhibition of the reactions. The polymerase enzyme is known to be sensitive towards ions such as Mg²⁺ and Ca²⁺. The most likely explanation would therefore be that low levels of Ca²⁺ remained in the samples after flocculation, and inhibited the PCR reaction. PCR could therefore not be used to confirm the presence of *Cryptosporidium* oocysts in samples that had been concentrated using calcium carbonate flocculation. Similar results could be expected after the concentrate had been further purified by flow cytometry.

Immuno-magnetic separation was used to concentrate *Cryptosporidium* oocysts from faecal samples. After concentration, the DNA was extracted with the QiAmp purification kit and the two PCR reactions were performed. The appropriate products were amplified for both of the primer sets.

In a further set of experiments, seeded samples were concentrated by flocculation and thereafter the oocysts were purified by means of immuno-magnetic separation before the DNA was extracted and PCR performed. In these samples the appropriate size products were amplified, indicating that PCR-based confirmation was possible only if flocculation followed by immuno-magnetic separation.

3.3 Conclusions

The study indicated that PCR-based methods have the potential to be used to confirm the presence of protozoan cysts and oocysts in water samples. Due to the problems experienced with the sensitivity of the technique and its incompatibility with most of the methods, which incorporate flocculation as a concentration step, it will be difficult to implement this technology on a routine basis in the near future.

4 CATCHMENT MONITORING FOR CRYPTOSPORIDIUM AND GIARDIA

The Pietermaritzburg catchment in KwaZulu-Natal was chosen for investigation of protozoon incidence in its rivers as the area is impacted by rural, peri-urban and urban settlements. Water sampling sites were chosen along the uMsunduze River and its tributaries as they make their way through the rural and peri-urban areas of Vulindlela and through Camps Drift (watersports area) and the city of Pietermaritzburg. In addition, influent, effluent and sludge samples were collected from Darvill wastewater works and the viability status of (oo)cysts determined. As the sludge is disposed onto land, the effect of desiccation on the viability of (oo)cysts present was also evaluated.

4.1 Materials and methods

4.1.1 Sampling and concentration of water, wastewater and sludge samples

River water sampling of selected sites in the Pietermaritzburg catchment was carried out between September 1998 and May 2000. Ten-litre water samples were collected in plastic bottles and stored at 0- 10° C until analysis within 48 hours of sample collection. The 10-litre water samples were concentrated according to the calcium carbonate flocculation method of Vesey et al. (1993) which has consistently high recoveries (64.0 - 76.0%) in tap and river water (Vesey et al., 1993; Shepherd and Wyn-Jones, 1996, Jarmey-Swan, unpublished data). However, the recovery for each water-sampling site was not determined during this study. After dissolution of the floc with sulphamic acid and prior to centrifugation, the pH of the concentrate was increased to 6.0 ± 0.5 . Following centrifugation, the pellet was assayed as described in 4.1.2.

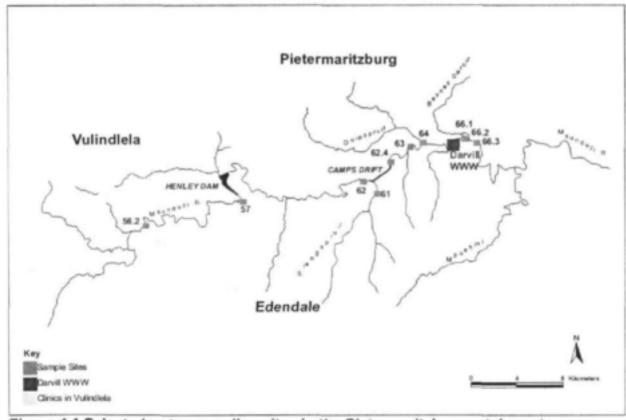


Figure 4.1 Selected water-sampling sites in the Pietermaritzburg catchment

Sewage influent samples (collected after grit screens had removed larger particulates) and treated effluent samples (following activated sludge and chlorination)(Darvill Final Effluent) were collected in 10-litre bottles from Darvill Wastewater Works, Pietermaritzburg. Effluent samples from an old discharge point which, may have been contaminated with a leak of raw sewage, were also sampled (Darvill Old Effluent). These samples were stored at 0-10°C until analysis within 48 hours of sample collection. Both sample types were concentrated by centrifugation at 3000g (10 min) and the pellet assayed as described in 4.1.2.

Primary sludge samples, following 5 days digestion and then by anaerobic digestion, were collected in 250 m ℓ plastic bottles between September 1998 and May 2000 from Darvill Wastewater Works, Pietermaritzburg. Twenty millilitre wastewater sludge samples were washed through the Visser helminth filter and the filter wash collected in beakers. The Visser helminth filter apparatus (Visser Filters, Pretoria, South Africa), which consists of three consecutive filter sleeves which fit together with a 100 μ m mesh inner filter enclosed by an 80 μ m mesh and then a 35 μ m mesh filter was used to remove larger particulates. The eluate passing through the 35 μ m filter was further concentrated by centrifugation at 3000 q (10 min) and the pellet assayed as described in 5.1.2.

4.1.2 Detection of Cryptosporidium and Giardia in water and sludge samples

The concentrated samples (250 - 500 $\mu\ell$) were stained in solution by the addition of an equal volume (250-500 $\mu\ell$) of fluorescein isothiocyanate (FITC) labelled monoclonal antibodies against *Cryptosporidium* and *Giardia* (Crypto/*Giardia* -Cel IF, Cellabs, New South Wales, Australia) and incubated at 37°C for 30 minutes. After incubation, the stained solutions were filtered through 1.2 \Box m pore size cellulose-acetate membranes and mounted on microscope slides for viewing. A Zeiss Axioplan epifluorescence microscope with selected excitation of 450 - 490 nm and barrier filter of 520 nm was used with X400 magnification to examine the stained samples. Presumptive oocysts and cysts were identified using the following criteria: (a) distinct apple green fluorescence of the (oo)cyst wall; (b) shape (*Cryptosporidium*: spherical; *Giardia*: oval); (c) size (*Cryptosporidium*: 4-6 μ m; *Giardia*: 5-15 μ m in width and 8-18 μ m in length).

4.1.3 Viability staining of oocysts and cysts in effluent and sludge samples

Random influent, effluent and pre- or post anaerobic digested sludge samples from Darvill Wastewater Works were further analysed to determine the viability status of *Cryptosporidium* oocysts or *Giardia* cysts following activated sludge treatment or anaerobic digestion. A pea-sized amount of the pellet was removed from the concentrated samples, resuspended in 2 mℓ of phosphate buffered saline (PBS; pH 7.4) and centrifuged at 2200 rpm (5 min). The supernatant was discarded and the remaining pellet subjected to a further two washings. The washed pellet underwent sucrose flotation (Roberts-Thompson et al., 1976) prior to TMR-FDA viability staining (Jarmey-Swan et al., 2000).

4.1.4 Desiccation of sludge samples

Anaerobic digested sludge samples (1 mℓ), previously determined to contain viable Giardia cysts, were pipetted in duplicate into Petri dishes and either placed in direct sunlight outside or kept at room temperature indoors. The viability status of the cysts present in the anaerobic digested sludge, disposed onto land, was determined, as described above, after exposure at the following time intervals: 2 hours, 4 hours, 24 hours and 1 week.

4.1.5 Rainfall and temperature data to determine seasonal variations

Daily average rainfall data and daily average maximum air temperature data were collected at Darvill Wastewater Works (a meteorological station), Pietermaritzburg, from September 1998 - May 2000. The data were used to establish the occurrence of Cryptosporidium and Giardia with regard to seasonal variations.

4.2 Results and discussion

4.2.1 Occurrence in the uMsunduze River Pietermaritzburg catchment

Giardia positive water samples occurred at eight of the eleven selected river sites in the uMsunduze River, Pietermaritzburg catchment while *Cryptosporidium* was detected on one occasion at four of the sites from September 1998 to May 2000, Table 4.1.

Giardia cysts were detected in 8% of river samples whilst Cryptosporidium oocysts were detected in 2% of samples. A considerable number of samples produced non-detect results. A much larger number of samples from sewage effluents were positive, with 63% and 6% respectively. Du Preez and Gericke (1999) detected Giardia cysts in 17% and Cryptosporidium oocysts in 8% of surface water, protected boreholes and partially treated water samples analysed. Other findings in South Africa have reported a higher occurrence of Giardia, which may be indicative of a higher prevalence of giardiasis in the population (Kfir et al., 1995; Gericke et al., 1994; du Preez et al., 1996). Harding and Genthe (1998) detected Giardia cysts in 50% of surface water samples collected in the Western Cape while Cryptosporidium oocysts were not detected. In contrast, researchers in the USA have found Cryptosporidium oocysts in higher numbers in water samples (Rose et al., 1991; Chauret et al., 1995) while Madore et al. (1987) found that the two parasites often coexist in polluted waters and the frequency of oocysts/cyst detection has been shown to be directly related to the degree of human activity in the watershed.

Table 4.1 Occurrence of *Cryptosporidium* and *Giardia* in the uMsunduze River Pietermaritzburg catchment from September 1998 - May 2000

Sample point uMsunduze River (unless stated other)	Sample size	Cryptosporidium positive (%)	Giardia positive (%)	Km distance between sample pts
56.2 Above Henley dam				
57 Henley Dam inflow weir	8	0	1 (12.5)	16.2
61 Slangspruit river above uMsunduze	18	1 (5.6)	3 (16.7)	22.4
62 Edendale weir	24	0	3 (12.5)	3.3
62.4 Camps Drift lower weir	13	0	2 (15.4)	2.7
63 Above Dorpspruit confluence	20	0	0	3.1
64 Dorpspruit River at Ohrtmann Road	22	1 (4.5)	1 (4.5)	1.5
66.1 Upstream Baynespruit	19	0	0	1.5
66.2 Upstream Darvill WWW effluent	29	1 (3.4)	2 (6.9)	1.3
66.3 Downstream Darvill WWW effluent	23	1 (4.3)	3 (13.0)	0.8
67 Motocross track	20	0	3 (15.0)	1.3
Total river sites	222	4 (2)	18 (8)	
626 Darvill WWW Old Effluent point	34	2 (5.9)	21 (61.8)	
719 Darvill WWW Treated effluent	48	3 (6.3)	31 (64.6)	
Total WWW sites	82	5 (6)	52 (63)	

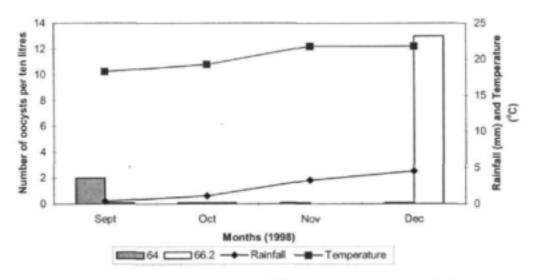


Figure 4.2 The occurrence of *Cryptosporidium*, with respect to daily average rainfall and temperature, in river samples in the Pietermaritzburg catchment during 1998

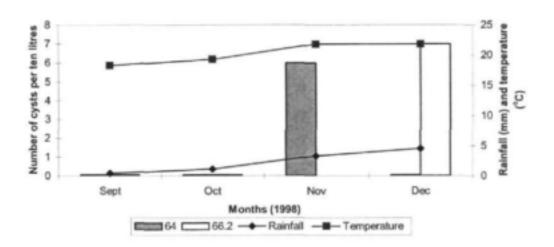


Figure 4.3 The occurrence of Giardia, with respect to daily average rainfall and temperature, in river samples in the Pietermaritzburg catchment during 1998

Cryptosporidium oocysts were detected during September and December 1998 at the Dorpspruit Ohrtmann Road (64) sampling point (2 oocysts 10 ℓ^{-1}) and the uMunduze River upstream of the Darvill effluent (13 oocysts 10 ℓ^{-1})(66.2) respectively (Table 4.1, Figure 4.2). The detection in the Dorpspruit may be due to the nearby informal settlement that utilise the river water and have pit latrines situated on the riverbank.

Giardia cysts were detected during November 1998 at the Dorpspruit Ohrtmann Road sampling site (6 cysts 10 ℓ ⁻¹) and the uMsunduze upstream of the Darvill maturation effluent (7 cysts 10 ℓ ⁻¹) during December 1998 (Figure 4.3). Giardia cysts were not detected during September or October 1998. Cysts were detected as the temperature and rainfall increased from below 1mm (< 20°C) to 5mm (22°C). The presence of Cryptosporidium oocysts and Giardia cysts at the uMsunduze upstream of the maturation effluent from Darvill (66.2)

indicates faecal contamination from the Baynespruit. This area has informal and formal settlements. Although the formal residential areas are sewered, the steep slopes may result in dislocated sewerage pipes, which could result in direct faecal contamination of the Baynespruit. In addition, illegal industrial discharge, which may include sewerage, is a common occurrence.

Cryptosporidium oocysts were not detected at any of the sites sampled from January 1999 to November 1999 whilst Giardia cysts were detected in large numbers during January and February 1999 in the Slangspruit River above the uMsunduze confluence (300 cysts 10 ℓ^{-1} and 23 cysts 10 ℓ^{-1} respectively) (61 Table 4.1, Figure 4.3).

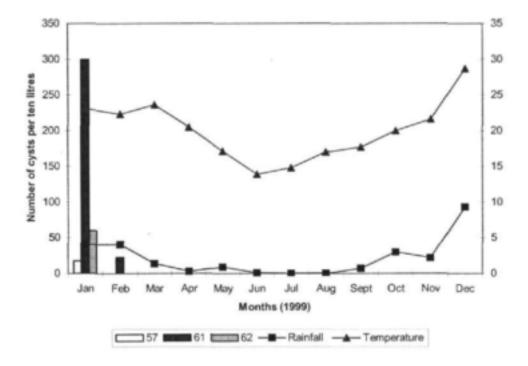


Figure 4.4 The occurrence of *Giardia*, with respect to daily average rainfall and temperature, in river samples in the Pietermaritzburg catchment during 1999

The few Giardia cysts (18 10 ℓ^1) which were detected at the uMsunduze Henley inflow weir (57)(Figure 4.4) during January 1999, indicate infection in the Vulindlela community which is comprised of dense rural / peri-urban settlements with poor services. In addition, the land is barren due to overgrazing and cattle and goats use the nearby rivers. Soil erosion also results in increased run-off during rainfall, which could transport surface material such as dung and stools into the rivers. This concentration is probably higher upstream of Henley Dam in which many cysts will have sedimented. The impact from the dense formal residential areas of Edendale and Imbali is indicated by the detection of cysts (up to 300 10 ℓ^1) at the Slangspruit river sampling point (61). This area experiences a number of sewer blockages and breakages and is unable to handle increased flow volumes during heavy rains, resulting in discharge to the river. The 60 10 ℓ^1 Giardia cysts detected at sample point 62 are representative of infection in the central Edendale area. This area is comprised of scattered informal settlements and formal sectors with semi-formal sewer systems or conservancy tanks, which are poorly serviced, often resulting in direct discharge to the river.

Giardia (7 cysts 10 ℓ⁻¹) was also detected in the uMsunduze Downstream of the Maturation Effluent from Darvill wastewater Works (66.3), possibly from the treated effluent from Darvill WWW.

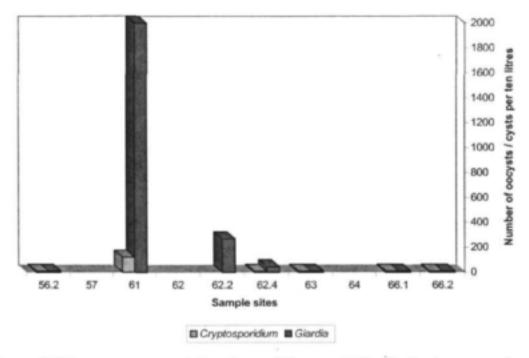


Figure 4.5 The occurrence of *Cryptosporidium* and *Giardia* in river samples in the Pietermaritzburg catchment following a sewer break during December 1999

Following a sewer break, due to heavy rainfall on that day (Temperature 26.9°C; Rainfall 39.5 mm), alongside the Slangspruit River (61) during December 1999, samples were collected a week after the incidence occurred. Analysis of the samples indicated that 120 *Cryptosporidium* oocysts 10 ℓ^{-1} and 2000 *Giardia* cysts 10 ℓ^{-1} were present in the Slangspruit inflow to Camps Drift (water sports impoundment). Camps Drift Upper weir contained 270 *Giardia* cysts 10 ℓ^{-1} and the Lower weir (62.4) 45 *Giardia* cysts 10 ℓ^{-1} with no *Cryptosporidium* oocysts detected. (Figure 4.5). The second day of sampling these were much reduced.

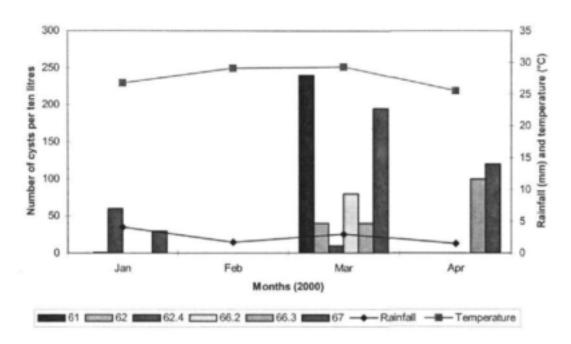


Figure 4.6 The occurrence of *Giardia*, with respect to daily average rainfall and temperature, in river samples in the Pietermaritzburg catchment during 2000

Increased rainfall during January 2000 (Daily average: 4.1 mm) resulted in *Giardia* cysts being detected (60 cysts 10 ℓ^{-1}) at Camps Drift Lower Weir (62.4). (Fig 4.6) The occurrence of cysts at the Motocross (67) sampling site could be due to Darvill Final Effluent, which is released into the uMsunduze River upstream of this sampling site. A high number of cysts occurred at the Slangspruit above the uMsunduze confluence (61) possibly due to sewer blockages or breaks in the dense formal residential areas of Edendale and Imbali while the occurrence of cysts at site 62 indicates infection in the central Edendale area.

4.2.2 Occurrence of Cryptosporidium and Giardia in wastewater

Cysts were detected in the following Darvill Final Effluent monthly samples analysed: (October -December 1998, January and June 1999), ranging from 12 - 312 cysts 10 ℓ^{-1} while oocysts were only detected on one occasion during December 1998 (8 oocysts 10 ℓ^{-1}). This is of concern as Darvill effluent enters the uMsunduze River which could contribute to infection of those using the river as a water source. (Oo)cysts numbers were found to increase as rainfall and temperature increased (Figure 4.7).

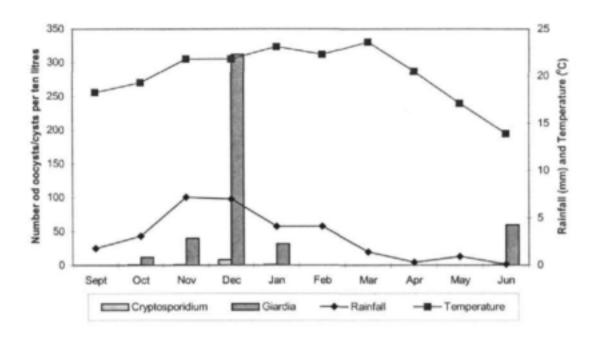


Figure 4.7 The occurrence of *Cryptosporidium* and *Giardia*, with regard to daily average rainfall and temperature, in monthly samples of Darvill Final Effluent

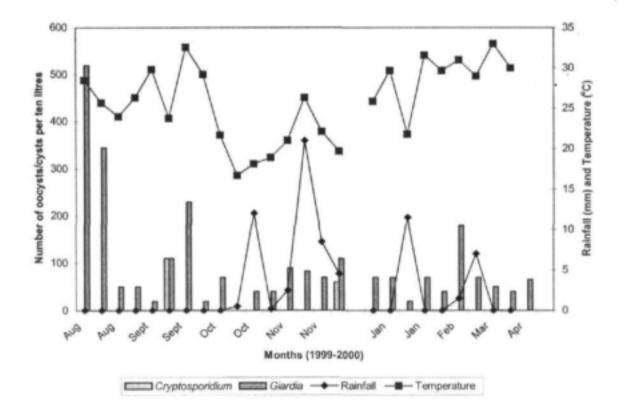


Figure 4.8 The occurrence of *Cryptosporidium* and *Giardia*, with respect to daily average rainfall and temperature, in weekly samples of Darvill Final Effluent

From August 1999, weekly sampling of Darvill Final Effluent was instituted (Figure 4.8). Giardia cysts occurred in twenty-five of the twenty-six weekly Darvill Final Effluent samples (96.2%). The cyst levels ranged from 0 cysts 10 ℓ^{-1} to 520 cysts 10 ℓ^{-1} . Cryptosporidium occysts were detected in 7.7% (2/26) of weekly Darvill Final Effluent samples. Occysts were detected during September 1999 (110 10 ℓ^{-1}) while 60 occysts 10 ℓ^{-1} were detected on occasion during December 1999 (Figure 11). Fewer (oo)cysts detected during the summer rainfall months between October and March, may have been possibly due to dilution of the sewage from increased storm water infiltration of sewers, which is known to be prevalent in the area.

Large numbers of *Giardia* cysts were detected in two Darvill sewage influent samples analysed during March and April 2000, ranging from 1.1-1.45 x 10^5 10 ℓ^{-1} , (*Cryptosporidium* oocysts were not detected on these occasions). Treated effluent samples collected on the same day, indicated up to 99.9% reduction in cyst numbers. Other researchers have determined the removal efficiency of *Cryptosporidium* oocysts and *Giardia* cysts by activated sludge to be between 88.5-93.8% in the final effluent (Bukhari *et al.*, 1997) while Madore *et al.* (1987) found the removal of oocysts by activated sludge approached 79.0%. Other South African researchers have detected up to 450 oocysts 10 ℓ^{-1} and 3 910 cysts 10 ℓ^{-1} in treated effluents (Kfir *et al.*, 1995).

Darvill WWW is unusual, as the plant treats wastewater by both the activated sludge process and anaerobic digestion of the sludge itself. It would appear that the majority of (oo)cysts are settled out in the raw sludge in the primary settling tanks prior to being treated by aerobic and then anaerobic digestion. A smaller number of (oo)cysts remain in the supernatant in the primary settling tanks, which is then passed on to the activated sludge process.

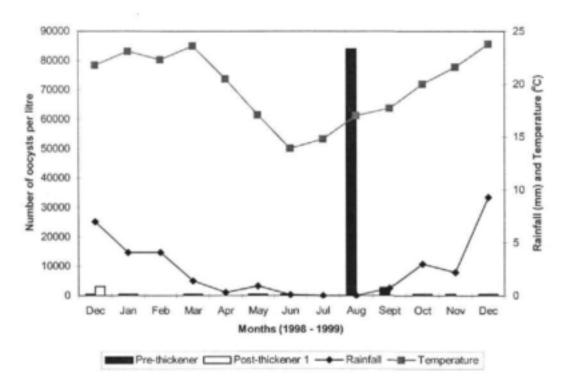


Figure 4.9 Occurrence of Cryptosporidium oocysts in the digested and anaerobically digested sludge samples from Darvill Wastewater Works

Cryptosporidium oocysts were detected in 16.7% (2/12) of the digested and one anaerobically digested sludge sample (20%)(1/5), which contained 2 900 oocysts ℓ^{-1} (Figure 4.9). The highest number of oocysts (84 000 ℓ^{-1}) was detected in the digested sludge during August 1999, but appeared to be removed by anaerobic digestion.

High numbers of *Giardia* cysts were detected in digested and anaerobically digested sludge samples (Figure 4.10). The highest number of cysts (1.2 million ℓ^{-1}) was detected in the digested sludge during January 1999. The anaerobically digested sludge was found to contain up to 61.7% fewer *Giardia* cysts than just digestion.

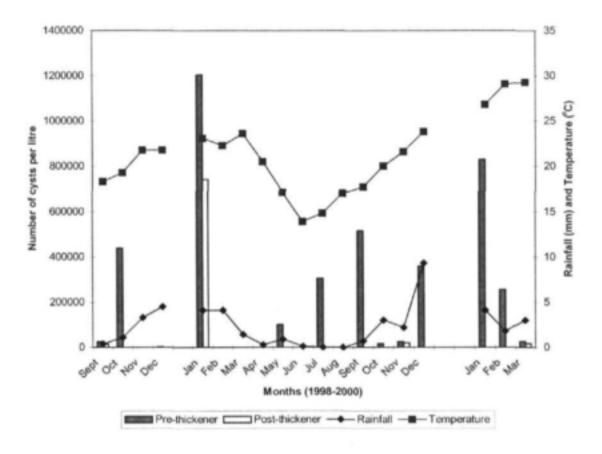


Figure 4.10 Occurrence of Giardia cysts in digested and anaerobically digested samples from Darvill Wastewater Works

4.2.3 Viability staining of oocysts and cysts in effluent and sludge samples

All of the three Darvill Old Effluent samples, which were analysed to determine viability, contained viable Giardia cysts whilst one also contained Cryptosporidium oocysts of which most were viable. One Darvill Final Effluent sample, which was analysed, also contained viable Giardia cysts while another contained a low number of cysts, which were nonviable. Only viable cysts were detected in the Darvill digested sludge sample.

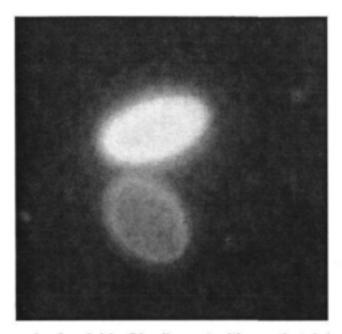


Plate 1 Photomicrograph of a viable Giardia cyst with a red staining wall and internal green fluorescence (top).

The non-viable (bottom) Giardia cyst stained red only (viewed under oil immersion by epifluorescence microscopy, fitted with a triple band filter with excitations at 400/ 450/ 510 nm)

4.2.4 Desiccation of sludge

Only non-viable Giardia cysts were detected in the sludge samples following desiccation in direct sunlight and at room temperature, even after short periods. Although Robertson et al. (1992) found only 3% of Cryptosporidium oocysts were viable after two hours at room temperature, longer periods of drying (> 4 hours) resulted in 100% death of the oocysts. Desiccation of sludge would appear lethal, providing the sludge is finely dispersed onto land, which would allow adequate drying. However, wind dispersal could play an important role in the transmission of Cryptosporidium and Giardia present on the sludge lands.

4.2.5 Collection of veterinary samples and data

The Regional Veterinary laboratories and local veterinarians were contacted to obtain samples of dung and details of any Cryptosporidium detected.

Methods

The Allerton Regional Veterinary laboratories and local veterinary pathology laboratory were visited and the project described to them and assistance sort with the collection and analysis of dung samples, especially from animals with diarrhoea such as calves. The significance of the protozoon parasites was explained and training was given on laboratory procedures especially the immuno-fluorescence method for their detection. Sample bottles were provided and the veterinary field technicians were asked to collect samples of diarrhoea from domestic animals in the rural areas, especially Vulindlela. Cattle dip tanks would be ideal for this as the cattle are rounded up periodically for this process.

Results and discussion

Despite numerous requests, no dung samples were ever submitted from the veterinarians or technicians. However, Allerton did provide results of their own analysis over the period of the study. This showed 27 samples of which only two were positive for *Cryptosporidium*, from the Lions river and Richmond areas, not the Pietermaritzburg area.

Apparently Cryptosporidium is not a problem for livestock in this area (or not identified as such), despite international reports that it is the most common cause of diarrhoea in calves, foals and lambs worldwide (Jarmey-Swan and Bailey 1996).

4.3 Conclusions

- As Cryptosporidium and Giardia were detected widely in river and wastewater samples, they are probably ubiquitous in the Pietermaritzburg area community and livestock.
- Occurrence was sporadic however, with Giardia cysts detected in 8% of samples from rivers and in higher numbers than Cryptosporidium occysts, which were only detected in 2% of samples.
- The highest numbers detected in rivers may have been due to broken sewers or run-off from land and appeared to be seasonal with increases in rainfall and temperature in summer. Very high numbers of the protozoa were detected two kilometres below a sewage break (Cryptosporidium 120/10ℓ, Giardia 2000/10ℓ), however, these did eventually disappear within a five kilometres distance downstream.
- Darvill Wastewater Works was found to remove up to 99.9% of Giardia cysts, after the activated sludge process, chlorination and maturation, in the final effluent.
- Some 70% of Darvill effluent samples were positive for the protozoa, however, containing up to 520 Giardia cysts 10 ℓ⁻¹ and 110 10 ℓ⁻¹ Cryptosporidium oocysts with up to 200 cysts 10 ℓ⁻¹ in the river downstream.
- The potential Darvill effluent loading could be some 2.5 Billion cysts and 0.5 Billion oocysts per day into the uMsunduze River (at a dry weather flow of some 50 M/day), which constitutes half the dry weather (winter) flow of the river.
- Sludge disposed onto land was found to contain up to 3000 oocysts €¹ and 700 000 cysts €¹ and needs to be monitored if used in sensitive locations (eg for sports field or agricultural applications). Desiccation experiments proved that viability could be reduced significantly.

- The anaerobically digested sludge was found to contain up to 62% fewer Giardia cysts than just aerobic digestion.
- Neither the activated sludge process, nor the anaerobic digestion of sludge, appeared to significantly affect the viability status of the Cryptosporidium or Giardia.
- Treated effluents need to be monitored prior to discharge into rivers, which may be a source of water for both humans and animals and possibly treated further.
- The occurrence of Giardia cysts throughout the year in the effluent samples indicates that giardiasis is endemic in the Pietermaritzburg population, while cryptosporidiosis infections may be sporadic, although possibly asymtomatic.
- It would appear that diseases caused by these pathogens are not prevalent in livestock in the area

5 OCCURRENCE OF CRYPTOSPORIDIUM AND GIARDIA IN A COMMUNITY

This aim of this study was to determine the likelihood of *Cryptosporidium* and *Giardia* causing diarrhoea in the Vulindlela/Pietermaritzburg catchment of the uMsunduze River. The extent of diarrhoeal diseases in the Vulindlela area was established using information obtained from clinics and hospitals, derived from a parallel WRC research project. An attempt was made to correlate the water source types used for domestic purposes, with the occurrence of diarrhoea in the community. Stool samples from primary school children were also examined for cysts and oocysts, as well as in another area.

5.1 Methodology

The information on the incidence of *Giardia* and *Cryptosporidium* gathered from the hospital was mostly incomplete however and a more focused approach, concentrating efforts in one area, was adopted. The Vulindlela area was chosen because it was densely populated with informal settlements. Medical services in the area consisted of small clinics, from which relevant information could be gathered.

5.1.1 Collection and examination of stool samples from schools

Parasitic infections afflict an estimated two million children in the province of KwaZulu-Natal. The Provincial Parasite Control Programme (PCP) was formed to examine primary school children for parasitic infections and to treat them with an anthelminthic drug. The PCP agreed to submit the stool samples they collected for further analysis for the presence of Cryptosporidium oocysts and Giardia cysts. The PCP treated children with the anthelminthic drug, Albendazole that has been shown to be as effective as Metronidazole in treating giardiasis (Hall and Nahar, 1993; Dutta et al., 1994). Subsequent stools from the PCP after treatment were therefore unlikely to be positive and were therefore not accepted.

217 stools collected in the Vulindlela area were examined for the presence of *Giardia* cysts. The formol-ether concentration technique and bright field microscopy method was used for the cysts, which were identified by their oval shape and size. *Cryptosporidium* oocysts were detected using Sheathers flotation (Moodley et al.,1991b) and bright field microscopy and were identified as pink, refractile round ovoid bodies. The presence of other parasites was also noted. 194 stool samples, obtained from schools in the Kranskop/Umvoti area north of Pietermaritzburg, were also examined using the methods described above.

5.2 Collection of clinical information regarding positive stool samples from hospital records

The Edendale hospital treats patients from the Vulindlela and Edendale areas of Pietermaritzburg. Permission was granted to access their records, including from their pathology laboratory, which were examined to determine:

- number of patients identified with Giardia or Cryptosporidium
- · home area of such patients
- age of the patients
- type of water source used

5.2.1 Collection of clinical information from rural clinics in the Vulindlela area

This information was gathered in conjunction with another WRC project (number 925) entitled "The impact of introducing treated water on aspects of community health in a rural community in kwaZulu-Natal", run in parallel in Vulindlela. Especially redesigned clinic data sheet books were printed, in conjunction with the Dept of Heath to include information on water sources and diarrhoea. The nursing staff were specially trained to use these data sheets and a colour chart to identify and classify the type of water source used was developed; the following information was gathered:

- Patients name
- · Age of patient
- Home area of patient
- Type of water source used
- Illness patient treated for
- · Consistency of stool sample (in the case of diarrhoea)

5.2.2 Parasite analysis of household water

A total of 20 household water samples were collected in 10-litre plastic bottles and returned to the laboratory for parasite analysis.

5.2.3 Collection of dung samples

Meetings were held with the Allerton Regional veterinary laboratory and the Stale Veterinarian to obtain dung samples from calves, foals and lambs with diarrhoea.

5.3 Results and discussion

5.3.1 Presence of Giardia and Cryptosporidium in stool samples

Collection of stool samples from primary school children in the Vulindlela area South-west of Pietermaritzburg took place during January 1999.

Table 5.1 Parasites present in stools received from schools in the Vulindlela area

Parasite	Number of positive stools (%)		
Cryptosporidium	0 (0)		
Giardia	12 (5.5)		
Ascaris lumbricoides,	65 (30)		
Trichuris trichura	26 (12)		
Entamoeba histolytica	9 (4.1)		

Cryptosporidium oocysts were not detected in any of the Vulindlela stool samples. Giardia cysts, however, were present in 5.5% of the samples.

Of the stools examined from schools in the Kranskop/Umvoti area, 9.3% (18 out of 194) were *Giardia* positive while none were positive for *Cryptosporidium*. Other parasites identified included Entamoeba coli, Entamoeba histolytica, Schistosoma haematobium, Schistosoma mansoni, Trichuris trichura, Ascaris lumbricoides, Necator americanus and Taenia spp. All stool samples received from the PCP were formed indicating that those children who were *Giardia* positive were asymptomatic and not ill and able to attend school.

5.3.2 Clinical information obtained from the hospital and pathology laboratory records

Information obtained from the Edendale hospital and Pathology laboratory was mostly incomplete and therefore lacking information.

- · The cause of diarrhoea was usually not identified
- Diarrhoeic stools were generally only analysed if dysentery, cholera or typhoid was suspected.
- Analysis of stools for giardiasis or cryptosporidiosis was only undertaken if the diarrhoea was chronic.
- Patients were often not formally admitted and therefore no record for such patients existed

Information regarding the number of patients with Giardia or Cryptosporidium, the area such patients originated from, their age and what type of water was used for consumption could not usually be ascertained, despite detailed examination of the records

The Pathology Laboratory at the Edendale hospital does, however receive diarrhoeic stools for *Cryptosporidium* and *Giardia* analysis if requested by the doctor. The laboratory indicated that 100-120 stool samples are analysed every month. Very few samples were positive for *Giardia* but approximately 3% were positive for *Cryptosporidium*. The *Cryptosporidium* positive stools are mostly obtained from AIDS patients and the results therefore probably have no implication for water use.

5.3.3 Clinical information obtained from clinics in the Vulindlela area

A total of 8077 patients visited five clinics for treatment, over a 12-month period and 39% (3172) were treated for diarrhoea. The majority of these patients originated from the Mafakatini, Zayeka, Inadi, Madwaleni, Gandaganda, Mgwagwa, Zondi and Mbubu locations in the Vulindlela area. Further information on why these areas showed high prevalence of diarrhoea could not be obtained although discussions were held with nurses at the respective clinics.

Figure 5.1 represents the percentage calculated for different age groups treated for diarrhoea. The results were derived from a random group of 600 patients seeking treatment

at the clinic in Vulindlela. Children between one and five years showed the highest incidence of diarrhoea. The second highest incidence was noted for persons 21-30 years old. Less than 10% of the age group 6 to 10 years presented with diarrhoea.

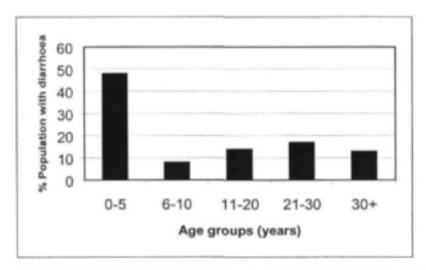


Figure 5.1 Percentage of the different age groups with diarrhoea in a population

Nurses at the clinics noted the type of water patients used for domestic in response to local photographs showing the different types and any occurrence of diarrhoea.

Table 5.2 Percentage of people (those who visited the clinics) with diarrhoea, using different sources of water

Water source	% with diarrhoea	% using source	No. using source
Well water	91	0.5	25
Spring	54	8	636
Umgeni tap water	44	23	1904
Borehole	44	7	589
River	42	6	512
Unknown source	31	17	1373
Protected spring	29	11	888
Piped water	27	3	242

Although only a small number of people indicated use of well water, the highest incidence of diarrhoea was recorded for this group (91%, 22 persons out of 25).

5.3.4 Correlating incidences of diarrhoea with the water source used

The information gathered from the Edendale hospital and Pathology laboratory was too fragmented to make any conclusion regarding the use of a specific type of water source and diarrhoea in terms of *Giardia* and *Cryptosporidium*. No correlation between water use and diarrhoea caused by *Giardia* or *Cryptosporidium* could be determined.

A large amount of data was generated by the clinic data sheets, although only a small part is presented here. The data showed that the incidence of diarrhoea in the population was very high.

However, possible correlations between the type of source water and diarrhoea were indicated (Table 5.2). The results indicated that unprotected water sources could contribute to the incidence of diarrhoea in this area. Even when Umgeni tap water was used, open containers were still filled and stored in the households where contamination could take place as only outside taps were provided. The patients may not actually be using the Umgeni tap water, however, since it was individually metered and charged for, when local sources are still available.

Section 4.6.6 clearly indicated that Giardia cysts and Cryptosporidium oocysts were present in the river water tested with counts obtained for Giardia being higher than for Cryptosporidium. Similar findings have been previously reported in South Africa. Only Giardia cysts were found to be present in the stool samples examined. Although Cryptosporidium oocysts were not detected the possibility that they were present cannot be ignored; this is especially true in light of the fact that the infective dose is very low.

The rural areas are not easily accessible with bad roads and safety factors making it difficult to collect samples. Difficulties were experienced in transporting samples to a laboratory within the prescribed time. Samples could not easily be collected and transferred to a laboratory for analysis without degradation of the parasites and other organisms. Nurses at the clinics could not be contacted and refrigerators were not available for storage of samples. Ethical issues further complicate collection and analysis of stool samples. The actual etiological agent of the diarrhoea could not be identified and therefore no correlation between water use and diarrhoea actually caused by *Giardia* or *Cryptosporidium* could be determined from the data.

5.3.5 Parasite analysis of household water

None of the 20 water samples collected from households that reported diarrhoea were positive for Giardia or Cryptosporidium.

5.3.6 Dung samples

Despite numerous requests, no dung samples were submitted by the Regional Veterinary laboratories, State Veterinarian, or their associated animal technicians. However the Regional Veterinary laboratories did provide results of their own analysis over the period of the study. This showed only 27 samples were examined for the parasitic protozoa over a three-year period, of which only two were positive for *Cryptosporidium*, from the Lions River and Richmond areas, outside the Pietermaritzburg area. However, a sample of calf scour (diarrhoea) procured from a private veterinarian outside the area, showed a very large number of *Cryptosporidium* (millions per ml).

5.4 Determination of human health risk associated with levels of (oo)cysts in river water

Risk assessment aims to provide a quantitative estimate of the probability of illness associated with environmental exposures. This investigation focused on human health risk assessment from Giardia cysts and Cryptosporidium oocysts present in river water.

The objective of this part of the study was to determine the potential human health risk associated with the ingestion of river water contaminated with Giardia cysts and Cryptosporidium occysts. The risk of Giardia and Cryptosporidium infections was quantified based on results obtained for different sampling sites on the uMsunduze River and its tributaries, Darvill sewage treatment works' final and old effluent.

5.4.1 Introduction to health risk assessment

Microbial health risk assessment is a method to predict the risk of infection from waterborne pathogens. Health risk assessment is the quantitative estimation of the probability of adverse effects associated with measured or predicted levels of hazardous agents (NRC, 1986). The assessment requires scientific information which will help estimate what the probability of the health effects of human exposure to potentially hazardous materials are. Risk is a combination of two factors: 1) the probability that an adverse effect will occur and 2) the consequences of the event (Schwab and Genthe, 1998).

An adverse event involves exposure to potentially hazardous substances or situations that can cause harm to the exposed individual. The hazard is the intrinsic 'dangerous' or 'harmful' property of a substance or situation. A hazard can include a chemical substance, floods, drought storms and microbiological organisms in the air, soil, food or water. Where the hazard is of microbiological nature impact can be described in terms of either infection or clinical illness (Rose et al., 1991). Exposure means contact between a person or population or ecosystem and the hazardous substance. Adverse events can only occur when there is contact with a harmful substance. Finally the dose is the amount of the hazardous substance that actually gets into the body where it can cause harm. The concentration of the pollutant in the air, soil, food or water as well as the amount of exposure determine the size of the dose (Schwab and Genthe, 1998).

The risk assessment process (US EPA, 1987; US Commission on RA & RM, 1997) consists of four distinguishable but interacting phases, generally referred to as:

- a. hazard identification,
- b. exposure assessment,
- dose-response assessment, and
- d. risk characterisation

The interrelation of these phases is depicted in Figure 5.2

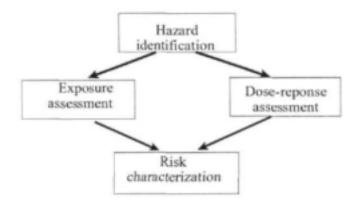


Figure 5.2 The Health Risk Assessment process

Hazard identification:

The goal of hazard identification is to establish whether exposure to a chemical or microbiological agent can cause an increase in the incidence of illness or infection. This is the first step in any risk assessment and determines whether the process should be continued or abandoned.

Exposure Assessment:

Exposure assessment has been defined as the process of measuring or estimating the intensity, frequency and duration of human exposure to a contaminant, which is present in the environment. The task of exposure assessment is to provide the actual exposure conditions required to predict risk, and to identify and predict the effects of the proposed control options (Severn, 1987).

Dose-Response Assessment:

Dose-response assessment is the process of characterising the relationship between dose of a hazardous agent and incidence of an adverse effect in the exposed population.

Risk Characterisation:

Risk characterisation has been defined as the process of calculating the incidence of the health effect under the conditions of exposure described in the exposure assessment, using the identified dose-response relationship (Deisler, 1987). A major component of risk assessment is an evaluation of all assumptions used and all sources of uncertainty.

5.4.2 Quantitative health risk assessment

Scope of the study

- Evaluate results obtained for the presence of Giardia and Cryptosporidium in river water samples (eleven sampling sites) and treated sewage effluent (two sampling sites)
- Quantify the potential health risk associated with Giardia and Cryptosporidium parasites
- Evaluate the significance and acceptability of the calculated risk

The application of risk assessment to problems relating to the microbial quality of water has been limited. The limitation is caused by the lack of epidemiological evidence linking drinking water contaminants to health effects in a population. It is also very costly to obtain such data. Inappropriate microbial risk assessment techniques have also been a contributing factor (Rodda, 1995). The risk to which a population is exposed to known levels of a microbial agent is subject and can be estimated by dose-response extrapolation models. Dose response information is available for approximately 16 pathogenic organisms (Rose and Gerba, 1991). Haas (1983) evaluated three probability models for ability to describe infection by low level exposure to enteric pathogens in drinking water (a simple exponential model, a modified exponential model (beta model) and a log-normal (log probit) model). The results of human exposure studies to a variety of bacteria, viruses and protozoa were evaluated in a dose response fashion. A best-fit curve defining the data sets was developed. It was concluded that the beta model best described the available data sets for bacterial infections, however a single-hit exponential model was used for Giardia as no improvement was observed with the beta-distributed model (Rose et al., 1990). This model is shown below:

P=1-exp(-rN)

Where

P = probability (risk) of infection from a single exposure

N = number of organisms ingested per exposure

r = is the fraction of microorganisms that are ingested which survive to initiate infections and is characterized by dose response curves.

The r values for Giardia lamblia and Cryptosporidium parvum are 0.019 an 0.004 respectively (Rose and Gerba, 1991 and Havelaar et al., 1999)

The above model estimates the daily risk of infection. This can be used to determine the risk over any specified period of exposure as follows:

$$P_x = 1 - (1 - P(N))^x$$

Where

P_x = probability (risk) of one or more infections over period x

X = number of days exposed

P(N) = daily risk

N = numbers of organisms ingested (Rose et al., 1991)

To determine the annual risk of contracting one or more infections as a result of exposure to a given microbial agent, X in the above model would be assigned a value of 365 (Gerba and Haas, 1988).

The US EPA has suggested that one infection/10 000 individuals/year (or 1x10⁻⁴) is acceptable risk for infectious agents acquired through drinking water (Rose and Gerba, 1991)

5.4.3 Hazard identification

Drinking water has long since been recognized as a potential vector for the transmission of communicable diseases (Benton et al., 1989; Craun, 1991). Although concentrations of pathogenic organisms in drinking water are often low and constitute only a low risk of infection, a significant number of consumers may be affected because drinking water reaches almost every member of a population.

In South Africa the risk of infection by waterborne pathogens is elevated because many populations have no or only limited access to treated drinking water. These populations often rely on surface water sources or partially treated water for drinking purposes, swimming and washing.

Over the last two decades the protozoan parasites Giardia and Cryptosporidium have become recognized causes of diarrhoea in man. These pathogens cause acute, sporadic gastroenteritis in healthy individuals. In immuno-deficient persons (young children, elderly persons, those receiving immunosuppressive treatment and Aids patients), the illness may be prolonged and become be life threatening.

5.4.4 Exposure assessment

During the two-year sampling period samples containing either of the parasites were collected during the months August to April. This trend demonstrated a definite seasonal occurrence (Spring, Summer and Autumn) with the highest numbers occurring during the months October to March. Results obtained are summarized in Table 5.3.

The calculated geometric mean gives an indication of numbers of parasites people are exposed when using the river water (Table 5.3).

5.4.5 Dose response

Calculation of the risk of infection from the ingested dose requires dose-response information. For both organisms the dose response relation for infection has been investigated in human feeding studies. Rendtorff (1954b) reported results clearly indicating high infectivity. A high proportion of subjects shed newly formed cysts after a low ingested dose (3-10 cysts).

Dose response data for infection with *Cryptosporidium parvum* in human volunteers have been published by (Du Pont et al., 1995). The dose for infection for *Cryptosporidium parvum* appear to be five times lower than that of *Giardia lamblia* (Haas and Rose 1994).

Table 5.3
Summary Statistics for Giardia cysts and Cryptosporidium oocyst per 10-litre sample

Sampling site numbers	Geom	etric	Maxin	num	Minin	num	Averag	je	Numb samp analy	les
	G	C	G	C	G	C	G	C	G	C
62.4	0.6	ND .	60	ND	ND	ND	5.4	ND	13	13
66.1	ND	ND	ND	ND	ND	ND	ND	ND	19	19
63	ND	ND	ND	ND	ND	ND	ND	ND	20	20
62	0.4	ND	60	ND	ND	ND	5	ND	24	24
66.3	0.6	1.2	100	120	ND	ND	6.4	6.2	23	23
56.2	ND	ND	ND	ND	ND	ND	ND	ND	26	26
57	0.4	ND	18	ND	ND	ND	2.3	ND	8	8
67	0.9	ND	195	ND	ND	ND	17.3	ND	20	20
66.2	0.3	0.2	80	13	ND	ND	3	0.8	29	29
61	1.2	0.1	300	10	ND	ND	31.3	0.6	18	18
64	0.1	0.1	6	2	ND	ND	0.3	0.09	22	22
Darvill Final Effluent	13.6	0.3	520	110	ND	ND	62.5	3.7	48	48
Darvill Old Effluent	14	0.3	248	190	ND	ND	60.1	7.4	34	34

ND = Not detected

5.4.6 Assumptions adopted for the risk evaluation

Informal settlements occur along the uMsunduze River and its tributaries. Microbial contamination from these populations and their cattle herds lead to potentially high levels of contamination in these waters. Sampling points below the Darvill sewage treatment works, where higher Giardia and Cryptosporidium counts were recorded, are also in close proximity of informal settlements. It is known that people make use of the river water as a source of drinking water and wash water. Individuals also use the river as a recreational area where they may accidentally ingest water. This is especially true for young children who are considered more prone to infection.

Results obtained during the three-year sampling period showed that cysts and oocysts were only present during the months August to April. The majority of samples found positive were those collected and analysed during November to March. These are the hottest months in this part of the country and consumption of water as well as recreational activities will increase during this time. The increased ingestion of water could therefore increase the probability of infection.

The health risk assessment was conducted using various assumptions:

The numbers of Giardia and Cryptosporidium assumed to be ingested are based on the geometric means calculated for ten litre volume samples (Table 5) with the assumptions that:

An individual ingests 1.5 litres of water on a single occasion

An individual ingests 100m? of water while swimming

Because the data set incorporates a large number of non-detect results, which may be a function of the low detection rate of the test method, the geometric mean was calculated with non-detect results substituted with either

Zero

1 cyst

2 cysts

5.4.7 Risk characterization

Evaluation of the geometric mean parasitic levels in the river water over the monitoring period showed that *Giardia* cysts were more prevalent than *Cryptosporidium* oocysts. Counts showed little variation between sampling sites with the exception of higher counts obtained for samples taken from the Darvill Wastewater Works (Darvill Final Effluent and Darvill Old Effluent).

Daily weekly and yearly risk were calculated using the geometric mean of counts obtained for the uMsunduze River down stream of Darvill Maturation River effluent (66.3), uMsunduzi up stream Maturation River effluent (66.2) and Darvill Final Effluent.

Rose and Gerba (1991) suggested that the geometric mean be used for calculation of mean yearly risks because it more closely resembles maximum daily risks than yearly risks based on arithmetic mean levels.

The detection method for Giardia and Cryptosporidium used (Vesey et al., 1993) during this study, allows recovery of only approximately 68% oocysts from river water. Approximately 30% of the cysts are therefore not accounted for. If one oocyst was detected the actual number present might have been three oocysts. This may implicate that the large number of non-detect results (98% for Cryptosporidium and 91.5% for Giardia) may not have been zero. The risk of infection therefore might be much higher. This has been taken into account by substituting non-detect results with one and two cysts.

The United States Environmental protection Agency has suggested that one infection per 10 000 individuals per year (or 1x10⁻⁴) as an acceptable risk for infectious agents acquired drinking water (Rose and Gerba, 1991).

Table 5.4 Daily, weekly and yearly risk of infection after ingestion of Giardia in 1.5\ell of water (non-detect results =0)

Sampling site no	Number of Giardia (geom. mean non-detect results =0)	P-assuming daily consumption of 1.5 ℓ of water	P-assuming weekly consumption 1.5 ℓ of water	P-assuming daily consumption 1.5 \ell water for year
66.3	0.06	1.14E-03	7.95E-03	3.40E-01
66.2	0.03	5.70E-04	3.98E-03	1.88E-01
Darvill Effluent	0.68	1.28E-02	8.65E-02	9.91E-01

Table 5.4 represents the estimates of risk of infection when the non-detect results were actually zero. The results show that only 0.03-Giardia ingested with 1.5ℓ of water on a single occasion, poses a risk of 5 in 10 000. This is higher than the acceptable risk of 10^{-4} suggested by the EPA.

Table 5.5 Daily, weekly and yearly risk of infection after accidental ingestion of Giardia in 100m ℓ of water (non-detect results =0)

Sampling site no	Number of Giardia (geom. mean, non- detect results =0)	P-assuming daily consumption of 100 m/ of water	P-assuming weekly consumption 100 mℓ of water	P-assuming daily consumption 100mℓ water one year
66.3	0.016	3.04E-04	2.13E-03	1.05E-01
66.2	0.013	2.47E-04	1.73E-03	8.6E-02
Darvill Effluent	0.14	2.66E-03	1.84E-02	6.21E-01

Table 5.5 represents the risk calculated when 100mℓ of water is accidentally ingested at sampling sites 66.3, 66.2 and the Darvill final effluent when the non-detect results are actually zero. A single ingestion of the river water at site 66.3 will cause infection in three persons in 10 000. The risk gradually increases with the number of ingestions and will be one in 10 if 100mℓ is ingested daily over a period of one year.

Table 5.6 represents the probability of infection for daily, weekly and yearly consumption of 1.5 ℓ of water for sampling points 66.3, 66.2 and Darvill final effluent. Based on the assumption that an individual ingests 1.5 ℓ of river water per day contaminated with 0.24 Giardia cysts the risk of infection was found to be 4 in a 1000 (Table 9). The probability of infection increases to 8 in 10 if an individual ingests 1.5 ℓ of water as daily event for 365 days (Table 5.6).

Table 5.6 Daily, weekly and yearly risk of infection after ingestion of *Giardia* in 1.5 ℓ of water (non-detect results =1)

Sampling site no	Number of Giardia (geom. mean, non- detect results =1)	P-assuming daily consumption of 1.5 ℓ of water	P-assuming weekly consumption 1.5 ℓ of water	P-assuming daily consumption of 1.5 \ell of water over one year
66.3	0.24	4.55E-03	3.14E-02	8.11E-01
66.2	0.20	3.79E-03	2.62E-01	7.50E-01
Darvill effluent	2.19	4.08E-2	2.54E-01	1.00E+00

Table 5.7 Daily, weekly and yearly risk of infection after ingestion of Giardia in 1.5 ℓ of water (non-detect results=2)

Sampling site no	Number of Giardia (geom. mean, non- detect results =2)	consumption of	P-assuming weekly consumption 1.5 ℓ of water	P-assuming daily consumption of 1.5 \ell of water over one year
66.3	0.44	8.33E-03	4.55E-02	9.53E-01
66.2	0.35	6.63E-03	4.55E-02	9.12E-01
Darvill effl.	2.79	5.16E-02	3.10E-01	1.00E+00

Table 5.7 represents the risks calculated for sampling points 66.3, 66.2 and Darvill Final Effluent. To demonstrate the limitations of the detection method non-detect results were substituted with two during calculation of the geometric mean.

Based on the assumption that an individual ingests 1.5 ℓ of river water per day, contaminated with 0.44 *Giardia* cysts, the risk of infection doubled (8 in a 1000) when compared to the daily risk when non-detect results were substituted with one (Table 9 and 10). The annual risk of infection for the Darvill effluent was one (100% probability).

Table 5.8 Daily, weekly and yearly risk of infection after accidental ingestion of Giardia in 100 ml of water (non-detect results=1)

Sampling	Number of	P-assuming daily	P-assuming	P-assuming daily
site no	Giardia (geom.	consumption of	weekly	consumption of
	mean, non-	100 mℓ of water	consumption	100 mℓ of water
	detect results =1)		100 mℓ of water	over one year
66.3	0.016	3.04E-04	2.1E-03	1.05E-01
66.2	0.013	2.47E-04	1.73E-03	8.62E02
Darvill Effluent	0.146	2.77E-03	1.92E-02	6.37E-01

Table 5.8 represents daily, weekly and annual risks based on the assumption that an individual swimming could accidentally ingests 100 mℓ of water. If 100 mℓ of the Darvill Effluent is ingested the daily risk of infection will be 3 in 1000 and the weekly risk 1 in 2000. When 0.016 cysts are present in the water (sampling site 66.3) the daily risk of infection is 3 in 10 000.

Table 5.9 Daily, weekly and yearly risk of infection after ingestion of Cryptosporidium in 1.5 ℓ of water (non-detect results =1)

Sampling site no	Number of Cryptosporidium (geom. mean non detect results =1)	P-assuming daily consumption of 1.5 \ell of water	P-assuming weekly consumption 1.5 \ell of water	P-assuming daily consumption of 1.5 \ell of water over one year
66.3	0.18	7.20E-04	5.03E-03	2.31E-01
Darvill Effluent	0.2	8.00E-04	5.58E-03	2.53E-01

Tables 5.9 and 5.10 represents the daily, weekly and yearly risks for *Cryptosporidium* oocysts in samples 66.3 and the Darvill final effluent. Calculations shown in Table 12 were conducted with non-detect results substituted with one and those in Table 13 substituted with two. The annual risk of infection, when an individual ingests 1.5 ℓ of water, is 2 in 10 when non-detect results are substituted with one (Table 5.9). The risk is twice as high when non-detect results are substituted with two (4 in 10)(Table 5.10).

Table 5.10 Daily, weekly and yearly risk of infection after ingestion of Cryptosporidium in 1.5 ℓ of water (non-detect results=2)

Sampling site	Number of	P-assuming	P-assuming	P-assuming
no	Cryptosporidium (geom. mean non detect results =2)	daily consumption of 1.5 ℓ of water	weekly consumption 1.5 ℓ of water	daily consumption of 1.5 \ell of water over one year
66.3	0.35	1.40E-03	9.75E-03	4.00E-01
Darvill Final	0.36	1.44E-03	1.00E-02	4.09E-01

Table 5.11 Daily, weekly and yearly risk of infection after accidental ingestion of Cryptosporidium in 100 m£ of water (non-detect results =0)

Sampling site	Number of	P-assuming	P-assuming	P-assuming
no	Cryptosporidium (geom. mean non detect results =0)	daily consumption of 100 mℓ of water	weekly consumption 100 m/ of water	daily consumption 100 mℓ of water over one year
66.3	0.012	4.80E-05	3.36E-04	1.74E-02
Darvill Effluent	0.013	5.20E-05	3.64E-04	1.88E-02

Table 5.11 represents the risk calculated when 100 mℓ of water is accidentally ingested at sampling site 66.3 and the Darvill Final Effluent when the non-detect results are actually zero. With the assumption that an individual will ingest only 100 mℓ on a single occasion, containing 0.012 *Cryptosporidium* oocysts, the risk is negligible. Annual risk however will be higher than the EPA recommended yearly risk of 10⁻⁴.

Table 5.12 Daily, weekly and yearly risk of infection after accidental ingestion of Cryptosporidium in 100 ml of water (non-detect results=1)

Sampling site	Number of	P-assuming	P-assuming	P-assuming daily
no	Crypto (geom. mean non detect results =1)	daily consumption of 100 mℓ of water	weekly consumption 100 mℓ of water	consumption 100 mℓ of water over one year
66.3	0.012	4.80E-05	3.36E-04	1.74E-02
Darvill Effluent	0.013	5.20E-05	3.64E04	1.88E-02

Table 5.12 represents daily, weekly and annual risks based on the assumption that an individual swimming could accidentally ingests 100 mℓ of water. If 100 mℓ of river water at sampling site 66.3 is ingested on a single occasion the risk of infection will be 4 in 100 000. There is no risk of infection if 100 mℓ of the Darvill final effluent is ingested on a single occasion. The risk of infection, however, increases when weekly and annual ingestion is considered (1.7 in 100 and 1.8 in 100 for sampling site 66.3 and Darvill effluent respectively).

5.4.8 Uncertainty analysis

Uncertainty is an inherent characteristic of all risk assessments and uncertainties in this study were the following:

Dose response: The dose response information for both Giardia and Cryptosporidium is based on healthy adult hosts. This is not the most important group from the public health perspective. Compared to newborns, elderly persons, and other risk groups the risk of infection calculated with these data may be underestimated.

Distribution of microorganisms in water: It was assumed that the occurrence of microorganisms is governed by random distribution.

Volume of sample analysed: Rodda et al. (1993) found that the greatest restriction on detection of low risk levels was the volumes of water analyzed, which are not sufficient to detect low risks. The problem could be alleviated by increasing the sampling frequency to estimate risk over a shorter time frame. During this study the sample volume analyzed was only ten litres. This indicates that if zero cysts occur only limited protection is ensured. If, for example, 0.5 cysts occurred in 10 litres there should be 1 in 20 litres.

That divided by 2 litres per person drinking water per day would mean that one person in 10 would be exposed to one cyst every day.

Exposure rate: The risk assessment was based on hypothetical exposure rate. Daily activities and health information were assumed and were not applicable to specific individuals or populations.

Volume of water ingested: When the concentration of pathogenic organisms in the water is known the dose (the amount an individual is exposed to) is determined by the volume of unboiled or treated water ingested. The volumes of river water on which calculations were based in this assessment are assumed volumes.

Viability and infectivity of cysts and oocysts: Only limited information on the viability and infectivity of Giardia and Cryptosporidium in environmental waters such as river water is currently available. This assessment assumed that all cysts or oocysts ingested via the river water are viable and capable of infection. This is probably an over estimation of the actual number of infections that would occur in a population.

5.5 Conclusions

From the findings of this study the following conclusions can be made:

- Reported incidence of diarrhoea was high in the Vulindlela area with 39% overall and 49% in children under 5.
- The cause of diarrhoea could generally not be established from hospital or laboratory records; of the approximately 100 stool samples analysed each month, very few were positive for Giardia but approximately 3% were Cryptosporidium (mostly AIDS related therefore probably not of significance for water use).
- Giardia cysts were present in the schoolchildren's stool samples analysed, with 9.5% prevalence from the Kranskop area and 5.5% from Vulindlela; Cryptosporidium oocysts were not detected, however.
- There appeared to be a relationship between unprotected water sources and incidence of diarrhoea in the Vulindlela area, from clinic records. 91% of those who said they were using well water and 54% using spring water had diarrhoea.
- No correlation between water use and diarrhoea actually caused by Giardia or Cryptosporidium could be determined from the data.
- The potential risk of infection for Giardia and Cryptosporidium in rivers below sewage effluent outfalls, if used for drinking and recreation, would appear to be high. Drinking the Dusi river downstream of the Darvill works on a daily basis would give an annual risk of infection from Giardia of one (100% probability) and from Cryptosporidium, 4 persons out of every 10.

Swimming would similarly pose a risk for 1.8 persons out of every 100 for Cryptosporidium. (Within the framework of the assumptions made to perform this risk assessment).

- A total of 20 household and source water samples were analysed from families who
 reported diarrhoea, but all were negative for Giardia and Cryptosporidium.
- It would appear from reports that the protozoan parasites are not a problem for livestock in this area or in South Africa in general, despite international information that it is the most common cause of diarrhoea in calves, foals and lambs worldwide

6 GENERAL DISCUSSION

Few studies have been undertaken in South Africa to determine the incidence or prevalence of cryptosporidiosis or giardiasis in the human population. Published reports have, however, highlighted the risk to young children (Schutte et al., 1981; Berkowitz et al., 1988; Walters et al., 1988; Moodley et al., 1991a). Seasonal and temporal trends of human infections have been observed in a number of investigations, from stool samples. Those trends often coincided with periods of maximum rainfall, which were suggested as reflecting seasonal agricultural practices and environmental and zoonotic transmission (Moodley et al., 1991a).

However, in this study in KwaZulu-Natal, the incidence of *Cryptosporidium and Giardia* in river water and sewage effluents did not appear to correlate (Pearson's correlation test) with climatic factors such as rainfall, season or year, in this case. This possibly indicates that water-borne transmission is probably not the predominant route and other factors such as personal hygiene, potable water supply, sanitation and education probably have a more significant impact (Jarmey-Swan et al., 2000). *Cryptosporidium and Giardia* were found to be present in human stool samples, with laboratory-confirmed incidences ranging from 2.9 - 3.7% of cases submitted for analysis, from hospital records.

Although (oo)cysts were detected only sporadically in rivers, laboratory detection methods suffer from losses of oocysts and cysts which generally tend to escalate with increasing turbidity. These losses are further exacerbated by the recovery efficiency being more variable when the number of organisms is low. It is also possible that a variation in (oo)cyst load or non-point source contamination occurred, which random sampling missed. This may have resulted in underestimation of both the percentage of positive samples and the concentration of (oo)cysts in the water samples (Smith and Hayes, 1997).

The study indicated that PCR-based methods have the potential to be used to confirm the presence of protozoan cysts and oocysts in water samples. However, it was found to be incompatible with most of the methods which incorporate flocculation as a concentration step, owing to problems experienced with the sensitivity of the technique. It would therefore be difficult to implement this technology on a routine basis in the near future, although the PCR worked with membrane filtration and IMS, as well as flocculation with IMS. Yarn wound cartridge filters were not assessed as they have become unpopular recently due to poor recoveries and are rarely used in South Africa today.

Researchers in the USA have found *Cryptosporidium* oocysts in higher numbers than *Giardia* in water samples (Rose et al., 1991; Chauret et al., 1995). Madore et al. (1987), however, found that the two parasites often co-existed in polluted waters and the frequency of oocysts/cyst detection has been shown to be directly related to the degree of human activity in the watershed. In contrast, this research has shown that *Giardia appears* in much higher numbers than *Cryptosporidium* and that their source in rivers is more likely to be of human origin via either raw sewage or treated effluent, than animal. Recent studies (Venter et al 2003), have shown that the human strain of *Cryptosporidium* appears to be much the more common in South Africa, which concurs with this study and is the opposite of the situation on other continents.

This study has indicated that *Cryptosporidium* does not appear to be a problem for livestock in KwaZulu-Natal or in South Africa in general, despite international reports that it is the most common cause of diarrhoea in calves, foals and lambs worldwide (Jarmey-Swan and Bailey 1996).

Of the patients who reported diarrhoea, 45% had connections to Umgeni tap water and therefore this could be more general hygiene than water related. The actual use of the Umgeni tap water was also not guaranteed, since it was individually metered and charged for and previous traditional sources could still be used.

The potential risk of infection for Giardia and Cryptosporidium posed by river water, downstream of wastewater works, would appear to be high. Drinking the Dusi river downstream of the Darvill wastewater works on a daily basis would give an annual risk of infection from Giardia of one (100% probability) and from Cryptosporidium, 4 persons out of every 10. Swimming would similarly pose a risk for 1.8 persons out of every 100 for Cryptosporidium. (Within the framework of the assumptions made to perform this risk assessment)

The US EPA has suggested that one infection/10 000 individuals/year (or 1x10⁻⁴) is acceptable risk for infectious agents acquired through drinking water (Rose and Gerba, 1991).

There is a need for a better understanding of sources of contamination in catchments particularly with respect to protists. Monitoring plans which rely on sampling on an infrequent basis at various sites on a "hit and miss" basis are not useful in determining the likely impact of diffuse sources of water contamination. Intensive monitoring of specific areas is the only way to get an accurate picture of their likely input as the presence of parasites is likely to be sporadic, reflecting infection in the community and rainfall events. This is also true of other potential sources of contamination, such as sewage treatment facilities and again intensive monitoring will give a much more realistic picture than infrequent monitoring. The data acquired from such monitoring is invaluable for a number of reasons, in particular to indicate which sources of contamination need to be addressed as a priority as well as providing data about which types of future development are likely to be potentially the most dangerous.

Extensive monitoring of catchments may appear to be an expensive option, but the long-term gains in helping to prevent future contamination of the catchments and drinking water are clear. No evidence could be found of any water borne outbreaks of *Cryptosporidium*, in the general population, comparable to those experienced in other countries, eg. the USA and Europe, above the general background of endemic diarrhoea from other sources. It would appear that these parasites may be endemic in the general population, where they may be widespread resistance, preventing clinical symptoms.

It is also not certain whether the public health and environmental health services would be sufficiently sensitive to detect these occurrences, even if they did in fact occur, amongst the cholera, dysentery and AIDS cases.

7 CONCLUSIONS

- PCR-based methods have the potential to be used to confirm the presence of protozoan cysts and oocysts in water samples but owing to sensitivity problems it would be difficult to implement this technology on a routine basis in the near future.
- Occurrence in rivers was sporadic with Giardia cysts detected in 8% of samples and in higher numbers than Cryptosporidium oocysts, detected in 2% of samples; mainly during the wet summer months (although this may have been due to sewer breaks).
- As Cryptosporidium and Giardia were detected widely in river and wastewater samples, they are probably ubiquitous in Pietermaritzburg area community and possibly livestock.
- Darvill Wastewater Works was found to remove up to 99.9% of Giardia cysts, after the
 activated sludge process, chlorination and maturation, in the final effluent.
- Some 70% of Darvill effluent samples were positive for the protozoa, however, containing up to 520 Giardia cysts 10 ℓ⁻¹ and 110 10 ℓ⁻¹ Cryptosporidium oocysts with up to 200 cysts 10 ℓ⁻¹ in the river downstream.
- The potential Darvill effluent loading could be some 2.5 Billion cysts and 0.5 Billion oocysts per day into the uMsunduze River (at a dry weather flow of some 50 Mℓ /day), which constitutes half the dry weather (winter) flow of the river.
- Sludge disposed onto land was found to contain up to 3000 oocysts ℓ⁻¹ and 700 000 cysts ℓ⁻¹ and needs to be monitored if used in sensitive locations; desiccation experiments proved that viability could be reduced significantly.
- Neither the activated sludge process, nor the anaerobic digestion of sludge, appeared to significantly affect the viability status of the Cryptosporidium or Giardia.
- Treated effluents, which could be a source of water for humans and animals, need to be monitored prior to discharge into rivers, and possibly treated further to remove cysts.
- The potential risk of infection for Giardia and Cryptosporidium posed by the river water, downstream of sewage effluents would appear to be high, if used for drinking or recreation.
- The occurrence of Giardia cysts throughout the year in the effluent samples indicates that giardiasis is probably endemic in the Pietermaritzburg population, while cryptosporidiosis infections may be sporadic and quite possibly asymtomatic.
- Giardia cysts were present in (black) schoolchildren's stool samples analysed, with 9.5% prevalence from the Kranskop area and 5.5% from semi-rural Vulindlela; Cryptosporidium oocysts were not detected, however.
- Reported incidence of diarrhoea was high in the Vulindlela area, with 39% overall and 49% in children under 5.
- A total of 20 household and source water samples were analysed from families who reported diarrhoea, but all were negative for Giardia and Cryptosporidium
- The actual cause of diarrhoea could generally not be established from local clinic, hospital or laboratory records; very few stools tested were positive for Giardia but approximately 3% were Cryptosporidium (mostly AIDS related therefore probably not of significance for water use).
- It would appear that diseases caused by these pathogens are not prevalent in livestock in this area or in South Africa in general, despite international reports they are the most common cause of diarrhoea in calves, foals and lambs worldwide.

8 RECOMMENDATIONS

8.1 Health education and guidelines

(For schools, Environmental Health Officers, Veterinarians, DWAF, farmers etc)

- The potential risk of infection downstream of wastewater effluents, for human consumption and recreation (swimming), would appear to re the community, especially children must be made aware of this and steps taken to restrict access.
- The effluents themselves and maturation ponds etc are often used, as they appear clean, for portable water, swimming and fishing. Access control for these areas should be considered, along with warning signs and pictures in various languages.
- Livestock is also at risk from the above
- Health education at schools should include the above and that even chlorination will not remove these protozoa.
- Direct faecal-oral transmission amongst children especially at crèches would seem to be important, therefore personal hygiene should also be emphasised at school.
- Screening of high-risk school children would enable carriers of intestinal parasites to be identified, as these infections can seriously affect cognitive ability.
- Access by livestock to areas of fresh sewage sludge should also be restricted and the sludge should not be used to fertilise crops that are eaten raw.
- If used as soil conditioner etc the sludge should be dried first and monitored for the protists before being transported off-site.
- Veterinarians should be encouraged to send dung samples to Veterinary laboratories for identification purposes.

8.2 General guidelines

(For Municipal Managers, Water Boards, DWAF, farmers etc)

- Tertiary treatment should be considered at wastewater works or alternative disinfectants eg UV, which would inactivate (oo)cysts.
- Computerisation of hospital, clinic and laboratory records to aid prevalence studies.
- Catchment management to ensure recreational and abstraction points are not close downstream to wastewater works
- Catchment surveys to identify water treatment plant abstraction points at risk from broken sewers or septic tank effluents; these should be monitored regularly.

8.3 Future research

- Intensive monitoring of specific areas is the only way to get an accurate picture of the presence of parasites, as these are likely to be sporadic, reflecting infection in the community and rainfall events.
- The data acquired from such monitoring is invaluable to indicate which sources of contamination need to be addressed as a priority as well as providing data about which types of future development are likely to be potentially the most dangerous.

- Risk assessment of each Water Treatment Plant is necessary to prioritise resources for monitoring and should comprise both process and catchment assessments.
- 4. The role of livestock (and possibly wild animals), as the source of the protozoan parasites in rivers has not been properly investigated, although indications are that this is relatively unimportant and surveys of feedlots and farms needs to be undertaken in this regard.
- Typing of the different strains is now possible and early data suggests that there are different types for animals and human sources.
- Investigation into resistance patterns in humans, to the parasitic protozoa could establish how widespread these infections are.

TECHNOLOGY TRANSFER

9.1 Workshops

- · A KZN Parasite Control Programme workshop, hosted by the Provincial Environmental Health department, was attended and an overview presented on the occurrence of Cryptosporidium and Giardia in faecal and water samples with particular reference to the Pietermaritzburg area.
- A KZN Regional Communicable Disease Control meeting was also attended at which a similar presentation was made to highlight the occurrence of these protists in KwaZulu-Natal.
- The Allerton Regional Veterinary laboratories and local veterinarians were visited and the project described to them and the significance of the protozoan parasites was explained.
- Training in laboratory methods of detection was given to Umhlatuze Water, Allerton Regional Veterinary laboratory, Talbot and Talbot etc.

9.2 Publications

Jarmey-Swan C, Bailey IW and Howgrave-Graham AR, 1997. Detection, occurrence and epidemiology of Cryptosporidium in KwaZulu-Natal, South Africa. In 1997 International Symposium on Waterborne Cryptosporidium Proc., Editors: C.R. Fricker, J.L. Clancy and P.A. Rochelle, 159-170.

Jarmey-Swan C, Gibbs RA, Ho GE, Bailey IW and Howgrave-Graham AR, 2000. A novel method for detection of viable Giardia cysts in water samples. Wat. Res. 34 (6), 1948-1951. Jarmey-Swan C, Bailey IW and Howgrave-Graham AR 2001. Ubiquity of the waterborne pathogens Cryptosporidium and Giardia in KwaZulu-Natal populations. Water SA January 2001

Jarmey-Swan C, Bailey IW and Johnson C, 2000. Occurrence and source of Cryptosporidium and Giardia in catchment areas and wastewater works in KwaZulu-Natal. In The Water Institute of Southern Africa (WISA) biennial conference and exhibition: Health Impacts Proc.

External Education Services, Umgeni Water, have compiled:

- · "Water, Health and Hygiene" video, which highlights ways of purifying water, hygiene advice and ways of preventing the spread of gastro-enteritis.
- · "Be clean-be healthy-be happy" poster is available in English and Zulu and provides rural and peri-urban communities with information on maintaining toilets and general hygiene practices.

9.3 Archiving of data

The raw data for the relevant sections is archived as follows:

Umgeni Water:

Occurrence of parasitic protozoa in a catchment area.

CSIR:

Occurrence of Cryptosporidium and Giardia in a community

University of Pretoria: Development of a PCR technique for Cryptosporidium and Giardia

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