

Occurrence of *Cryptosporidium* spp. in South African irrigation waters and survival of *Cryptosporidium parvum* during vegetable processing

By

Géraldine L.M.C. Duhain

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DECLARATION

I declare that the dissertation herewith submitted for the degree MSc Food Science at the University of Pretoria has not previously been submitted by me for a degree at any other university or institution of higher education.

Géraldine L.M.C. Duhain

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DEDICATION

I dedicate this dissertation to my parents, Françoise and Pierre, and to my siblings, Louise, Guillaume and Julien.

ABSTRACT

OCCURRENCE OF *CRYPTOSPORIDIUM* SPP. IN SOUTH AFRICAN IRRIGATION WATERS AND SURVIVAL OF *CRYPTOSPORIDIUM PARVUM* DURING VEGETABLE PROCESSING

By Géraldine Duhain

Supervisor: Prof. Elna M. Buys

Co-supervisor: Prof. Amanda Minnaar

Department: Food Science

Degree: MSc Food Science

Surface waters used for irrigation purposes in South Africa have been found to be of poor microbiological quality and to be contaminated with human pathogens. These pathogens can be transferred from contaminated water onto fresh produce and potentially cause human infections. *Cryptosporidium* and *Giardia* are waterborne parasitic protozoa that have been found in surface waters worldwide. They can cause morbidity in infected individuals and be lethal when infecting people with compromised immunity. This study was divided into two phases.

The first phase was a field survey aimed at determining the incidence of human pathogens *Cryptosporidium*, *Giardia* and *Salmonella* spp. in rivers used for irrigation purposes in South Africa as well as on vegetables irrigated with these rivers. The rivers selected were from three different provinces of South Africa. The relationship between faecal indicators and the presence of *Cryptosporidium* oocysts and *Giardia* cysts was also investigated. Out of the 30 water samples analysed, 43% were positive for *Cryptosporidium* oocysts, 23% tested positive for *Giardia* cysts and 27% were positive for *Salmonella* spp. However, no *Cryptosporidium* oocysts or *Giardia* cysts were found on the vegetables analysed. No significant differences in the

prevalence of *Cryptosporidium* and *Giardia* and indicator parameters were observed between the three rivers. Using a logistic regression model, no significant correlations were observed between the incidence of faecal indicators and the presence of *Cryptosporidium* oocysts and *Giardia* cysts.

In the second phase of the study, the individual and combined effects of chlorine, blanching, blast freezing and microwave heating on *Cryptosporidium parvum* oocysts inoculated on green peppers were investigated. The viability of the oocysts after treatments was determined using the vital dye propidium iodide. Stained oocysts were counted with a flow cytometer. Chlorine treatments did not significantly affect the viability of the oocysts. Blast freezing affected the viability of 20% of the oocysts. Both microwave heating and blanching affected 93% of oocysts. Combined treatments with chlorine and blast freezing did not affect the viability of the oocysts significantly compared to the control. Combined treatment with chlorine and microwave heating was significantly more effective than microwave heating alone and affected 98.1 % of the oocysts. The data indicate that *C. parvum* oocysts are sensitive to heat and, to some extent, to freezing temperature but are resistant to chlorine.

The results of the survey show the presence of *Cryptosporidium* and *Giardia* in irrigation waters and thus a possible health risk associated with the consumption of raw vegetables as those can become contaminated via the irrigation water. The results of the challenge tests indicate that *C. parvum* oocysts on vegetables are inactivated by blanching and microwave heating but survive blast freezing and exposure to chlorine. Boiling and microwave heating of vegetables should be sufficient to kill *C. parvum*. On the other hand, ready-to-eat vegetables could be at risk of carrying live *C. parvum* oocysts as the use of chlorine in washing bath is not expected to inactivate *C. parvum* oocysts present on vegetables.

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CHAPTER 1: INTRODUCTION AND PROBLEM STATEMENT

According to Beuchat (2002), foodborne infection outbreaks associated with the consumption of raw vegetables have been increasing world-wide. One of the reasons for this increase is the changes in consumers' dietary habits. People are becoming more concerned about their health and have increased their consumption of fresh and minimally processed fruits and vegetables. They demand ready-to-eat fresh products, free from chemical preservatives (Zink, 2009). Also, in order to increase the vitamin intake of populations in developing countries, nutritionists recommend these populations grow their own vegetable gardens and consume their own vegetables (Zachariah, Teck, Buhendwa, Labana, Chinji, Humblet and Harries, 2006). Microbiological results from irrigation water and vegetables in South Africa have however indicated that the downside of consuming more fresh vegetables is the possible increased exposure to pathogens and thus increased risks of infection, especially if the vegetables are consumed in the raw state (Britz, Sigge and Barnes, 2008).

Because water resources in South Africa are limited, most farmers rely on untreated surface water for irrigation of their vegetable crops. However, surface water can be of poor microbiological quality and could contaminate the vegetables with pathogenic organisms since contaminated water is deposited onto the surface of the crops during irrigation (Robertson and Gjerde, 2001a). High levels of *Escherichia coli*, indicator of faecal pollution as well as bacterial pathogens *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella* spp. have been recovered in rivers from Western Cape, Mpumalanga, Gauteng and North West Province (Britz *et al.*, 2008). Moreover, diarrhea outbreaks of waterborne sources have been reported in Delmas and Standerton therefore showing the impact of water pollution on the population's health in South Africa (NICD, 2007; NICD 2008).

Apart from the pathogens already mentioned, pathogenic protozoa can also be found in untreated surface water. *Cryptosporidium* and *Giardia* are two of the most common and resistant human waterborne parasitic protozoa (Chaidez, Soto, Gortares and Mena, 2005). They both have been associated with severe outbreaks of diarrhea in humans. They cause morbidity in healthy individuals and can be lethal in susceptible population. *Cryptosporidium* and *Giardia* are regularly isolated from raw water in the Gauteng area (Monique Grundlingh, Rand Water, personal communication, 2008). In a survey done by Kfir, Hilner, du Preez and Bateman (1995), *Cryptosporidium* and *Giardia* were found in South African surface water, treated effluent and drinking water. Because of the high cost and low recovery rate of the isolation methods for *Cryptosporidium* and *Giardia*, it is important to assess the correlation between the presence of faecal indicator organisms such as *E. coli*, coliforms and incidence of *Cryptosporidium* and *Giardia*. Few studies have been done in South Africa to determine the simultaneous incidence of *Cryptosporidium* and *Giardia* and faecal indicators. Therefore one of the objectives of this study is to determine whether the presence of faecal indicators can be linked to the presence of *Cryptosporidium* oocysts and *Giardia* cysts in irrigation water and on vegetables in South Africa.

Most reported cryptosporidiosis and giardiasis outbreaks have been associated with drinking water. However, *Cryptosporidium* oocysts have been found on the surface of raw vegetables in both developing and developed countries (Robertson and Gjerde, 2001b). The transmissive stage of these parasites can survive for long periods of time especially in moist environment such as those surrounding fresh produce. Furthermore *Cryptosporidium* oocysts and *Giardia* cysts are resistant to chlorine at the concentration and contact times usually used for washing vegetables and thus might not be eliminated by washing (Chaidez *et al.*, 2005). The cysts and oocysts could therefore survive on both washed and unwashed vegetables and cause foodborne infections. Also, the presence of the pathogens on export produce could lead to serious economical losses if fruits are rejected and exportation suspended (Britz, Sigge and Barnes, 2008).

Apart from being washed, vegetables can also be blanched and frozen before being retailed. Once bought, vegetables can also be cooked either in hot water or in a microwave before being consumed. These treatments might affect the viability of *Cryptosporidium* oocysts. Little is known about the hurdle effect of combined treatments on the survival of oocysts on vegetables. This study will thus investigate the individual as well as combined effects of chlorine, blanching, freezing and microwaving on *Cryptosporidium* oocysts structure when inoculated on fresh produce.

CHAPTER 2: LITERATURE REVIEW

2.1 WATER SITUATION IN SOUTH AFRICA

Water is indispensable to life and is essential for food production, hygiene, sanitation and for economic development. However, water resources in South Africa are scarce and limited since a large part of South Africa is located in a semi-arid part of the world. The average annual rainfall for the country is 495 mm, and ranges from 100 mm/year in the western desert to about 1200 mm/year in the eastern part of the country (FAO, 2005). Water resources in South Africa are made up of surface water and ground water. Surface water resources include rivers, dams, pans, wetlands and dolomite eyes fed by underground water sources. The river network in South Africa can be divided into four major systems, namely the Orange river system which includes the Caledon and the Vaal rivers, the Limpopo system in the North which includes the Olifants and Crocodile rivers, the Tugela river and the Olifants river system in the south-western part of the country (FAO, 2005). Groundwater is the term used to describe underground water resources, which flow between soil pore spaces and within underground rock formations (NWPEO, 2008).

Renewable, water is a finite resource, which requires careful management and protection. It is important for water to be of acceptable quality for human and other uses. Water is highly susceptible to pollution and continued deterioration of water quality in some parts of South Africa has been reported (DACE, 2004). Bacteriological contamination of water sources is wide spread in the country and can lead to waterborne disease outbreaks. One of the major sources of microbiological pollutions are the many informal settlements with no or poorly maintained sanitation facilities that have been established near rivers. Runoff waters from these areas carry high levels of faecal matter of human origin that can possibly contain human pathogens (Britz, 2007). Increased urban development has

caused an increased pollution load. This together with lack of adequate water treatment plants and sewerage work maintenance has led to an increase in sewage effluent being released into surface water in recent years (Groenewald, 2010a, 2010b). Pollutants from mining, pesticides used in agriculture, leach from waste disposal facilities and uncontrolled dumping near water sources are contributing to the increased pollution of our surface waters (Groenewald, 2009).

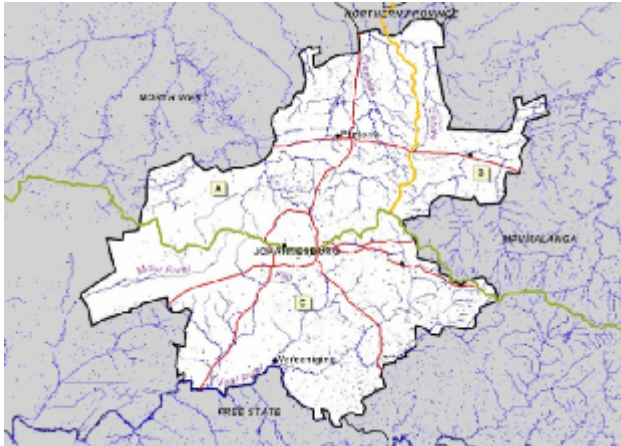
The demand on South Africa's water resource is increasing due to urban and agricultural development. Climate change is expected to increase even further the variability and intensity of rainfall putting even more pressure on the already scarce water resources. River flow in some parts of the country is expected to drop by up to 58% by the end of the century (De Wit and Stankiewicz, 2006). Already 11 of the 19 water management areas in South Africa are facing a water deficit (based on 2000 figures) (MSER, 2003).

The water situation in the provinces included in this study, namely the Gauteng, North West Province and Mpumalanga, will now be discussed in more details.

2.1.1 GAUTENG PROVINCE

Gauteng Province includes three water management areas namely the Crocodile West-Marico, Upper Vaal and Olifants river (Figure 1a). Due to the high demand for water, the province can not only rely on natural surface runoff and ground water but must import raw water from outside the province (DACE, 2004)

a)



b)



c)

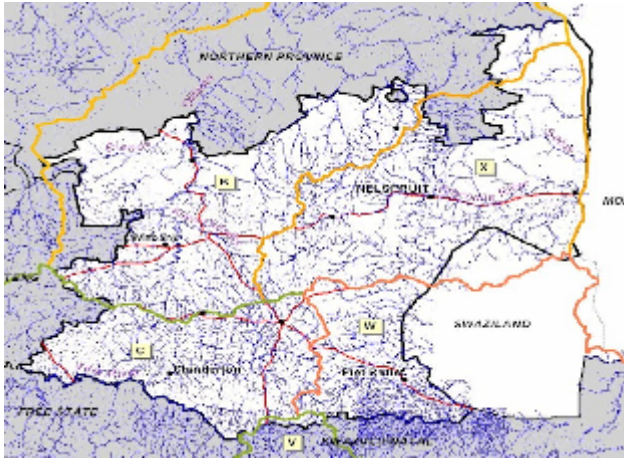


Figure 1: Map showing the major rivers of the Gauteng province (a), the North West province (b) and the Mpumalanga province (c) (DWA, 2010)

Demand for water in Gauteng is increasing due to the increasing population density and economic development. The mineral industry and growing agricultural activities put an extra strain on the water resources. Furthermore, increased runoff from increasing urbanisation is causing a loss to the ground water recharge potential (DACE, 2004). Aquifer de-watering and re-watering caused by mining activities is a big problem in Gauteng. This causes water to migrate from elevated regions to lower lying areas thus leaving some areas without water (DACE, 2004).

The microbiological quality of the Klip river was monitored in this study. Agriculture is one of the main users of water from the Klip river catchments and it was estimated that 4 400 ha of land could be irrigated in the catchments (Kotze, 2002). Treated sewage effluent from Johannesburg's southern wastewater treatment work are used to irrigate crops in the upper Klip river while sewage sludge from the East Rand Water Care Works are used for irrigation in the area of Klip river and Rietspruit (Kotze, 2002). Maize, fodder crops and vegetable such as carrots, spinach, cabbage, onions, potatoes and salad greens are the main irrigated crops grown in the Klip river catchments area (Kotze, 2002).

The quality of surface water in Gauteng has been found to be poor due to chemical pollution from mines and microbiological pollution from informal settlements and faulty

sewage treatment works (DACE, 2004; Groenewald, 2010a). The sulphate/chloride ratio, which is used to indicate the effect of mining on water salinity, has been found to be elevated in the Blesbokspruit, Klip river and Wonderfonteinspruit showing the polluting effect of mining in those areas. High levels of total dissolved solids were also found in those waters indicating discharge from industrial and mining activity. The levels of faecal coliforms in the Klip river and Blesbokspruit have been found to be elevated since 2000. This shows pollution of these rivers with faecal material that could most likely be carrying human pathogens (DACE, 2004).

In 2003, the ecological status of rivers in Gauteng was described as poor to fair. This means that the natural functioning of the rivers had been disrupted and the rivers ecosystem extensively used (River Health Program, 2003). The fish population, which is used as a good indication of the long term influences of pollution on a river, has been described as poor in the Upper Klip river, Natalspruit river, Lower Klip river, Suikerbosrand river, Rietspruit river and Blesbokspruit river (DACE, 2004).

Most of the major dams in Gauteng suffer from high levels of eutrophication which is caused by enrichment of water with plant nutrients, especially nitrogen and phosphorus compounds. Hyper eutrophication can cause serious health effects. The process of eutrophication takes thousands of years to occur naturally but in this case unnatural eutrophication was a direct result of agricultural and urban runoff, municipal and industrial wastewater effluents, and septic tanks that all contribute organic nutrients (DACE, 2004).

2.1.2 NORTH WEST PROVINCE

The four management areas in North West are the lower Vaal, middle Vaal, upper Vaal and Crocodile Marico river (Figure 1b) (NWDACE, 2008).

The ground water in North West is of particular importance as 80% of the water used in rural and agricultural activities comes from ground water (NWDACE, 2008). Depletion

of the ground water resources is caused by use of water for irrigation, drinking and stock watering as well as by removal or transfer of water associated with mining activities and industrial development (NWDACE, 2008). Irrigated agriculture is the biggest user of water in North West and requires 418 million m³ per annum, most of which come from ground water. Vegetable crops grown under irrigated conditions in the North West Province include potatoes, cabbage, peppers, beans and tomatoes (NWPG, 2010).

Ground water in North West was found to be of reasonable quality according to a study done by Zitholele Consulting (2008). However high nitrate and total dissolved solids concentrations were found in some regions. High level of sulphate due to acid mine drainage from gold mines in the far West Rand were also observed (NWDACE, 2008).

The surface water resources in North West province are under pressure due to population growth, mining, agricultural and industrial water uses (NWDACE, 2008). The surface water resources are scarce as many surface water systems are non perennial. More surface water is available in the east of the province than in the west. The main sources of surface water pollution in the North West province are diffusion of fertilizers run-off, insecticides and herbicides from agricultural land and storm water runoff from urban surfaces such as roads as well as diffusion from dense settlements. The rivers in North West province are also polluted by effluent from mining and industrial activities (NWDACE, 2008).

High levels of nitrate and nitrites were found in the rivers of the North West province which is indicative of high nutrient status. This is associated with agriculture and industrial pollution e.g. discharges of sewage effluent in the rivers (DACE, 2004; NWDACE, 2008). High levels of nitrite can lead to eutrophication and algal bloom in dams. Eutrophication impacts negatively on tourism and recreational sport as it leads to unpleasant odours, kills the fish and causes a potential health risk for the recreational water users. High faecal coliforms levels have been observed in rivers in North West province between 2002 and 2007. The faecal coliform counts were above the Department of Water Affairs and Forestry (DWAF) standard of 2000 faecal coliforms count/100ml

for water for domestic and recreational water. The counts were also above the World Health Organisation (WHO) standard of 1000 faecal coliform count/100 ml in irrigation water (NWDACE, 2008; WHO, 1989). Faecal coliforms indicate pollution of the rivers with faecal matter which indicates possible contamination with human pathogens (Scott, Rose, Jenkins, Farrah and Lukasik, 2002). Effluents from sewage treatment plants as well as runoff from poorly sanitised settlements are the probable cause for these high faecal coliform counts. The North West province has the highest number of informal settlements of the country as 23% of the household are described as informal (Statistics South Africa, 2007). Sanitation in these settlements is poor and water runoffs from these areas contain high levels of faecal matter.

2.1.3 MPUMALANGA PROVINCE

The sources of four of South Africa's major river systems are found in Mpumalanga, namely the Olifants river system, the Orange river system (Vaal river), the Inkomati river system (Crocodile, Sabie, Sand and Komati rivers), and the Pongola river systems (Figure 1c) (MSER, 2003).

Most of the water available in Mpumalanga comes from surface water (65%). Water transfers from outside the province provides 19% of the total available water while only 6% of the water comes from ground water (MSER, 2003). Return flow from mining, industrial, urban sectors and from irrigation account for 10% of the total water available in Mpumalanga. In Mpumalanga, 46% of the water available is used by farmers to irrigate their crops (MSER, 2003). However, the demand for irrigation water is unevenly distributed throughout the province with 28% of water requirement for irrigation coming from the middle Olifant river area alone in the North East of the Province. This is where the Loskop dam and Loskop dam irrigation scheme are located (MSER, 2003). Wheat, vegetables, tobacco, peanuts, cotton and citrus fruit are cultivated and irrigated under the

Loskop dam irrigation scheme in Mpumalanga (Loskop Irrigation Board, 2009). However, increased pollution levels in the Olifant river have been reported in the media creating concerns about the safety of fresh produce and the risk of losing exportation deals (Groenewald, 2010b).

There has been a general decrease in water quality in Mpumalanga since 1996 as shown by various quality indicators such as water nutrient level, total dissolved solids, pH and metal concentration (MSER, 2003). The eutrophication levels in Mpumalanga's dams were within acceptable levels in 2002. The Loskop and Witbank dams were described as mesotrophic while the Middelburg dam was oligotrophic which means that these dams contain low levels of organic matter. The Grootdraai dam, however, was found to be eutrophic which means it contains higher levels of nutrients and algae. High levels of total dissolved solid content have been found in the upper Vaal and Olifants water management areas (MSER, 2003). These high levels usually indicate discharge from industrial and mining activities (DACE, 2004). High levels of sulphate and heavy metals were also observed in the surface waters of the province and these levels exceeded standard set in the South African drinking water quality guidelines for industrial and irrigated agricultural use (MSER, 2003).

2.2 WATER USE IN IRRIGATION

Agriculture practices in South Africa are limited by the availability of water. Due to low rainfall and high incidence of dry spells, farmers in most part of the country are dependent on irrigation as 65% of the country does not receive sufficient rainfall for successful rain-fed crop production (FAO, 2005). It is thus not surprising that irrigation is the main user of water in South Africa accounting for 62% of water withdrawal in the country (MSER, 2003; FAO, 2005). The main irrigated crops are fodder crops, wheat, maize, vegetables and pulses. Horticulture is a major user of water for irrigation; vegetable production alone accounted for 8% of total harvested irrigated cropped area in South Africa in 2000 (FAO, 2005).

Because clean water resources in South Africa are limited, farmers often have to use water sources of poor quality to irrigate their crops. Water used for irrigation can be classified according to their composition and where they originate from (Britz, 2007). Raw water refers to untreated water from surface water sources such as rivers and dams or groundwater from wells. Wastewater refers to water that has already been used for domestic or industrial use. Wastewater can be divided into sullage and black water. Sullage includes household grey water but excludes water from toilets. Black water refers to toilet waste. In poor populations living in informal settlements, wastewaters contain high concentrations of human excreta and thus possibly high levels of human pathogens. Storm water refers to water generated from runoffs from house roofs, paved areas and roads during rainfall. Effluents from water treatment plants are returned to the nearest natural water surface to make a contribution to the river ecology. Storm water and effluent from water treatment plants contribute to the flow and microbiological quality of surface waters. This contribution can be beneficial or detrimental depending on the efficiency of the water treatment plant and the quality of the effluent being discharged in the rivers (Britz, 2007). Unfortunately the poor maintenance and over loading of water treatment plants have been reported in South Africa. Dumping of raw sewage and effluent of poor quality into rivers has thus resulted in contamination of rivers with high levels of faecal material (Groenewald, 2009).

The use of water of poor microbiological quality to irrigate fresh produce could constitute a public health hazard as human pathogens can be transferred to crops during irrigation (Armon, Gold, Brodsky and Oron, 2002). This is of particular importance if water containing pathogens is used to irrigate crops that will not be cooked prior to consumption (Tyrrel, 1999).

2.3 SOUTH AFRICAN FRUIT AND VEGETABLE INDUSTRY

In 2003, agriculture contributed 3.8% to the GDP and employed 8% of the labour force in South Africa (FAO, 2005). South Africa is a net exporter of food products. Maize is South Africa's main farm export while other major crops include sugar cane, wheat,

potatoes, groundnut, citrus fruit and grapes (FAO, 2005). According to the National Department of Agriculture (DOA, 2009), the total quantity of the most important vegetables sold on fresh produce market increased from 2 046 300 tons in 1997 to 2 115 400 tons in 2008. In 2008, the major contributors of important vegetables sold on fresh produce markets were potatoes (1853 000 tons), tomatoes, (426 000 tons), onions (375 000 tons), green mielies (323 000 tons) and pumpkins (226 000 tons) (DOA, 2009).

South Africa is the major and leading exporter of fruits and vegetables in Africa. South Africa holds 31% percent of share in the European Union (EU) market and is now the largest third-country supplier of fruits and vegetable to the EU. Just behind is Turkey with 29% share and Morocco with 22% share in the fruit and vegetable EU market (Diop and Jaffee, 2005). Several countries in Sub Saharan Africa export fruits and vegetables; however three countries dominate the market. South Africa, Cote d'Ivoire and Kenya account for 90 % of international export of fruits and vegetables in Sub Saharan Africa with South Africa as the leading exporter (Diop and Jaffee, 2005). South Africa, together with Chile and Argentina dominate the exports market for apples, grapes and pears to the European Union. South Africa is also a major exporter of fruits and vegetables to the United States (Diop and Jaffee, 2005).

It is clear that fruits and vegetables play a major role in South Africa's economy. South African farmers and retailers are concerned over the state of the rivers and the impact it could have on the quality and safety of their products. Major retailers are worried to loose exportation deals with Europe due to the presence of pathogens on their fresh produce (Tempelhoff, 2010). It is thus important to avoid contamination of fresh produce with pathogens as outbreak of foodborne illnesses as a result of consumption of South African fresh products would lead to banning export of such produce to overseas countries which would lead to severe economical losses (Arnade, Calvin and Kuchler, 2009).

2.4 WATERBORNE PROTOZOA PATHOGENS

2.4.1 CRYPTOSPORIDIUM

Cryptosporidium is an obligate, intracellular, eukaryotic protozoon from the phylum Apicomplexa. The first cases of cryptosporidiosis were reported in 1976 and cryptosporidiosis infections have now been reported in over 90 countries and six continents (Fayer, Morgan and Upton, 2000).

Cryptosporidium spp. infects both human and animals and over 152 species of mammals have been reported to be infected with *Cryptosporidium* (Fayer *et al.*, 2000). *Cryptosporidium hominis* is the specie that causes most human cryptosporidiosis and *Cryptosporidium parvum* is typically found in ruminants but can also infect humans (Dawson, 2005). Other species of *Cryptosporidium* have also been reported to infect humans and zoonotic transmission from animal to human is thus possible (MMWR, 2010).

Cryptosporidium has a two-stage life cycle consisting of a reproductive stage and an environmentally resistant oocyst stage. Oocysts are spore-like survival structures, resistant to environmental stresses and commonly used disinfectants such as chlorine (Moriarty, Duffy, McEvoy, Caccio, Sheridan, McDowell and Blair, 2005). The great resistance of *Cryptosporidium* oocysts to chemical disinfectants is attributed to their oocysts walls which are made of a double layer of protein-lipid-carbohydrate matrix (Templeton, Lancto, Vigdorovich, Liu, London, Hadsall and Abrahamsen, 2004). Oocysts are spherical in shape, measure between 4 to 6 µm and contain four sporozoites. Amylopectin is the polysaccharide found in coccidian protozoa and provides energy for excystation and penetration of host cells (Harris, Adrian and Petry, 2003). Small amounts of amylopectin are also used during the dormancy stage and *Cryptosporidium* oocysts have been found to be unable to infect host once the amylopectin store has been depleted (Vetterling and Doran, 1969).

Oocysts are the stage transmitted from an infected host to a susceptible host by the faecal oral route (Fayer *et al.*, 2000). Oocysts can be transmitted from person to person, from animal to human, through drinking contaminated water or eating contaminated food and

from swimming in contaminated water (Fayer *et al.*, 2000; MMWR, 2010). Once ingested, oocysts release the reproductive stage: the sporozoites. Sporozoites attach themselves to the intestinal epithelium and multiply, damaging the mucous membrane of the intestinal lining and causing diarrhea (Dawson, 2005). New oocysts are then formed and shed in the faeces of infected host. *Cryptosporidium* has a low infective dose and the ingestion of as few as 10 oocysts has been shown to cause infections. The infective dose varies from species to species but the average infective dose has been calculated to be 87 oocysts (Okhuysen, Chappell, Crabb, Sterling and Dupont, 1999). The incubation period varies between 3-7 days (Tzipori and Ward, 2002). *Cryptosporidium* infections are usually self limiting in healthy individuals but can become chronic and life threatening in individuals with weakened immune systems (Dawson, 2005; Lane and Lloyd, 2002). Populations especially at risk of infections are children, malnourished persons, and individuals with compromised immunity including AIDS patient, transplant recipient, patients receiving chemotherapy for cancer, institutionalized patients and patients with immunosuppressive infectious diseases (Fayer *et al.*, 2000). Up to 2002, no drug therapy had been found effective against *Cryptosporidium* infections. Treatment for cryptosporidiosis is mainly symptomatic and supportive (Fayer *et al.*, 2000). In 2002, Nitazoxanide was the first and only broad spectrum anti-parasitic drug approved for use in the United State for the treatment of cryptosporidiosis (MMWR, 2010).

2.4.2 GIARDIA

Giardia lamblia is a waterborne, flagellated protozoon from the order Diplomonadida and was first discovered in 1681 by Antoine Van Leeuwenhoek (Lane and Lloyd, 2002). The *Giardia* species infecting humans are *Giardia intestinalis* and *Giardia duodenalis* sometime called *Giardia lamblia*. *Giardia lamblia* is endemic throughout the world with a high incidence in the tropics and subtropics. It infects humans, mammals, reptile and birds (Lane and Lloyd, 2002). Giardiasis is now recognized as a traveler's disease worldwide. It is one of the most common source of intestinal infection in the developed world as well as a serious cause of infection in developing countries (Stevens, 1982;

Swaminathan, Torresi, Schlagenhauf, Thursky, Wilder-Smith, Connor, Schwartz, VonSonnenberg, Keystone and O'Brien, 2009).

Giardia lamblia has a two stage life cycle and alternates between trophozoite and cysts. The trophozoite is the vegetative reproductive stage. It is pear shape and 9.5 to 21 μm long by 5 to 15 μm wide, has four pairs of flagellae and two nuclei. The trophozoite has a ventral sucking disk made of microtubule which helps with attachment to the intestinal mucosal layer of its host (Wolfe, 1992). Cysts are the survival stage and the infectious form that is found in the environment (Lane and Lloyd, 2002). They are ovoid, measure 8 to 12 μm long by 7 to 10 μm wide and contain four nuclei. Cysts have a hyaline cyst wall which makes them resistant to chlorination and they can survive for several months in cold surface water (Lane and Lloyd, 2002).

Infection occurs when cysts are ingested with contaminated food or water. Cysts pass through the stomach undamaged and excyst once they reach the duodenum where the alkaline pH is favourable for the growth of the trophozoites. Two trophozoites are released from each mature cyst. Some trophozoites then attach themselves to the intestinal villi with the help of the sucking disks while others swim freely in the duodenum and ileum (Lane and Lloyd, 2002). Trophozoites replicate asexually by binary fission inside the intestine, damaging the epithelial cells and causing the symptoms of giardiasis. New cysts are then formed which are discharged in stools of infected hosts and then transmitted to new host through food or water (Thompson, 2000). Humans are the main reservoir of *Giardia* but animals can carry species of *Giardia* similar to those infecting humans (Wolfe, 1992). Calves are also a big reservoir of *Giardia* with a high incidence of infection worldwide (Olson, Thorlakson, Deselliers, Morck and McAllister, 1997). *Gardia duodenalis* which includes *G. lamblia* naturally infects humans, beavers, coyotes, cattle, cats and dogs (Wolfe, 1992). Species of *Giardia* originating from animals have been reported to be infectious to humans as well (Wolfe, 1992). Shedding of high numbers of cyst in the environment by livestock is thus a public health concern as this could lead to zoonotic infections (Lane and Lloyd, 2002).

Giardiasis infection occurs by faecal-oral contamination with cysts or indirectly through consumption of contaminated food or water. Ingestion of 100 or more cysts is required to ensure infections in humans but as few as 10 cysts have resulted in infections in volunteers. This is due to the fact that a single ingested cyst will divide and quickly multiply to infectious levels (Lane and Lloyds, 2002). Travellers often become infected by drinking contaminated ground or surface water. The incubation time varies between 9 to 15 days and the symptoms of the disease are variable but include nausea, low fever, watery diarrhea and malabsorption (Wolfe, 1992; Thompson, 2000). The acute stage lasts between 3 to 4 days and usually clears spontaneously in individuals with healthy immune system. However, chronic infection may develop and last two or more years or even become fatal in individuals with compromised immunity (Wolfe, 1992). Yet, around 13% of infected adults and up to 50% of infected children remain asymptomatic (Wolfe, 1992). Several drugs are available to treat the infection. In South Africa the use of metronidazole is recommended (National Department of Health, 1998).

2.5 WATER CONTAMINATION WITH *CRYPTOSPORIDIUM* AND *GIARDIA*

Cryptosporidium and *Giardia* have mainly been associated with waterborne outbreaks. *Cryptosporidium* oocysts are ubiquitous in surface water worldwide (WHO, 2008) and have been found in untreated surface water (Ong, Moorehead, Ross and Isaac-Renton, 1996), ground water (D'Antonio, Winn, Taylor, Gustafson, Current, Rhodes, Gary and Zajac, 1985), treated drinking water (D'Antonio *et al.*, 1985), recreational water and even bottled water (Franco and Neto, 2002). Kfir, Hilner, du Preez and Bateman (1995) investigated the presence of *Cryptosporidium* and *Giardia* in surface water in South Africa. They found 30% of samples tested positive for *Giardia* cysts while 2.9% of samples tested positive for both *Giardia* and *Cryptosporidium* (Kfir *et al.*, 1995).

The first confirmed documented waterborne outbreak of cryptosporidiosis occurred in Texas in 1984 and was caused by unfiltered ground water supply (D'Antonio *et al.*,

1985). The number of reported cases of cryptosporidiosis has been increasing ever since and the centre of disease control in the United State has reported an increased from 6 479 cryptosporidiosis cases in 2006 to 10 500 cases in 2008 (MMWR, 2010). On the other hand, giardiasis cases are on a slight decline and 19 140 cases of giardiasis were reported in the United State during 2008 while 19 238 cases were reported in 2006. More cases of cryptosporidiosis were reported in summer than in winter. This was mainly attributed to the increase in recreational water-associated outbreaks due to *Cryptosporidium* oocysts' resistance to chlorine (MMWR, 2010). Recreational water-associated outbreaks of giardiasis are also well documented and *Giardia* was found to be responsible for 3.7% of recreational water-associated gastroenteritis outbreaks in the United States between 1997 and 2006 (MMWR, 2010).

Humans and animals act as a reservoir of *Cryptosporidium* and *Giardia*. In a study carried out in 1986 in a Durban hospital, South Africa, *Cryptosporidium* was the second most common organism detected among children admitted with diarrhoea (Van den Enden, 1986). In Limpopo, South Africa, a *Cryptosporidium* incidence of 18% was observed in school children and hospital patients (Samie, Bessong, Obi, Sevilleja, Stroup, Houpt and Guerrant, 2006). It has been estimated that 32% of AIDS patient have suffered from cryptosporidiosis at some stage in their life (Hunter and Nichols, 2002 according to Moore *et al.*, 2007). Cattle and calves are big reservoirs of the parasites. High prevalence of *Giardia* and *Cryptosporidium* has indeed been reported (Olson *et al.*, 1997; Lefay, Naciri, Poirier and Chermette, 1999). Furthermore, higher *Cryptosporidium* seroprevalence has been observed among dairy farmers compared to other farmers which suggest zoonotic transmission from cattle to human (Fayer, Dubey and Lindsay, 2004).

Large amount of cysts or oocysts are shed in faeces of infected hosts. As much as 10^{10} *Cryptosporidium* oocysts or 10^9 *Giardia* cysts can be released daily from a host during acute or chronic infection (Tzipori and Ward, 2002). Excretion of infectious *Cryptosporidium* oocysts can carry on up to 50 days after the diarrhea has stopped while shedding of infectious *Giardia* cysts can carry on for months (Tzipori and Ward, 2002; MMWR, 2010). In South Africa, water sources located near farms or informal

settlements are thus more likely to become contaminated with *Cryptosporidium* oocysts and/or *Giardia* cysts. Water sources can become contaminated through run off water from infected fields or poorly sanitized settlements and direct contamination by infected animal and human faeces is also likely to occur. Rivers downstream of cattle farms have indeed been found to contain higher levels of *Cryptosporidium* oocysts and *Giardia* cysts than water upstream of the farms (Ong, Ross and Isaac-Renton, 1996).

The many outbreaks associated with drinking water confirm that purification systems can fail to eliminate *Cryptosporidium* oocysts and *Giardia* cysts (Fayer, Morgan and Upton, 2000). Improperly designed conventional water treatment using sand filtration and chlorine systems may not remove all oocysts or cysts. A typical example of water contamination is the Milwaukee outbreak in 1993. It was caused by *Cryptosporidium* oocysts passing through one of the drinking water treatment facilities and resulted in more than 400 000 ill people and several deaths (Bailey, Jarmey-Swan, Venter and du Preez, 2004). This was the largest documented waterborne outbreak in United States history. More recently, *Cryptosporidium* oocysts were found in water supplies in Ireland in 2007. The water came from a lake and then underwent treatments with coagulation and/or filtration. Heavy rainfall was suspected to have caused an increased pathogen load in the lake due to run off from farms and *Cryptosporidium* oocysts were not completely eliminated by the water treatment facilities (Pelly, Cormican, O'Donovan, Chalmers, Hanahoe, Cloughley, McKeown and Corbett-Feeney, 2007). *Giardia* has also been associated with drinking water outbreaks and was responsible for 10.6% of drinking water-associated outbreaks in the United States between 1997 and 2006 (MMWR, 2010). *Giardia* has been involved in drinking water associated outbreaks in the US in 2007 and in Norway in 2004 (Daly, Roy, Blaney, Manning, Hill, Xiao and Stull, 2009; Robertson, Forberg, Hermansen, Gjerde, Alvsvag and Langeland, 2006).

2.6 FOODBORNE OUTBREAKS OF CRYPTOSPORIDIOSIS AND GIARDIASIS

Although outbreaks of food-related cryptosporidiosis and giardiasis are less reported than waterborne infections, oocysts and cysts are able to survive in wet or moist contaminated food (Schlundt, Toyofuku, Jansen and Herbst, 2004). *Cryptosporidium parvum* was found to be the causative agent of 1699 foodborne infection cases in England and Wales between 1996 and 2000 (Adak, Meakins, Yip, Lopman and O'Brien, 2005). In the United States of America *Cryptosporidium* was the fourth most causative agent of foodborne infections in 2004, causing 637 infections (CDC, 2006). Food associated outbreaks of giardiasis are rarer than cryptosporidiosis foodborne outbreaks (MMRW, 2010). *Giardia* was found to be responsible for 18 foodborne outbreaks in the European Union during 2006 (EFSA, 2006) while the CDC reported 16 outbreaks in the United State between 1998 and 2007 (MMRW, 2010). Table 1 summarises reported cases of foodborne cryptosporidiosis and giardiasis outbreaks.

Cryptosporidium oocysts have been found on the surface of raw vegetables in various countries (Monge and Chinchilla, 1996; Robertson and Gjerde, 2001b). According to Chaidez *et al.* (2005) contaminated irrigation water is one of the major sources of contamination for fresh produce. It was found in a recent study by Macarisin, Bauchan and Fayer (2010) that *Cryptosporidium parvum* oocysts were able to adhere to spinach plants after contact with contaminated water and were also able to infiltrate the plant through the stomatal opening. This is the first study showing attachment and internalization of oocysts on vegetables. Fresh produce irrigated with water contaminated with *Cryptosporidium* oocysts could thus become contaminated. The physical structure of the fruit or vegetable's surface seems to influence the amount of oocysts or cysts retained on the product. The presence of hairy structures on the surface of the product might increase the amount of cysts or oocysts retained on the fruit or vegetable (Armon, Gold, Brodsky and Oron, 2002; Kniel, Lindsay, Summer, Hackney, Pierson and Dubey, 2002). Fruits and vegetables can also become contaminated with oocysts or cysts during production when farm workers work with soiled hands or when contaminated fertilisers are used (Fayer, 2000). The presence of cattle near vegetable fields has also been linked to the contamination of vegetables with *Cryptosporidium* (Rzezutka, Nichols, Connelly, Kaupke, Kozyra, Cook, Birrel and Smith, 2010). Flies could be another possible source of contamination of fruits and vegetables as viable *Cryptosporidium* oocysts and *Giardia*

cysts have been recovered from flies by Szostakowska, Kruminis-Lozowska, Racewicz, Knight, Tamang, Myjak and Graczyk (2004). Oocysts are able to survive in the soil for up to 12 weeks (Olson, Goh, Phillips, Guselle and McAllister, 1999) and *Giardia* cysts can survive for more than 2 months in cool conditions (Bingham, Jarroll and Meyer in 1979 according to Adam, 1991). The cool and moist surface of fruits and vegetables thus provides suitable conditions for the survival of *Cryptosporidium* oocysts and *Giardia* cysts. The multiple possible sources of contamination indicate that sufficient viable oocysts could contaminate fresh produce and be ingested by susceptible hosts.

Table 1: Reported foodborne outbreaks of cryptosporidiosis and giardiasis

Product	Year	Country	Organism	Infected cases	Reference
Unpasteurised milk	2001	Australia	<i>Cryptosporidium</i> spp.	8	Harper, Cowell, Adams, Langley and Wohlsen, 2002
Salad bar: whole carrots, grated carrots and red peppers	2005	Denmark	<i>Cryptosporidium</i> spp.	99	Ethelberg, Lisby, Vestergaard, Enemark, Olsen, Stensvold, Nielsen, Porsbo, Plesner and Molbak, 2009
Salad bar	2008	Finland	<i>Cryptosporidium</i> spp.	72	Ponka, Kotilainen, Rimhanen-Finne, Hokkanen, Hanninen, Kaarna, Meri and Kuusi, 2009
Parsley	2008	Sweden	<i>Cryptosporidium</i> spp.	21	Insulander, Jong and Svenungsson, 2008
Sliced raw vegetables	1990	United State of America	<i>Giardia lamblia</i>	26	Mintz, Hudson-Wragg, Mshar, Cartter and Hadler, 1993
Cold noodle salad	1985	United State of America	<i>Giardia lamblia</i>	16	Petersen, Cartter, Hadler, 1988
Fruit salad	1986	United State of America	<i>Giardia lamblia</i>	9	Porter, Gaffney, Heymann and Parkin, 1990

Ice	1990	United State of America	<i>Giardia lamblia</i>	28	Quick, Paugh, Addiss, Kobayashi and Baron, 1992
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2.7 INDICATOR ORGANISMS

Testing environmental samples for specific pathogens can be expensive and time consuming. Also, the detection of specific pathogens in food or water can be difficult due to their presence in low numbers (Scott *et al.*, 2002). This is why the practice of monitoring indicator organisms has become widespread (Britz, 2007). An indicator organism in food is described as a microorganism or group of them that indicate that the food has been exposed to conditions that might introduce hazardous organisms and/or allow their growth (DOH, 2010). Ideally, indicator organisms should have survival characteristics similar to those of pathogens of concern and should be found in higher number than the pathogens themselves. Indicator organisms should correlate in number to the degree of pollution and they should not be found in the absence of pollution. Finally, indicator organisms should be relatively safe to work with in the laboratory (Scott *et al.*, 2002).

Cryptosporidium oocysts and *Giardia* cysts are spread through contaminated faeces. Run-off water from informal settlements, farms and effluent from faulty water treatment plants can thus contaminate rivers and dams with *Cryptosporidium* oocysts and *Giardia* cysts. According to Beuchat (1996), human specific enteric pathogens such as *Salmonella* spp., *Shigella* spp., Hepatitis A virus and Noroviruses are most likely to be found in water contaminated with human faeces. Animal faeces can also be source of various serotypes of *Salmonella*, *E. coli*, *Giardia* and *Cryptosporidium* (Beuchat, 1996). Also, most pathogens associated with fresh produce originate from the enteric environment. That means that they are found in the intestinal tracts and fecal material of humans and animals (Harris, Farber, Beuchat, Parish, Suslow, Garrett and Busta, 2003).

This is why indicator organisms are used to indicate faecal pollution and the possible presence of pathogens.

Faecal coliforms are commonly used as indicator organisms to indicate faecal contamination and to predict the presence of enteric pathogens (Rhodes and Kator, 1988; Tallon, Magajna, Lofranco and Leung, 2005) since the presence of thermo tolerant coliforms and *E.coli* has been found to have significant predictive values for the presence of entero-pathogens (Horman *et al.*, 2004). *E. coli* has long been used as an indicator of faecal pollution. It meets many of the criteria of a good indicator organism as it is not normally pathogenic to humans and is present in a higher number than the pathogens it predicts. In warmer climates, *E. coli* is however able to replicate in contaminated soil which may decrease its reliability as an indicator under such condition (Desmarais, Solo-Gabriele and Palmer, 2002). It has now been observed that the ecology, prevalence and resistance to stress of faecal coliforms differ from those of many pathogens (Scott *et al.*, 2002). The use of other indicator organisms such as *Enterococcus* spp., *Clostridium perfringens* and bacteriophage has thus been suggested (Scott *et al.*, 2002; Harwood, Levine, Scott, Chivukula, Lukasik, Farrah and Rose, 2005). The enterococcus group is a subgroup of faecal streptococci and has been found to be good indicators of faecal pollution especially in marine environments and recreational waters (Griffin, Lipp, McLaughlin and Rose, 2001). *Clostridium perfringens* is an enteric, gram-positive, anaerobic, spore-forming, pathogenic bacterium and is found in human and animal faeces (Scott *et al.*, 2002). It has the ability to survive for a longer period of time in the environment and has thus been used to predict the presence of viruses or remote fecal pollution (Payment and Franco, 1993). Bacteriophages have been suggested as indicators of enteric viruses as their morphology and survival characteristic are similar to those of enteric viruses (Havelaar, Van Olphen and Drost, 1993).

Protozoa are more resistant to environmental stresses than bacteria. *Cryptosporidium* and *Giardia* have a distinct resting stage, the oocyst or cyst respectively, which is able to survive for a long time in the environment. So once water has been contaminated with either *Cryptosporidium* and or *Giardia*, these parasites will remain viable in the water for

longer than bacteria. According to Rose and Slifko (1999), *Cryptosporidium* oocysts are able to survive in river water for 176 days at 5-10°C. *Giardia* cysts were shown to remain viable for 56 days in river water (DeRegnier, Cole, Schupp and Erlandsen 1989), while *E. coli* has been shown to die off in rivers after only 4 to 11 days at 4°C (Flint, 1986). Water contaminated with *Cryptosporidium* or *Giardia* might therefore not necessarily test positive for *E. coli* due to the longer survival of these protozoa in water. In previous studies, no correlation was found between the concentration of indicator organisms and presence or absence of *Cryptosporidium* and *Giardia* in surface water and water effluent from water treatment plants (Harwood *et al.*, 2005; Horman, Rimhanen-Finne, Maunula, von Bonsdorff, Torvela, Heikinheimo and Hanninen, 2004). It has also been suggested that monitoring of faecal coliforms levels on fresh produce might not adequately reflect the occurrence of *Cryptosporidium* and *Giardia* due to coliforms' high susceptibility to chemical disinfection compared to the two protozoa (Harwood *et al.*, 2005). The monitoring of several indicators, such as *Clostridium perfringens*, as well as some key pathogens was suggested as a more accurate alternative to the one-indicator system to predict the presence of human pathogen in water (Harwood *et al.*, 2005).

2.8 MICROBIAL SOURCE TRACKING

The role of water and food as a vehicle for transmission of cryptosporidiosis and giardiasis is now well recognised. *Cryptosporidium* oocysts and *Giardia* cysts can contaminate fresh produce via various routes including contaminated irrigation water (Amahmid, Asmama and Bouhoum, 1999; Armon *et al.*, 2002). Knowing the exact source of contamination of fresh produce would make the management and remediation of the pollution more effective (Simpson, Santo Domingo and Reasoner, 2002). Several microbiological, genotypic and phenotypic techniques are available to determine the exact source of contamination. The presence of small differences within different groups of microorganisms are detected and then used to identify the host or environment the organisms come from (Scott *et al.*, 2002). Genetic methods are based on the assumption that progeny of a specific population adapted to a specific environment will be genetically identical and that therefore a group of organisms originating from a particular

host or environment will possess similar genetic fingerprints which will differ from organisms adapted to different hosts or environments (Scott *et al.*, 2002). Similarly, phenotypic methods identify phenotypic differences between organisms focusing on a particular trait the organisms might have acquired from exposure to different host species or environments (Scott *et al.*, 2002).

Foodborne outbreaks of cryptosporidiosis and giardiasis have been reported in both developed and developing countries (Robertson and Gjerde, 2001). The exact food responsible for outbreaks is sometime difficult to identify due to the long incubation period and global sourcing of ingredients (Smith *et al.*, 2006). Although 15 species and more than 40 genotypes of *Cryptosporidium* have been isolated from specific hosts and environmental samples only some species of *Cryptosporidium* are known to cause human infections namely *C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. muris*, *C. suis* and *cervine* genotype (Ruecker, Braithwaite, Topp, Edge, Lapen, Wilkes, Robertson, Medeiros, Sensen and Neumann, 2007). *Cryptosporidium hominis* and *C. parvum* are the most common species isolated from humans although *C. hominis* has been responsible for more outbreaks than *C. parvum* (Smith, Caccio, Tait, McLauchlin and Thompson, 2006). Existing techniques commonly used to isolate *Cryptosporidium* from water and food samples do not identify the species or genotype of *Cryptosporidium* present in the sample and thus cannot accurately assess the risk for human infection (Ruecker *et al.*, 2007). In order to effectively assess the risk to human health associated with the presence of *Cryptosporidium* oocysts in water or food, the species and/or genotypes present in the sample must be identified (Ruecker *et al.*, 2007). Molecular characterization of oocysts isolated from food or water samples can also be used to determine the host faecal sources of contamination (Ruecker *et al.*, 2007). The most commonly found species in surface and waste waters are *C. parvum* and *C. hominis* as well as many species of *Cryptosporidium* infecting only specific animals and not pathogenic to human (Smith *et al.*, 2006).

2.9 SALMONELLA SEROTYPING

Salmonellas are one of the most important causes of foodborne illness worldwide and even though most *Salmonellas* are regarded as potential human pathogens, their characteristics and the severity of the illness that they cause differ between species and serovars (Adams and Moss, 2004). Species within the *Salmonella* genus are differentiated according to the Kauffman-White serotyping scheme (Garrity, 2005). The Kauffman-White scheme describes *Salmonella* on the basis of their somatic (O), flagellar (H) and capsular (Vi) antigens. The nomenclature of the *Salmonella* genus is different from the nomenclature of other genera as different serovars of *Salmonellas* are named as if they were distinct species (Adams and Moss, 2004). Previously, *Salmonellas* to be described were given species names derived from the disease that they caused in human or animals, e.g. *Salmonella cholerae-suis*. Due to the limitation of this approach, *Salmonellas* were then given the name of the geographical location where they were first isolated. E.g. *Salmonella montevideo*. Since 1966, this approach is only applied to serovars of the subspecies I (*S. enterica* subsp. *enterica*) which makes up 59 % of the 2400 serovars known and the majority (>99%) of human isolate. For the rest of *Salmonella* subspecies, the serovar formula is used after the name of the subspecies. E.g. *S. enterica* subsp. *salamae* ser. 42:g, t:-. This system now adopted internationally (Adams and Moss, 2004). Note that the serovar is capitalised and not italicised and should be preceded by the word “serovars” or “ser.” (Brenner, Villar, Tauxe and Swaminathan, 2000). Serovars can further be divided into biovars and phagovars. Biovars are strains of the same serovars that display different sugar fermentation patterns (Garrity, 2005). Biovars are determined by the presence of absence of enzymes that enable the fermentation of specific sugars. Phagovars are strains of the same serovars that display different sensitivity to series of bacteriophages (Garrity, 2005).

2.10 EFFECT OF VEGETABLE PROCESSING ON *CRYPTOSPORIDIUM* OOCYSTS

Foodborne infection outbreaks associated with the consumption of raw fruits and vegetables have been increasing (Beuchat, 2002). In 2004, 190 fresh produce associated outbreaks of food poisoning in United States were reported by the Centre for Disease

Control and Prevention (CDC) (More *et al.*, 2007). An *E. coli* O157:H7 outbreak associated with baby spinach resulted in 205 confirmed illnesses and three deaths (FDA, 2007). In 2010, Shiga toxin producing *E. coli* O145 was found on Romaine lettuce infecting 30 people and causing kidney failure in 3 patients (CDC, 2010). In 2009, 235 people became infected by eating raw alfalfa sprouts contaminated with *Salmonella* ser. Saintpaul (CDC 2009). In May 2011, an outbreak of Shiga toxin-producing *E. coli* O104:H4 causing hemolytic uremic syndrome was reported in Europe (ECDC, 2011). According to European Food Safety Initiative (EFSA, 2011), the outbreak has caused the death of 49 people and infected more than 4000. The causative agent of this outbreak was traced back to fenugreek seeds (EFSA, 2011). The protozoa cyclospora has also been associated with several foodborne outbreaks associated with vegetables and herbs therefore showing that protozoa can also cause fresh produce associated outbreaks (Insulander, Svenungsson, Lebbad, Karlsson and De Jong, 2010). Lopez, Rodson, Arrowood, Orlandi, da Silva, Bier, Hanauer, Kuster, Oltman, Baldwin, Won, Nace, Eberhard and Herwaldt, 2001).

Processed vegetables such as frozen vegetable have also been associated, albeit more rarely, with foodborne outbreaks (Lund, Baird-Parker and Gould, 2000). In 1997, an outbreak of hepatitis A virus linked to the consumption of frozen strawberries infected 213 people in the state of Michigan (Hutin, Pool, Cramer, Naina, Weth, Williams, Goldstein, Gensheimer, Bell, Shapiro, Alter and Margolis, 1999). In 2000, frozen raspberries were implicated with a cyclosporiasis outbreak in Pennsylvania (Ho, Lopez, Eberhart, Levenson, Finkel, da Silva, Roberts, Orlandi, Johnson and Herwaldt, 2002). These outbreaks show that frozen fruits and vegetables can also be possible carriers of pathogenic organisms.

Even though *Cryptosporidium* oocysts do not grow on food they only need to survive on food to cause an infection. Because *Cryptosporidium* oocysts cannot grow or multiply on the surface of vegetables, any *Cryptosporidium* oocysts present on vegetables at consumption is there because contamination occurred during production or processing. Irrigation water, fertilisers, contaminated soil and animal faeces can contaminate

vegetables with *Cryptosporidium* oocysts during production on the farm (Millar, Finn, Xiao, Lowery, Dooley and Moore, 2002). Contaminated wash water and infected food handlers can be sources of contamination during processing. Control of oocysts in food can be achieved through the implementation of HACCP system specifically designed for the elimination or reduction of viable oocysts in food (Millar *et al.*, 2002). It is thus important to understand the factors affecting the viability of *Cryptosporidium* oocysts on fresh produce as well as on frozen vegetables and to find effective ways of inactivating *Cryptosporidium* oocysts present on vegetables. The only intervention step aimed at reducing microbial loads during the production of minimally processed vegetables is the washing step with disinfectants. On the other hand, three main processing steps can lead to a reduction in microbial load during the manufacture of frozen vegetables. Those are the washing (using disinfectants), the blanching (hot water or microwaving) and the freezing steps. No previous studies have looked at the individual as well as combined effects of processing on *Cryptosporidium* viability when inoculated on vegetables. This study will therefore investigate the effect of individual processing steps as well as the hurdle effect of combination of steps on the viability of *Cryptosporidium* oocysts in frozen vegetables.

2.10.1 EFFECT OF CHLORINE

Washing of vegetables in water baths containing disinfectants is often the only step aimed at reducing microbial load on fresh produce during the preparation of ready-to-eat minimally processed vegetables (Pirovani, Piagentini, Guemes and Arkwright, 2004). Chlorine is the disinfectant mostly used at concentrations of 50 to 200 mg/L in the form of liquid or hypochlorite salt (Sanz, Giménez, Olarte, Lomas and Portu, 2002). The effect of chlorine on microbial load depends on the available free chlorine concentration which is affected by various processing conditions such as initial chlorine concentration and water-to-product ratio (Pirovani *et al.*, 2004). The free chlorine concentration in washing baths reduces over time as the chlorine reacts with organic compounds (Pirovani *et al.*, 2004). It was also observed that microbial reduction increases as the initial chlorine concentration and water-to-produce ratio increase (Pirovani *et al.*, 2004). The surface

structure of vegetables, such as the presence of grooves and hollows as well as the presence of hydrophobic waxy cuticles, affects the contact between water and microorganisms and can affect the effectiveness of the disinfectant (Adams, Hartley and Cox, 1989). The resistant structure of *Cryptosporidium* oocysts makes it difficult to kill with disinfectants (such as chlorine) commonly used in fresh produce processing. It was found by Korich, Mead, Madore, Sinclair and Sterling (1990) that *Cryptosporidium* oocysts need to be exposed to 80 ppm free chlorine for two hours to be inactivated. In another study by Fayer (1994), *Cryptosporidium* oocysts were found to be infectious after exposure to undiluted laundry bleach for 120 min. It was also found that exposure to 8 to 16 g of free chlorine per liter for 24 hours was required to kill oocysts independently of temperature or pH (Robertson and Smith, 1992). No literature could be found on the mechanism of action of chlorine on *Cryptosporidium* oocysts but Young and Setlow (2003), suggested that hypochlorite kills *Bacillus subtilis* spores by damaging the spore's inner membrane but does not affect the DNA. Chlorine might have a similar effect on *Cryptosporidium* oocysts since oocysts also have a thick cell wall like bacterial spores do.

2.10.2 EFFECT OF BLANCHING

Blanching of vegetables by immersion into hot water or use of steam is commonly used as a pre-processing step in the manufacture of frozen vegetables (Brewer, Begum and Bozeman, 1995). Blanching performs the following functions. It inactivates enzymes that could cause undesirable changes during freezing storage. It enhances or fixes the green colour of some vegetables. It induces wilting in leafy vegetables and so facilitates packing. Finally, it reduces the number of microorganisms on the food (Splittstoesser, Bowers, and Wilkison, 1980). This latter function of blanching will be investigated in this study.

It has been observed that *Cryptosporidium* oocysts are sensitive to extreme temperatures and studies have shown that pasteurisation treatment is sufficient to kill oocysts (Harp, Fayer, Pesch and Jackson, 1996). When suspended in water or milk, oocysts seem to be sensitive to heat and die when subjected to high-temperature-short-time pasteurisation

treatment of 71.7°C for 15 s (Harp *et al.*, 1996). Holding the oocysts at 45°C for 20 min was also found sufficient to render the oocysts non infective (Anderson, 1985). Blanching of vegetables could therefore be an effective way of eliminating oocysts from vegetables since blanching is usually carried out between 75°C and 95°C for 1 to 10 min depending on the size of the vegetable pieces and on the type of vegetable (Barbosa-Canovas, Altunakar, Mejia-Lorio, 2005).

The medium surrounding the microorganisms affects their sensitivity to heat and makes them more or less resistant. The presence of water makes microbial cells less resistant to heat and it was found that dried cells are more resistant to heat than moist cells (Jay, 2000). Denaturation of protein is the main mechanism of death by heat and occurs more readily in the presence of water which allows for thermal breaking of peptide bonds. This process requires more energy in the absence of water and this could explain the greater resistance of microbial cells to heat in the absence of water (Jay, 2000). Vegetables contain a high percentage of water e.g. broccoli contains around 91% and peppers around 92% water (Bastin and Henken, 1997). The high moisture content of fresh vegetable might thus affect the survival of *Cryptosporidium* oocysts during blanching by making the oocysts more susceptible to the heat treatment.

2.10.3 EFFECT OF FREEZING

The effects of freezing on microorganisms present on vegetables vary greatly from strain to strain and depend on the type of freezing employed (Jay, 2000). Previous studies have shown that the effect of freezing on *Cryptosporidium* oocysts is dependent on the time-temperature combination used as well as on the type of freezing employed. Fayer and Nerad (1996) found that cryogenic freezing at -70°C for 1 h, -20°C for 24 h or -15°C for 168 h was sufficient to render the oocysts non infectious. However cryogenic freezing is not commonly used for the commercial production of frozen vegetables and the temperature used for storage is generally above -20°C (Barbosa-Canovas *et al.*, 2005). According to Harrison and Croucher (1993) in Barbosa-Canovas *et al.*, (2005), the legal

limits for cold store temperature, distribution temperature and retail display temperature are -18°C, -15°C and -12°C respectively. Slow freezing was found to be less effective than fast freezing and 152 hours at -22°C was required to kill 90% of oocysts in a study by Robertson *et al.* (1992). The exact mechanism of action of freezing on *Cryptosporidium* oocysts is unknown. Freezing affects microorganisms in various ways. During freezing ice crystals form and deplete the amount of water available to the cells creating a low water activity environment. The viscosity of cellular matter increases due to the water being concentrated in ice crystal (Jay, 2000). Freezing causes losses in cytoplasmic gases such as oxygen and carbon dioxide and causes changes in pH of cellular matter. Freezing also causes some denaturation of protein as consequences of the lower water content and concentration of electrolytes (Lovelock, 1957).

2.10.4 EFFECT OF MICROWAVE HEATING

Microwave blanching of vegetables can be used in the industry as an alternative to hot water blanching during the manufacture of frozen vegetables (Kidmose and Martens, 1999; Ramesh, Wolf, Tevini and Bognar, 2002). Furthermore microwave ovens are now found in most households and are a convenient way to cook or heat up food. Many convenience vegetable products can be cooked at home in their packaging using microwave ovens and then consumed directly from the package without further washing. It is therefore important to determine the effect of microwave processing on the viability of *Cryptosporidium* oocysts. The microwave radiation and generated heat can be an effective way of killing pathogens present on vegetables if the products are microwaved for a sufficient amount of time. It was found by Ortega and Liao (2006) that 20s in a microwave on full power (1 100 Watts) or 30s at 650 Watts was sufficient to inactivate *Cryptosporidium* oocysts if a temperature of 80°C was reached. Due to variability in temperature achieved by different microwave ovens, they found that the cooking time was not a good indicator of inactivation efficiency but that the temperature reached inside the microwave was a more accurate indicator of oocysts inactivation (Ortega and Liao, 2006). The exact mechanism of action of microwave on *Cryptosporidium* oocysts is unknown but the killing effect of microwave in general is mainly attributed to the heat

generated even though non-thermal effects have also been observed (Banik, Bandyopadhyay and Ganguly, 2003). As discussed previously, heat mainly affects microorganisms by causing denaturation of proteins (Jay, 2000). With regards to the non-thermal effects of microwaves, it has been proposed that the microwaves cause ions to accelerate and collide with other molecules or cause dipoles to rotate and line up rapidly with the microwave's rapidly alternating electric field which would cause changes in secondary and tertiary structure of microorganism's proteins (Banik *et al.*, 2003).

2.11 CONCLUSIONS

Cryptosporidium and *Giardia* are waterborne and foodborne pathogens and can contaminate fresh produce via various routes. Due to the poor quality of available irrigation water in parts of South Africa, irrigation water might be a possible route of contamination of fresh produce with *Cryptosporidium* and *Giardia*. Quality assessment of irrigation water and fresh produce need to be carried out to determine the economic consequences as well as potential public health risks associated with the use of irrigation water of poor quality in South Africa. The validity of using indicator organisms to predict the presence of *Cryptosporidium* and *Giardia* in environmental samples must also be established.

Survival of *Cryptosporidium* and *Giardia* on fresh produce has the potential to cause severe foodborne outbreaks. Literature available on the effect of treatments on *Cryptosporidium* viability under different conditions is limited. Little information and no previous work have been done on the combined effect of washing with chlorine, blanching and freezing on *Cryptosporidium* viability. This study investigates the individual and hurdle effect of vegetable pre-processing and processing steps on the viability of *Cryptosporidium* oocysts inoculated on green pepper.

2.12 HYPOTHESES

1. Since both *Cryptosporidium* and *Giardia* are transmitted through faeces of infected hosts (Tzipori and Ward, 2002), the presence of faecal indicators (i.e. faecal coliforms and *E. coli*) in water can be used to predict the presence of *Cryptosporidium* or *Giardia* in water and on vegetables irrigated with such water (Horman, 2004).
2. Because of their thick wall structure made of a double layer of protein-lipid-carbohydrate matrix (Templeton *et al.*, 2004), *Cryptosporidium* oocysts have been found to be resistant to chemical disinfectants (Lorenzo-Lorenzo *et al.*, 1993). Chlorine will thus not affect the membrane permeability of oocysts inoculated on green pepper and can therefore not be expected to inactivate *Cryptosporidium* oocysts present on vegetables. Heating (Fayer, 1994; Anderson, 1985), microwave heating (Ortega and Liao, 2006) and freezing (Fayer and Nerad, 1996) on the other hand have been found to affect the viability of oocysts. Blanching, microwave heating and freezing will thus alter the membrane permeability of oocysts inoculated on peppers and render the oocysts non-infective. Treatments combining chlorine with blanching, microwaving and/or freezing will therefore be more effective than chlorine treatments alone.

2.13 OBJECTIVES

1. To determine simultaneously the incidence of indicator organisms, *E. coli*, coliforms and *Salmonella* spp., as well as the incidence of *Cryptosporidium* and *Giardia* in the Moses river in Mpumalanga, Skeerpoort river in North West and Klip river in Gauteng during 10 consecutive months
2. To determine simultaneously the incidence of indicator organisms, *E. coli*, coliforms and *Salmonella* spp., as well as the incidence of *Cryptosporidium* and *Giardia* on irrigated vegetables sampled before harvest.
3. To determine the correlation between indicator parameters (i.e. temperature, pH, *E. coli*, coliforms and *Salmonella* spp.) and the presence of *Cryptosporidium* and *Giardia* in irrigation water.
4. To determine the individual effects of chlorine, blanching, freezing and microwave heating treatments on the viability of *Cryptosporidium* oocysts inoculated on green peppers.
5. To determine the combined effect of chlorine and freezing treatment as well as combination of chlorine and microwave heating treatment on the viability of *Cryptosporidium* oocysts inoculated on green peppers.

CHAPTER 3: RESEARCH

3.1 INCIDENCE OF *CRYPTOSPORIDIUM*, *GIARDIA* AND INDICATOR ORGANISMS IN SOUTH AFRICAN RIVERS USED FOR IRRIGATION OF FRESH PRODUCE

ABSTRACT

Cryptosporidium and *Giardia* are protozoa that can cause severe gastrointestinal infections and can be lethal in individuals with compromised immunity. In South Africa, few studies have investigated the incidence of *Cryptosporidium* oocysts and *Giardia* cysts in surface water used for the irrigation of fresh produce. In this study, three rivers used for irrigation purposes from the Mpumalanga, North West and Gauteng provinces were analysed for the presence of *Cryptosporidium* oocysts, *Giardia* cysts, *Salmonella* spp., faecal indicators and physicochemical parameters from June 2009 to April 2010. The possible relationship between faecal indicators and the presence of *Cryptosporidium* oocysts and *Giardia* cysts was also investigated. Out of the 30 water samples analysed, 43% were positive for *Cryptosporidium* oocysts, 23% tested positive for *Giardia* cysts and 27% were positive for *Salmonella* spp. No significant differences in the prevalence of *Cryptosporidium* and *Giardia* and indicator parameters were observed between rivers ($p>0.05$). Using a logistic regression model, no significant correlations were observed between incidence of faecal indicators and the presence of *Cryptosporidium* oocysts and *Giardia* cysts. The presence of *Cryptosporidium* oocysts and *Giardia* cysts in irrigation water indicates a risk of infection to consumers who eat these produce as *Cryptosporidium* and *Giardia* can come in contact and attach on the surface of fresh produce during irrigation. The use of solely faecal indicator organism for monitoring surface water quality is not sufficient to accurately predict the presence of *Cryptosporidium* and *Giardia* and assess the microbiological safety of irrigation water.

3.1.1 INTRODUCTION

In South Africa, most farmers rely on irrigation water for the production of their crop as 65% of the country does not receive enough rainfall for rain fed crop production (FAO, 2005). Surface waters are thus widely used as a source of irrigation water for the production of fruits and vegetables. However, studies have indicated high levels of faecal contamination and presence of human pathogens in South African rivers (Britz, Sigge and Barnes, 2008). The use of water of poor microbiological quality for the irrigation of fresh produce could lead to contamination of fruits and vegetables with pathogenic organisms such as *Cryptosporidium* and *Giardia* since contaminated water is deposited onto the surface of the crops during irrigation (Macarasin, Bauchan and Fayer, 2010). This is of public health significance especially since the number of fresh produce associated outbreaks is on the rise worldwide due to the increased consumption of fresh produce, international trade and increased number of susceptible individuals (Beuchat, 2002). Contamination of fruits and vegetables with pathogenic organisms could also have a negative impact on South African export and lead to economic losses.

Cryptosporidium and *Giardia* are two of the most common and resistant human waterborne parasitic protozoa worldwide (Chaidez, Soto, Gortares and Mena, 2005). They have been isolated from raw water, treated effluent and drinking water in South Africa (Monique Grundlingh, Rand Water, personal communication, 2008; Kfir, Hilner, du Preez and Bateman, 1995). *Cryptosporidium* has been shown to attach to the surface of vegetables following irrigation and becomes internalized making its removal difficult to achieve (Macarasin *et al.*, 2010). Most reported cryptosporidiosis and giardiasis outbreaks have been associated with drinking water. However, *Cryptosporidium* oocysts have been found on the surface of raw vegetables in both developing and developed countries (Robertson and Gjerde, 2001 a).

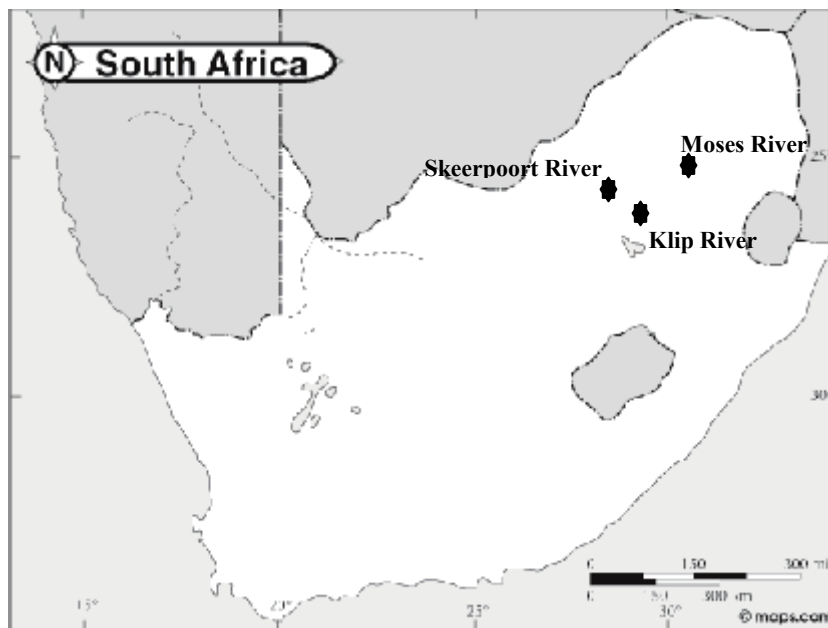
Due to the socio-economic consequences the contamination of fresh produce could have, it is crucial to determine the level of contamination of irrigation water with human pathogens. This study determined the occurrence of *Cryptosporidium* and *Giardia* in three South African rivers used for the irrigation of fresh produce. The cost of the isolation methods for *Cryptosporidium* and *Giardia* is high and the recovery rate of these methods is low. It is therefore necessary to assess the correlation between the presence of commonly used faecal indicator organisms such as

Escherichia coli and faecal coliforms and incidence of *Cryptosporidium* and *Giardia* in order to find a reliable indicator for those two protozoa. No studies have been done in South Africa to determine the simultaneous incidence of *Cryptosporidium* and *Giardia* and faecal indicators. Therefore the other objective of this study was to determine whether the presence of faecal indicators or bacterial pathogens such as *Salmonella* spp. can be linked to the presence of *Cryptosporidium* oocysts and/or *Giardia* cysts in irrigation water.

3.1.2 MATERIALS AND METHODS

3.1.2.1 SAMPLING SITES

Three sites were selected: the Moses river in Mpumalanga, the Skeerpoort river in North West and the Klip river in Gauteng (Figure 2). The sites monitored in this study were selected because these rivers are used as irrigation water sources for the irrigation of fruits and vegetables. Furthermore, the sites were suspected to be contaminated with *Cryptosporidium* and *Giardia* as high levels of faecal coliforms have been found previously in these waters (Britz, Sigge and Barnes, 2008; DACE, 2004). The presence of faecal coliforms in water indicates contamination with faecal matter. Since *Cryptosporidium* oocysts and *Giardia* cysts are spread through contaminated faeces (O'Donoghue, 1995), these waters could also be contaminated with the two protozoa.



● Indicates river sampling site

Figure 1: Map of South Africa showing the sampling sites (Newsbank Reference Maps, 2010)

3.1.2.2 WATER SAMPLING

Water samples were taken monthly from the three sampling sites over a period of ten months from June 2009 until April 2010. One litre water samples were collected aseptically in sterile glass bottles for bacteriological analyses and 50 litres samples were collected into ten litres plastic containers for *Cryptosporidium* and *Giardia* analyses. Samples were taken from the river bank, 20 to 30 cm beneath the water surface. The samples were transported directly to the Department of Food Science at the University of Pretoria and were analysed within 12 hours.

3.1.2.3 FRESH PRODUCE SAMPLING

Broccoli irrigated by the Loskop Dam irrigation Scheme was sampled over a period of three weeks prior to harvest. Lettuce from Skeerpoort farm was sampled over a period of three months at the same time as the water samples were taken. Three lettuce or broccoli heads were selected

according to accessibility from the edge of the field, transported to the lab in cooler boxes and analysed within 12 hours.

3.1.2.4 DETERMINATION OF PHYSICOCHEMICAL PARAMETERS

The temperature of the samples was measured with a portable device (Checktemp 1, Hanna Instrument, Inc. Woonsocket, R1, USA) when collecting the samples at the sampling site. The pH of each water sample was measured in the laboratory using the 211 Microprocessor pH meter (Hanna Instruments, Inc. Woonsocket, R1, USA).

3.1.2.5 ISOLATION OF *CRYPTOSPORIDIUM* OOCYSTS AND *GIARDIA* CYSTS

Cryptosporidium and *Giardia* were isolated using filtration, immuno-magnetic separation and staining with fluorescein-iso-thiocyanate (FITC) and 4',6-diamidino-2-phenylindole (DAPI) according to the Method 1623: *Cryptosporidium* and *Giardia* in water by Filtration/IMS/FA by United States Environmental Protection Agency (Anonymous, 2005).

Each 50 L raw water sample was filtered through a Pall Envirocheck™ 1µm HV filter capsule (Separation, Randburg, South Africa) using a pump. The Envirocheck™ filter capsule was filled with 125 ml prepared elution buffer containing Laureth-12 (PPG Industries, Gurnee, IL), 1 M Tris pH 7.4, EDTA and silicone antifoam agent (Merck, South Africa) and shaken on a shaker (Grant Instrument Ltd, England) at 300 rpm.

The elution buffer was centrifuged at 1100 x g for 16 min. The supernatant was then aspirated and discarded using pipettes. The pellet in the centrifuge tube was re-suspended by vortexing for 10 to 15 seconds. The pellet was then transferred into flat-side Leighton tube (Dehteq, Kyalami, South Africa) using pre-rinsed pipettes.

One ml Buffer A from the IMS kit (Invitrogen Dynabeads GC Combo, Dehteq, Kyalami, South Africa) and 1 ml Buffer B supplied with the IMS kit was added to each Leighton tube. 100 µl of

Cryptosporidium Dynabeads™ and 100 µl of *Giardia* Dynabeads™ were added to the each Leighton tube.

The Leighton tubes were placed on the rotator mixer (Rotamix Atrbiotech, US) and left to rotate at 15 rpm for 1 h at room temperature. Immuno-magnetic separation was then performed using the MPC-1 magnet (Dehteq, Kyalami, South Africa) and MPC-S magnet (Dehteq, Kyalami, South Africa) according to the method's instruction (Anonymous, 2005).

Samples on microscope slides were fixed with methanol and stained with 50 µl of *Cryptosporidium* FITC and 50 µl *Giardia* FITC stain (Davies Diagnostic Pty Ltd, Randburg, South Africa) and incubated at 37°C for 30 min. The samples were then stained with 50 µl DAPI working solution (Davies Diagnostic Pty Ltd, Randburg, South Africa) and left for 2 min at room temperature.

Each slide was scanned under fluorescence microscope (Zeiss Axiovert 200, Germany) in a systematic way at 65 x magnifications. Oocysts and cysts with apple green fluorescent cell walls and spherical or ovoid in shape were identified as positive for FITC. The UV filter block was then used for the DAPI examination. Oocysts and cysts that had been identified using the green filter were then examined under UV filter for confirmation. *Cryptosporidium* and *Giardia* were identified as DAPI positive if they exhibit up to 4 distinct sky-blue internal nuclei.

Positive control

Positive controls were used to verify the efficiency of the method. ColorSeed™ and EasySeed™ (BTF Pty Limited, Biomerieux, Sydney, United Kingdom) were used as positive control to determine the recovery rate of the method. Each ColorSeed™ or EasySeed™ capsule contains an exact amount of *Cryptosporidium* oocysts given with the certificate of analysis. The ColorSeed™ and EasySeed™ were used according to the manufacturer's instruction.

Cryptosporidium oocysts recovery rate was calculated using the formula:

***Cryptosporidium* recovery (%) =**

$$\frac{\text{Cryptosporidium detected}}{\text{Number of Cryptosporidium in ColorSeed™ as per Certificate of Analysis}} \times 100$$

3.1.2.6 NESTED POLYMERASE CHAIN REACTION (PCR)

DNA was extracted from microscope slide using Pinpoint slide DNA isolation System (Zymo Research, USA) followed by 15 cycles of freeze-thawing (1 min in liquid nitrogen followed by 1 min at 65°C in thermocycler) to facilitate bursting of oocysts wall and extraction of DNA. Amplification of *Cryptosporidium* DNA isolated from microscope slides was performed according to the method described by Nichols, Campbell and Smith (2003).

3.1.2.7 ISOLATION OF BACTERIAL PATHOGENS

Method ISO 4833 (International Organization for Standardisation, 1991) was used for the enumeration of aerobic colony count on Plate Count Agar plates (Biolab, Wadeville, South Africa) incubated at 37°C for 48 h.

Total coliforms, faecal coliforms and *E. coli* were enumerated using the most probable number method MFHPB 19 (Government of Canada, 2002). Samples were incubated in Lauryl Tryptose (LST) broths (Oxoid LTD, Basingstoke, Hampshire, England) at 35°C for 24 h. Positive broths were inoculated in Brilliant Green Bile 2% broth (Oxoid, Basingstoke, Hampshire, England) and incubated at 35°C for 24 h. Positive BGLB tubes were inoculated into EC broth (Oxoid, Basingstoke, Hampshire, England) and incubated in water bath at 45°C for 24 h. *E. coli* was enumerated on Eosin Methylene Blue agar (Levine) (EMB) plate (Oxoid, Basingstoke, Hampshire, England) incubated at 35°C for 18-24 h. *E. coli* colonies were confirmed on selective *E. coli*/coliform chromogenic medium (Oxoid, Basingstoke, Hampshire, England) incubated at 35°C for 18-24 h.

The presence of *E. coli* O157 DNA, shigatoxic *E. coli* and entero-hemorrhagic *E. coli* O26, O103, O145 and H7 DNA was determined with real time PCR using the GeneSystems (Pall GeneSystem, Bruz, France).

Salmonella spp. was isolated according to the method ISO 6579 (International Organization for Standardisation, 1993). Enrichment was done in Rappaport-Vassiliadis (RVS) enrichment broth (Oxoid, Basingstoke, Hampshire, England) incubated at 42°C for 24 h and in selenite cystine broth base (Oxoid, Basingstoke, Hampshire, England) incubated at 37°C for 48 h. *Salmonella* spp. were isolated on Phenol Red/Brilliant Green Agar and onto XLD Agar (Oxoid, Basingstoke, Hampshire, England) incubated at 35°C for 20-24 h. *Salmonella* spp. colonies were confirmed on *Salmonella* chromogenic medium (Oxoid, Basingstoke, Hampshire, England). *Salmonella* spp. colonies were serotyped by the ARC-Onderstepoort.

3.1.2.8 FRESH PRODUCE ANALYSIS

Cryptosporidium oocysts and *Giardia* cysts were isolated from fruit and vegetable samples according to the method described by Robertson and Gjerde (2000). The fresh produce sample was placed in stomacher bag with 200 ml of wash solution made of 50 ml of elution solution and 150 ml of water. The stomacher bag was stomached for 5 min. The wash water was then analysed for *Cryptosporidium* and *Giardia* according to EPA method 1623 described above.

Bacterial analysis of fresh produce was done by placing 10 g of produce in 90 ml of water in a stomacher bag. The bag was stomached and the filtrate was used for further analyses as describe in section 3.1.2.5.

3.1.2.9 STATISTICAL ANALYSIS

Analyses of variance, performed using Statistica Software for Windows Version 7 (Tulsa, Oklahoma, USA, 2003), was used to test for significant differences

in physicochemical and microbiological quality at 95% confidence interval between the different water sites and produce. Percentages prevalence was calculated for each pathogen analysed. Arithmetic means and standard deviations were calculated for MPN of faecal coliforms and *E. coli*, temperature and pH for each sampling site.

A binary logistic regression model analysis was performed using SAS® V9.2 running under Window XP (SP3) as supplied by SAS Institute Inc, SAS Campus drive (Cary, North Carolina). The analysis was performed to determine whether indicator parameters, faecal coliforms, aerobic plate counts, pH and temperature predicted the probability of the occurrence of pathogens in water samples. The dependant variable, the pathogen (i.e. *Cryptosporidium*, *Giardia* or *Salmonella* spp.), was treated as a binary variable. That means that a score of 0 was assigned when the pathogen was not detected and a score of 1 was assigned when the pathogen was detected. The independent variables, the indicator parameters, were continuous.

3.1.3 RESULTS

3.1.3.1 INCIDENCE OF INDICATOR ORGANISMS AND CHARACTERIZATION OF PROTOZOA PATHOGENS IN THE KLIP, MOSES AND SKEERPOORT RIVERS

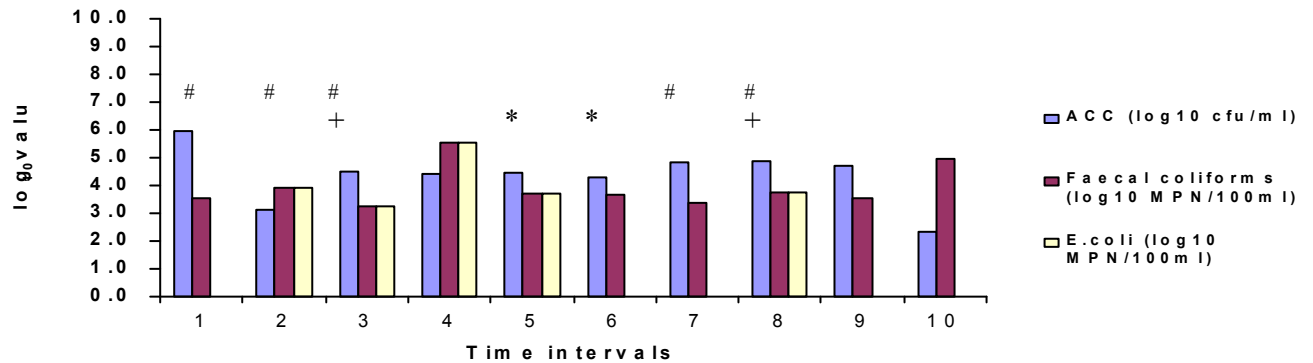
The aerobic colony counts in the Klip river ranged from 2.3 to 6 log₁₀ cfu/ml with an average of 5 log₁₀ cfu/ml (Figure 3). Levels of faecal coliforms and *E. coli* ranged from 3.2 to 5.5 log₁₀ counts/100 ml (Figure 3). *E. coli* was not found in 5 of the samples despite high faecal coliform counts. From the 10 water samples taken from the Klip river, 7 samples were positive for at least one pathogen and 2 samples were positive for 2 pathogens (Figure 3). *Salmonella* spp., *Salmonella* ser. Enteritidis and *Salmonella* ser. Adeoye were isolated from 2 out of the 10 samples taken from the Klip river during the summer months (Table 3). *Cryptosporidium* oocysts were present in 5 samples and *Giardia* cysts in 2 samples taken from the Klip River. *Cryptosporidium* and *Giardia* were isolated from the Klip river during both winter and summer months (Figure 3). Water samples positive for *Cryptosporidium* oocysts were confirmed with PCR (Figure 4)

The recovery efficiency for *Giardia* was significantly lower ($p < 0.05$) than the recovery efficiency for *Cryptosporidium* with 15.3% recovery for *Cryptosporidium* and 9.2% recovery for *Giardia*. The average recovery efficiency for the 10 positive controls for the isolation of *Cryptosporidium* and *Giardia* was lower than reported in some studies (Robertson and Gjerde, 2001b).

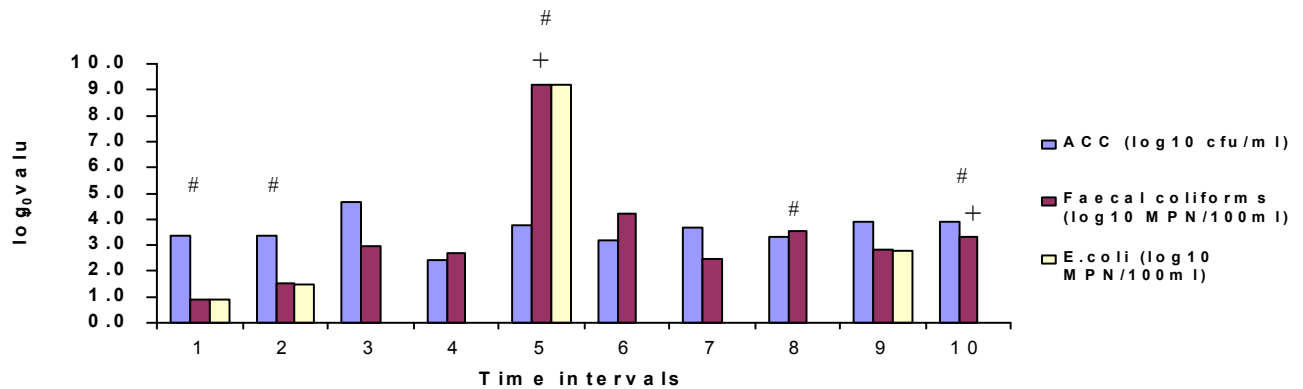
Aerobic colony counts in the Skeerpoort river ranged from 2.4 to 4.7 $\log_{10}$ cfu/ml (Figure 3). Faecal coliforms counts in the Skeerpoort river varied greatly between samples and ranged from 0.9 $\log_{10}$ /100 ml in winter to more than 9.2 $\log_{10}$ /100 ml in summer. *E. coli* was isolated from 4 out of the 10 samples and was found in samples with both high and low faecal coliform counts. The *E. coli eae intimin* was detected in the Skeerpoort river in March 2010 although *E. coli* 0157:H7 was not detected (Table 3). The *eae intimin* must have originated from other enterohemorrhagic *E. coli* (EHEC) not included in the GeneDisc analysis. None of the samples tested positive for *Salmonella* spp. but 5 samples tested positive for *Cryptosporidium* oocysts. *Giardia* cysts were found in 2 of the samples (Figure 3). *Cryptosporidium* oocysts were found during both winter and summer while *Giardia* cysts were only isolated in summer. *Cryptosporidium* oocysts were found in water samples containing low (0.9 $\log_{10}$ counts/100 ml) as well as high ($> 9.2 \log_{10}$ counts/100 ml) levels of faecal coliforms (Figure 3).

The aerobic colony counts in the Moses River ranged from 2.4 to 4.5 $\log_{10}$ cfu /ml (Figure 3). Faecal coliforms ranged from 2.5 to 4.8 $\log_{10}$ counts/100 ml and 7 out of the 10 samples had faecal coliform levels above 3 $\log_{10}$ counts/100ml. An increase in faecal coliforms was observed during the beginning of summer (sampling interval 4-6) which coincides with the first rainfall after a dry winter (Figure 3). *E. coli* was isolated from 8 out of the 10 samples. No *E.coli* was found in two of the samples for which the faecal coliforms counts were high (4 and 4.8 $\log_{10}$ counts/100 ml). *E. coli* shigatoxin 1 was detected in the Moses river in April 2010 although *E. coli* 0157:H7 was not detected (Table 3). Out of the 10 water samples analysed, 8 tested positive for at least one pathogen and 4 samples were positive for 2 pathogens (Figure 3). *Salmonella* spp. was found in 6 samples. *Salmonella* ser. Gaminara, *Salmonella* ser. Fulica, *Salmonella* ser. Infantis, *Salmonella* subsp. I and *Salmonella* subsp. II were isolated from the Moses river (Table 3). *Cryptosporidium* oocysts and *Giardia* cysts were isolated from 3 of the samples (Figure 3).

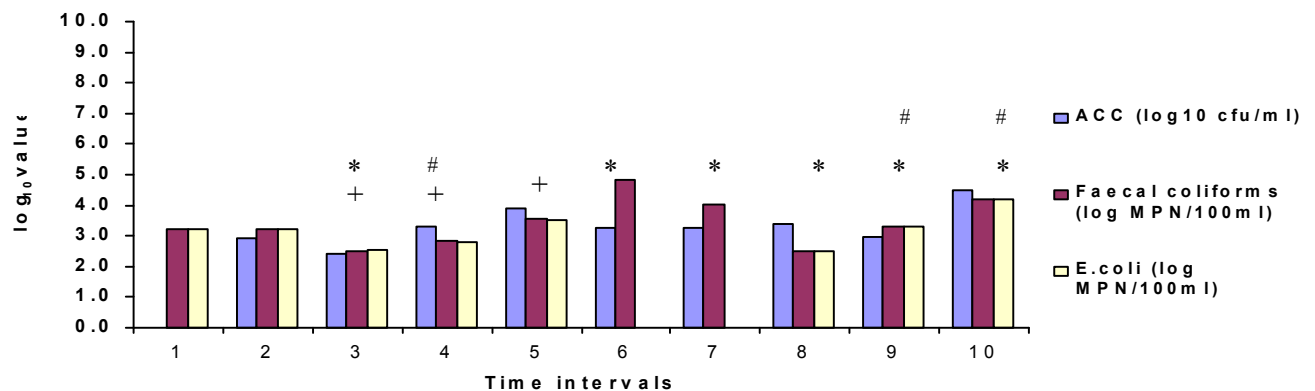
A



B



C



* Positive for *Salmonella* spp. # Positive for *Cryptosporidium* + Positive for *Giardia*

Figure 2: Aerobic colony count, faecal coliforms counts, *E. coli* counts and incidence of *Salmonella* spp., *Cryptosporidium* and *Giardia* in the Klip (A), Skeerpoort (B) and Moses rivers (C) for the 10 sampling intervals between June 2009 and April 2010

The two protozoa were isolated from different water samples and only one sample contained both *Cryptosporidium* oocysts and *Giardia* cysts. Both protozoa were isolated from water that contained both low and high levels of faecal coliforms and were found in the Moses river during winter as well as summer months.

3.1.3.2 INCIDENCE OF INDICATOR ORGANISMS AND CHARACTERIZATION OF PROTOZOA PATHOGENS IN BROCCOLI AND LETUCE

The aerobic plate counts for the lettuce heads were higher than those of the broccoli but not significantly so ($p=0.24$) (Table 2). Aerobic plate counts from both vegetables were acceptable according to the guideline set up by the University of Ghent for vegetables intended for raw consumption (Uyttendaele, Jacxsens, De Loy-Hendrickx, Devlieghere, Debevere, 2010) (Table 2). Faecal coliform counts were low on both vegetables. *E. coli* were isolated from the lettuce but not from the broccoli head. None of the samples were positive for *Salmonella*, *Cryptosporidium* or *Giardia*.

Table 1: Indicator parameters and percentages of samples positive for pathogens for the vegetable samples analysed.

	No. of samples	Aerobic colony count Log cfu / g [#]	Faecal coliforms / 100 g [#]	<i>E. coli</i> / 100 g [#]
Broccoli	3	3.18 (1.02)	44 (74)	0 (0)
Lettuce	3	4.38 (0.98)	37 (14)	22 (22)

[#] Standard deviations in brackets

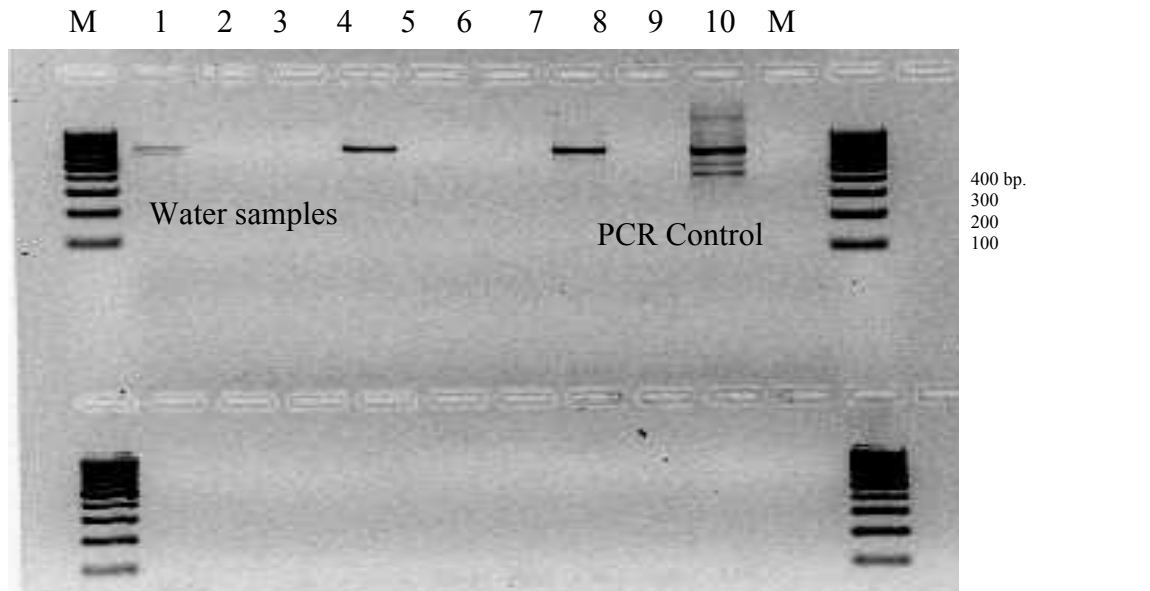


Figure 3: Amplified *Cryptosporidium* DNA on agarose gel. Lane 1-6: slides from water samples, 7-8 positive control slides, 9 PCR positive control, 10 PCR negative control, lanes M:100 bp DNA ladder

3.1.3.3 LINK BETWEEN INDICATOR ORGANISMS AND THE PRESENCE OF *CRYPTOSPORIDIUM* AND *GIARDIA* IN THE THREE RIVERS.

The minimum and maximum temperature of the three rivers ranged from 13.4°C, 12°C and 8.4°C in winter to 22.5°C, 27.8°C and 26.1°C summer for the Klip, Moses and Skeerpoort rivers respectively. No significant differences in temperature between rivers were observed ($p>0.05$) (Table 3).

The pH of the Klip river and Moses river were within normal range and varied between 6.58 and 8.02 for the Klip river and between 6.23 and 8.21 for the Moses river. The average pH of the Skeerpoort river was significantly higher ($p=0.003$) than the pH of the Moses and Klip river and ranged from 7.2 to 8.63 (Table 3).

Aerobic colony counts were on average higher in Klip river than in the Skeerpoort and Moses river. Faecal coliform counts in Skeerpoort river were lower on average than in the Moses and Klip river. These differences were however not statistically significant ($p>0.05$) (Table 3).

Fewer pathogens were isolated from the Skeerpoort river than from the other two rivers. Only 50% of the samples taken from the Skeerpoort river contained at least one pathogen and 2 samples tested positive for 2 pathogens. There were however no significant differences ($p>0.05$) between the three rivers in terms of incidence of pathogens and levels of indicator organisms.

This ten month study took place over three seasons, starting in winter 2009 and finishing in summer 2010. More samples were found positive for *Cryptosporidium* in summer than in winter and spring while more positive *Giardia* samples were found in spring than in winter and summer. However, those differences in incidence between seasons were not statistically significant ($p>0.05$) (data not shown).

Table 2: Indicator parameters and percentage of water samples (n=30) positive for pathogens for the samples collected between June 2009 and May 2010 from the Klip, Moses and Skeerpoort rivers

	No. of samples	Indicator parameters					% of samples positives for pathogen			
		Temperature	pH ⁴	Aerobic colony count/ ml	Faecal coliforms / 100ml	<i>E. coli</i> / 100ml	<i>Salmonella</i> spp.	<i>Cryptosporidium</i>	<i>Giardia</i>	Any pathogen
Klip ¹	10	18.8	7.32	122667	47590	36 990	20	50	20	70
Moses ²	10	20.6	7.36	5622	10740	2 640	60	30	30	80
Skeerpoort ³	10	20.0	8.06	8135	2670	315	0	50	20	50
Average	30	19.76 (5.44) ⁵	7.58 (0.59)	46 849	20 333	13 315	27	43	23	66

¹ *Salmonella* ser. Enteritidis and *Salmonella* ser. Adeoye were isolated from the Klip river

² *Salmonella* ser. Gaminara, *Salmonella* ser. Fulica, *Salmonella* ser. Infantis, *Salmonella* subsp. I and *Salmonella* subsp. II and *E. coli* Shigatoxin 1 were isolated from the Moses river.

³ *eae intimin* was isolated from the Skeerpoort river

⁴ Anova performed between rivers (n=10). Skeerpoort pH was significantly different from Klip and Moses pH (p=0.003).

⁵ Standard deviations indicated in brackets

Table 3: Percentage of water samples from the Klip, Moses and Skeerpoort rivers (n=30) positive for *Salmonella* spp., *Cryptosporidium* and *Giardia* at various levels of faecal coliforms in samples

Faecal coliform count/100 ml	No of samples (%)	% of positive samples			
		<i>Salmonella</i> spp.	<i>Cryptosporidium</i>	<i>Giardia</i>	Any pathogen
<100	2 (6.7) [#]	0	100	0	100
100-1000	7 (23.3)	28	14	28	42
1000- 10 000	14 (46.7)	21	57	28	78
>10 000	7 (23.3)	42	28	14	57
Total	30 (100)	26	43	23	66

[#] Standard deviations in brackets

Table 4: Percentage of water samples from the Klip, Moses and Skeerpoort rivers (n=30) positive for *Salmonella* spp., *Cryptosporidium* and *Giardia* at various levels of *E. coli* in samples

<i>E. coli</i> count/100 ml	No of samples (%)	% of positive samples			
		<i>Salmonella</i> spp.	<i>Cryptosporidium</i>	<i>Giardia</i>	Any pathogen
<1	12 (40) [#]	25	25	0	50
1-1000	6 (20)	33	50	33	83
1000- 10 000	9 (30)	22	55	44	77
>10 000	3 (10)	33	66	33	66
Total	30 (100)	26	43	23	66

[#] Standard deviations in brackets

Table 5: Logistic regression analysis of relationship between indicator parameters and presence of *Cryptosporidium* and *Giardia* (p-value)

Indicator parameter	p-value	
	<i>Cryptosporidium</i>	<i>Giardia</i>
Temperature	0.8193	0.7845
pH	0.4865	0.6598
Aerobic colony count	0.4112	0.8302
Faecal coliforms	0.6174	0.6767
<i>E. coli</i>	0.2141	0.3038

Table 4 shows the percentage of positive water samples for pathogens at various levels of faecal coliforms when looking at the three rivers together. Two water samples contained less than 100 faecal coliforms/100 ml. *Salmonella* spp. and *Giardia* were absent from those samples and only *Cryptosporidium* oocysts were isolated from those 2 samples. Between 100 and 1000 faecal coliforms/100 ml were found in 7 samples. The incidence of pathogens in those samples was low. The highest incidence of *Cryptosporidium* oocysts was found in samples containing between a 1000 and 10 000 faecal coliforms/100 ml. *Giardia* cysts were however found at equal rate in samples containing between 100 and 1000 faecal coliforms/100 ml and between 1000 and 10 000 faecal coliforms/100 ml. The incidence of *Salmonella* spp. was the highest in samples with faecal coliform counts above 10 000 counts/100ml. However, the overall incidence of pathogens was lower in samples containing more than 10 000 faecal coliforms/100 ml than in samples containing between 1000 and 10 000 faecal coliforms/100 ml although more than 50 % of the samples containing more than 10 000 faecal coliforms/100 ml were positive for either *Salmonella* spp., *Cryptosporidium* or *Giardia*.

Table 5 shows the percentage of water samples positive for pathogens at various levels of *E. coli* in water samples when looking at the three rivers together. Level of *E. coli* of less than 1 count/100 ml was found in 40% of the samples. In those samples the incidence of pathogens was low, and no *Salmonella* spp. was found. However, half of those samples tested positive for at least one pathogen. Levels of *E.coli* between one and 1000 counts/100 ml water were found in 20% of the samples. The incidence of pathogen in those samples was high and 83% of the samples tested positive for at least one pathogens. Between 1000 and 10 000 *E. coli*/100 ml were found in 30% of samples. The highest incidence of *Giardia* cysts was observed in these samples. The highest incidence of *Cryptosporidium* oocysts was observed in samples containing more than 10 000 *E. coli*/100 ml.

Predictive relationships between indicators and pathogens were evaluated using binary logistic regression. Results from each river were analysed separately by rivers or as pooled data set (all rivers together) to determine if the concentration of any of the indicators were correlated with the presence of *Salmonella* spp. *Cryptosporidium* or *Giardia*. The incidence of *Salmonella* spp.,

Cryptosporidium and *Giardia* did not correlate significantly with any of the indicator parameters. Aerobic colony counts, faecal coliform counts, *E. coli* counts, temperature and pH had no significant effect on the presence or absence of the pathogens (Table 6).

3.1.4 DISCUSSION

Cryptosporidium oocysts, *Giardia* cysts and *Salmonella* spp. were isolated from surface water used for irrigation of vegetables. The presence of these human pathogens in South African irrigation water may have serious public health implications as irrigation water can be a potential source of contamination of fresh produce as the pathogens can come in contact and attach to the surface of the crops (Armon *et al.*, 2002; Macarisin *et al.*, 2010).

The aerobic plate counts and faecal coliform counts of the Klip river were higher than those of the Skeerpoort and Moses rivers which indicates higher microbiological pollution in the Klip river (DOH, 2010). All samples from the Klip river were above the WHO guideline for irrigation water which limits the level of faecal coliforms to 1000 counts/100 ml (WHO, 1989) while the Moses and Skeerpoort river had faecal coliform level above the WHO standard in many but not all samples. This means that the Klip river has been contaminated with higher levels of faecal matter from human or animal origin than the two other rivers although the faecal contamination levels of the Moses and Skeerpoort were also high. The Klip, Moses and Skeerpoort rivers could have become contaminated due to run off water from informal settlement located near the rivers and from run off from nearby farms. The presence of high level of faecal coliforms in water indicates faecal pollution and possible contamination of the river with human enteric pathogens (Tallon *et al.*, 2005). Water containing high levels of faecal coliforms should thus not be used for irrigation purposes due to the risk of transfer of pathogen from the water to the fresh produce (Armon *et al.*, 2002).

Salmonella ser. Enteritidis, which was found in the Klip river, is a known human pathogen and has been the source of foodborne human infection outbreaks in the past (Garrity, 2005; Hennessy, Craig, Slutsker, White, Besser-Wiek, Moen, Feldman, Coleman, Edmonson, MacDonald and Osterholm, 1996). It is the most common serotype of *Salmonella* reported in the

UK and most outbreaks have been associated with the use of raw eggs in food products (Adams and Moss, 2004). *Salmonella* ser. Gaminara which was isolated from the Moses river is mostly associated with poultry (Garrity, 2005). It has however been the aetiological agent of a foodborne outbreak caused by infected orange juice (Cook, Dobbs, Hlady, Wells, Barrett, Puhr, Lancette, Bodager, Toth, Genese, Highsmith, Pilot, Finelli and Swerdlow, 1998). The presence of *Salmonella* spp. in irrigation water is a potential human health risk as pathogens can be transferred to fresh produce during irrigation (Tyrrel, 1999). No *Salmonella* spp. was found in the Skeerpoort river. This could be because the pH of the Skeerpoort river was more alkaline than the pH of the Moses and Klip river. The optimum pH for the survival of *Salmonella* being 7 (Adams and Moss, 2004), *Salmonella* spp. might not have survived in the Skeerpoort river due to its higher pH. The temperatures recorded did not differ significantly between rivers and were within the growth range of enteric bacteria (Adams and Moss, 2004).

The results of this study indicate the widespread presence of *Cryptosporidium* oocysts and *Giardia* cysts in surface water as the two protozoa were found in all three rivers. The incidence of *Cryptosporidium* and *Giardia* was the same in the Klip and Skeerpoort rivers despite much higher levels of faecal coliforms in the Klip river than in the Skeerpoort river. This suggests that the use of faecal coliforms as indicator for the presence of *Cryptosporidium* and *Giardia* is not reliable. The average incidence of *Cryptosporidium* oocysts in the 30 water samples was higher than the incidence of *Giardia* cysts. A similar trend has been observed in Norway and Portugal (Robertson and Gjerde, 2001b; Lobo, Xiao, Antunes and Matos, 2009). The opposite was however observed in a study on the prevalence of *Cryptosporidium* and *Giardia* in South African water by Kfir *et al.* (1995), where a higher incidence of *Giardia* cysts than *Cryptosporidium* oocysts was recorded in surface waters. The incidences of the two protozoa obtained in the present study are within the range of results obtained from other surveys undertaken in the UK (Watkins, Francis, Kay and Lewtrell, 2001) and in Norway on the occurrence of *Cryptosporidium* and *Giardia* in raw water (Robertson and Gjerde, 2001b). The presence of both protozoa in the same sample was observed in 5 out of the 30 samples (17%) analysed. This contrasts with the results from Kfir *et al.* (1995) where only 2.9% of surface water sampled in South Africa was positive for both protozoa. However, Kfir *et al.* (1995) used the settling

technique for concentrating cysts and oocysts while centrifugation was used in this study. The difference in methodology might have influenced the recovery rates.

On average, more *Cryptosporidium* and *Giardia* were isolated during spring and summer but the two protozoa were found in the Klip and Moses rivers during both summer and winter which suggests lack of seasonality. However, *Giardia* was only isolated from the Skeerpoort river during spring and summer which coincide with the rainy season. While lack of seasonality was reported in other studies (Robertson and Gjerde, 2001b), higher incidence of *Cryptosporidium* and *Giardia* in surface water has been observed after rainfall in some studies as heavy rainfall causes run off of top soil and contaminated debris into rivers (Muchiri, Ascolillo, Mugambi, Mutwiri, Ward, Naumova, Egorov, Cohen, Else and Griffiths, 2009; Atherholt, LeChevallier, Norton and Rosen, 1998). The sampling sites chosen for the Klip and Moses rivers happened to be watering points for cattle in the area. This means that direct contamination of the water with animal faeces was likely to happen throughout the year thus masking the possible effects of rainfall and seasons on the occurrence of *Cryptosporidium* and *Giardia*. On the other hand, no farm animals were seen in the vicinity of the Skeerpoort sampling site and *Giardia* was only isolated from the Skeerpoort river in summer. The effect of rainfall on the occurrence of *Cryptosporidium* and *Giardia* could thus be observed here. Determination of viability and infectivity of the oocysts would be important to assess the health risk associated with the presence of *Cryptosporidium* and *Giardia* protozoa in irrigation water.

There were no significant differences in the levels of indicator organisms and incidence of pathogens between the three rivers. This indicates widespread contamination of surface water with faecal matter and human pathogens independent of the location of the river.

The microbiological quality of the vegetables analysed was acceptable as the aerobic plate counts, faecal coliform counts and *E. coli* counts were low and no pathogens were isolated from the vegetables. No *Cryptosporidium* or *Giardia* was isolated from the vegetable samples despite the high incidence of these two protozoa in the water used for their irrigation. The low bacterial counts and absence of pathogens observed on the broccoli might be attributed to the fact that the broccoli had been irrigated only partially with water from the Loskop Dam canal, which is

supplied by the Moses and other rivers, and the rest of the irrigation water had come from a borehole. The absence of *Cryptosporidium* and *Giardia* from the vegetables might also be due to the low recovery rate of the method and the possibility of false negatives cannot be ruled out.

The presence of high faecal coliform counts in the water sampled did not necessarily indicate the presence of high levels of *E. coli*. It was indeed observed that *E. coli* was absent from some samples which had high faecal coliform counts and was sometimes present in water samples with low faecal coliform counts. The relationship between faecal coliform counts and presence of pathogen or *E. coli* counts and presence of pathogens was thus investigated.

No predictive relationship between levels of faecal coliform or *E. coli* and presence of *Cryptosporidium* or *Giardia* in surface water could be drawn from the results of this study. No correlation was found between temperature and pH and presence of *Cryptosporidium* or *Giardia* either. Similar poor correlation between faecal indicators and presence of pathogens has also been demonstrated in other studies (Harwood *et al.*, 2005). The absence of correlation between faecal indicators and *Cryptosporidium* and *Giardia* could be due to the different rates of survival of protozoa compared to those of bacterial faecal indicator and could also be due to the difference in recovery rate and detection limits (Scott *et al.*, 2002). The lower microbial density of *Cryptosporidium* and *Giardia* in surface water coupled with low recovery rate could have lead to failure to detect them due to sampling volume that was too small. Higher sampling volumes have indeed been used in other studies leading to higher recovery (LeChevallier, Norton and Lee, 1991).

The lack of correlation between indicator organisms and the presence of *Cryptosporidium* and *Giardia* suggests that the monitoring of environmental water samples with only indicator organisms is not sufficient to accurately predict the microbiological quality and safety of the water in terms of *Cryptosporidium* oocysts and *Giardia* cysts. *Cryptosporidium* and *Giardia* were indeed found in irrigation water that could have been classified as of acceptable microbiological quality if only faecal indicator counts were taken into account. The presence of these pathogens in irrigation water in South Africa indicates the potential for human infection acquisition through the consumption of fresh produce irrigated with those waters.

3.1.5 CONCLUSIONS

This study demonstrates the widespread presence of *Cryptosporidium* and *Giardia* in surface water used for irrigation of fruits and vegetables in three rivers from different provinces of South Africa. The presence of these pathogens in irrigation water has serious public health implications as these pathogens have been shown to attach and survive on the surface of fresh produce following irrigation with contaminated water. Commonly used faecal indicators failed to reliably predict the presence or absence of *Cryptosporidium* and *Giardia* in the water analysed. The use of only faecal indicator organisms for monitoring surface water quality is not sufficient to accurately predict the presence of *Cryptosporidium* and *Giardia* and assess the microbiological safety of irrigation water. Identification of the sources of water contamination and more understanding of the ecology of *Cryptosporidium* and *Giardia* and their distribution in the environment in comparison to those of indicator organisms is needed in order to identify good predictors for the presence of *Cryptosporidium* and *Giardia* in water.

3.2 EFFECT OF CHLORINE, BLANCHING, FREEZING AND MICROWAVE HEATING ON *CRYPTOSPORIDIUM PARVUM* VIABILITY

ABSTRACT

Cryptosporidium parvum oocysts have been found on the surface of vegetables in both developed and developing countries. Vegetables can become contaminated with *C. parvum* via various routes including irrigation water. This study investigated the effect of individual treatments of chlorine, blanching, blast freezing and microwave heating as well as combined treatments of chlorine and freezing, and chlorine and microwave heating on the viability of *C. parvum* oocysts inoculated on green peppers. The viability of the oocysts after the treatments was assessed by determining the percentage oocysts stained with propidium iodide (PI) using a flow cytometer. Based on the PI staining, the chlorine treatments did not affect the viability of the oocysts. Blast freezing significantly inactivated 20% of the oocysts. Microwave heating and blanching significantly inactivated 93% of oocysts. Treatment of oocysts with chlorine followed by blast freezing treatment did not affect the viability of the oocysts significantly. Treatment of oocysts with chlorine and microwave heating were significantly more effective than microwave heating alone and inactivated 98.1 % of the oocysts ($p < 0.01$). The study indicates that *C. parvum* oocysts are sensitive to heat (hot water blanching and microwave heating), to some extent sensitive to blast freezing treatments but are resistant to chlorine. The use of chlorine during vegetable processing is therefore not a critical control point for *C. parvum* oocysts and should not be expected to inactivate *C. parvum* oocysts present on vegetables. Consumption of raw or minimally processed vegetables can therefore constitute a health risk as *C. parvum* oocysts are resistant to chlorine and can therefore still be found viable on ready-to-eat, minimally processed vegetables. However, cooked vegetables (in hot water or in a microwave) should be safe for consumption regardless of the pre-treatments or processing procedures followed.



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

3.2.1 INTRODUCTION

The consumption of vegetables has increased worldwide. People are becoming more concerned about their health and want to consume raw or lightly cooked vegetables (Pollack, 2010). Also due to the busy modern lifestyle, consumers have less time to prepare their vegetables at home and prefer to buy already washed, ready-to-eat raw vegetables or frozen vegetables pieces that just need to be cooked briefly in hot water or microwaved for even more convenience (Zink, 2009).

The number of foodborne infection outbreaks associated with fresh produce has however been increasing worldwide (Rose and Slifko, 1999). Foodborne infections associated with frozen produce have also been reported (Ho *et al.*, 2002). Foodborne cryptosporidiosis outbreaks have been reported in many countries and *Cryptosporidium* oocysts have been found on the surface of vegetables in both developed and developing countries (Robertson and Gjerde, 2001a; Ponka *et al.*, 2008; Ethelberg *et al.*, 2009). Furthermore, during the first phase of this study, *Cryptosporidium* and *Giardia* were found in water used for irrigation in South Africa. Since *Cryptosporidium* oocysts can be found in irrigation water and on fresh produce, it is important to understand the effect each processing step has on *Cryptosporidium* and whether oocysts can be killed during washing in chlorinated water, blanching and/or freezing. Previous studies have investigated the effect of treatments on *Cryptosporidium* oocysts inoculated in water or milk (Fayer, 1994). However, little is known about the effect of vegetable processing on *Cryptosporidium* or the effect of combined treatments on the survival of *Cryptosporidium* oocysts associated with vegetables.

Washing of vegetables in a chlorinated water bath is often the only step aimed at reducing microbial load on fresh produce during the preparation of ready-to-eat vegetables (Adams *et al.*, 1989). These products are usually sold as washed and ready to consume without any further washing from the consumer's side before consumption. Furthermore, these products are often eaten raw (e.g. in salads) and not cooked. Washing (in chlorinated water), blanching (in hot water or using microwaves) and blast freezing

steps are used in the production of frozen vegetables. Blanching is mainly used to inactivate enzymes but this step also reduces microbial load on the produce (Brewer, Begum and Bozeman, 1995; Archer, 2004). The initial blast freezing step is aimed at increasing the shelf life of the product by slowing metabolic activities of the product as well as those of the microorganisms still present on the vegetable and thus could affect the viability of *Cryptosporidium* oocysts. Furthermore, at home cooking of fresh or frozen vegetables using microwave ovens often occurs and many convenience vegetable products are now available on retail in a microwaveable packaging. At home microwave cooking of vegetables could therefore also have an effect the viability of *Cryptosporidium* oocysts (Ortega and Liao, 2006).

This challenge test was performed on green peppers to simulate the condition of vegetables' surfaces. Green peppers were chosen because their smooth surface facilitated the recovery of the oocysts after treatments and subsequent enumeration with a flow cytometer. The selection of treatment combinations was based on the assumption that chlorine alone would not be effective against *C. parvum* while the hot water blanching treatment would be effective against *C. parvum* as shown in the literature. Therefore the hurdle effect of combination of chlorine treatment with freezing or chlorine treatment with microwave heating was investigated as little literature was available about the effect of these treatments on *C. parvum* viability.

The individual effects of chlorine, hot water blanching, microwave heating and blast freezing as well as the combined effect of chlorine and freezing, and chlorine and microwave heating on the viability of *Cryptosporidium* oocysts inoculated on green peppers were investigated during this study.

3.2.2 MATERIALS AND METHODS

3.2.2.1 EXPERIMENTAL DESIGN

- The effects of 100 ppm and 200 ppm chlorine treatments on the viability of *C. parvum* oocysts inoculated on green pepper pieces were compared with each other and with the control samples.
- The individual effects of 200 ppm chlorine, hot water blanching, blast freezing and microwave heating on the viability of *C. parvum* inoculated on green pepper pieces were compared with each other and with the control samples.
- The effects of a 200 ppm chlorine treatment followed by a blast freezing treatment were compared to the control samples as well as to the individual effect of chlorine and blast freezing treatments on the viability of *C. parvum* oocysts inoculated on green pepper pieces.
- The effects of a 200 ppm chlorine treatment followed by a microwave heating treatment were compared to the control samples as well as to the individual effects of chlorine and microwave heating treatments on the viability of *C. parvum* oocysts inoculated on green pepper pieces.

3.2.2.2 *C. PARVUM* OOCYSTS INOCULATION ONTO GREEN PEPPER

Live *Cryptosporidium parvum* oocysts (5×10^6 oocysts in 8 ml PBS) were obtained from Waterborn Inc. (New Orleans, LA, USA). Eighty micro litres of the stock inoculum was inoculated onto 2 g green pepper pieces previously washed with ethanol and placed inside 5 ml sterile plastic tubes. Each inoculated piece was left to dry inside its tube at

4°C for 1 h before being treated. Six pieces of green pepper were inoculated for each treatment in order to have six replicates of each treatment.

3.2.2.3 EFFECT OF CHLORINE ON THE SURVIVAL OF *C. PARVUM* OOCYSTS INOCULATED ON GREEN PEPPER

The period of exposure used for the chlorination treatments were chosen to mimic the washing treatments used in the industry during the processing of frozen green pepper strips. The effect of chlorine at two different concentrations was studied.

All glassware used was first washed in chlorine-free distilled water. Chlorine solutions, 100 ppm and 200 ppm were prepared from 3.5% hypochlorite. Chlorine, 1 ml 100 ppm, was added to six tubes containing inoculated green pepper pieces. One ml 200 ppm chlorine was added to another 6 tubes. After 40 s, 50 µl 10% Na₂S₂O₃ was added to each tube to neutralize the free chlorine (Korich *et al.*, 1990). Complete neutralisation of chlorine was tested with potassium iodide starch paper (Macherey-Nagel GmbH & Co KG, Düren, Germany).

3.2.2.4 EFFECT OF BLANCHING ON THE SURVIVAL OF *C. PARVUM* OOCYSTS INOCULATED ON GREEN PEPPER

The time/temperature combination used for the blanching treatment was chosen as an average temperature and time used to blanch vegetables in industry (Puupponen-Pimia *et al.*, 2003). Six plastic tubes containing inoculated green pepper pieces were placed in a 96°C water bath for 3 min and then cooled in 4°C water for 3 min to stop the heating process.

3.2.2.5 EFFECT OF BLAST FREEZING ON THE SURVIVAL OF *C. PARVUM* OOCYSTS INOCULATED ON GREEN PEPPER

The time/temperature combination used for the freezing treatment was chosen to mimic the initial blast freezing treatment used in the industry during the processing of frozen green pepper strips. Six plastic tubes containing inoculated green pepper were placed in a blast freezer (Linde, Munich, Germany) at -20°C for 4 min and then thawed in 4°C water for 3 min.

3.2.2.6 EFFECT OF MICROWAVE HEATING ON THE SURVIVAL OF *C. PARVUM* OOCYSTS INOCULATED ON GREEN PEPPER

The microwave heating treatment used in this study was chosen to imitate the cooking instructions given on the packaging of fresh vegetables and frozen vegetables in the retail in South Africa.

Six tubes containing inoculated green pepper pieces were microwaved in a domestic microwave oven (Daewoo model KOR_145Q, Korea) at 2450 MHz with a maximum output power of 850 Watts for five minutes. The samples were then cooled down in 4°C water for 3 min to stop the heating process.

3.2.2.7 HURDLE EFFECT OF COMBINED TREATMENTS ON THE SURVIVAL OF *C. PARVUM* OOCYSTS INOCULATED ON GREEN PEPPER

Chlorination and blast freezing treatment of oocysts

Six plastic tubes containing inoculated green pepper pieces were first treated with 200 ppm chlorine as described in 3.2.2.3 and then immediately frozen as described in section 3.2.2.5.

Chlorination and microwaving treatment of oocysts

Six plastic tubes containing inoculated green pepper pieces were first treated with 200 ppm chlorine as described in 3.2.2.3 and then immediately microwaved and cooled down as described in section 3.2.2.6.

3.2.2.8 CONTROL

A control sample was used to determine the propidium iodide (PI) fluorescence emitted by live stained *C. parvum* oocysts. 6 green peppers pieces were inoculated, kept at 4°C and then recovered and stained the same way as the treated samples.

3.2.2.9 RECOVERY OF *C. PARVUM* OOCYSTS AFTER TREATMENTS

Four millilitres buffer made of peptone buffer saline and 0.01% Tween 20 was added to each tube. The tubes were mixed using a vortex for 30 s to wash the oocysts off the green pepper pieces. The content of each tube was then transferred to a 5 ml centrifuge tube through a 35 µm pore size nylon filter placed in a funnel. The tubes were centrifuged at 2500 x g for 10 min. The brake was set at level 025 (Cook *et al.*, 2007). The supernatant was aspirated with a micropipette to leave 1 ml pellet in each tube. The tubes were kept at 4°C until staining.

3.2.2.10 STAINING

Three microlitres *Cryptosporidium* labeled fluorescein-iso-thiocyanate (FITC) (Davies Diagnostic, Randburg, South Africa) was added to each tube one hour prior to analysis, vortexed and kept in a cooler box.

Five minutes before analysis with flow cytometer, 500 µl (PI) (Beckman Coulter Miami, Florida, USA) was added to each tube.

3.2.2.11 FLOW CYTOMETRY ANALYSIS

The viability of *C. parvum* oocysts after the treatments was measured by determining the percentage cells positive for the nucleic acid dye propidium iodide with a flow cytometer. A strong correlation has been found between oocysts infectivity and nucleic acid staining intensity (Neumann, Gyurek, Gammie, Finch and Belosevic, 2000).

Analyses were performed on a Epic Altra flow cytometer (Beckman Coulter, Miami, Florida) equipped with water cooled enterprise laser and using the Expo 32 MultiCOMP software (Beckman Coulter, Miami, Florida). Cells were identified by FITC fluorescence and forward scattering. The percentage of cells positive for both FITC and PI was determined.

3.2.2.12 STATISTICAL ANALYSIS

Single factor Analysis of Variance (ANOVA) was used to determine whether the individual and combined treatments had a statistically significant effect (99% confidence interval) on the viability of *Cryptosporidium parvum* when compared to the control samples. The significant level of the ANOVA test was set at $p < 0.01$. The least significant difference test (LSD test) was performed to determine the significant difference between the means of the treatments. The t-test, independent by group, was performed to compare each treatment against the control or against another treatment. Analyses were performed using Statistica Software for Windows Version 7 (Tulsa, Oklahoma, USA, 2003).

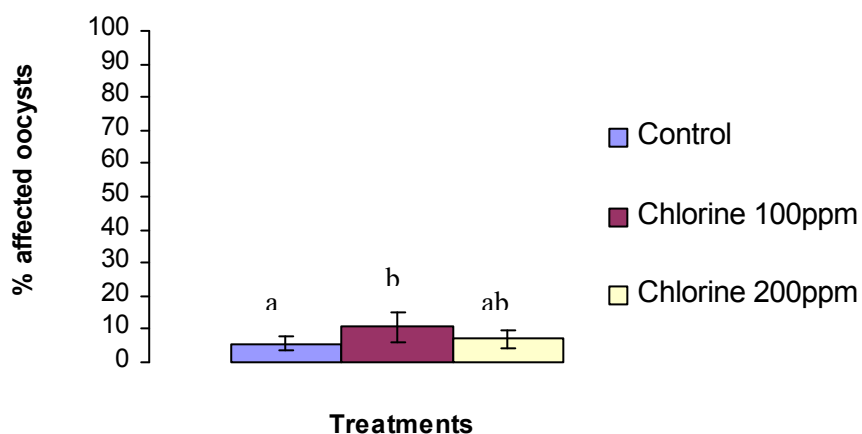
3.2.3 RESULTS

3.2.3.1 EFFECTS OF CHLORINE CONCENTRATION ON *CRYPTOSPORIDIUM PARVUM* VIABILITY

The average percentage of oocysts stained by PI after being treated with 100 ppm chlorine (10.8%) was significantly higher ($p < 0.01$) than the control samples (Table 8). The percentage oocysts stained by PI after the 200 ppm chlorine treatment (7 %) was in the other hand not significantly different from the control samples ($p = 0.39$) (Table 8). There was however no significant difference in the percentage of oocysts stained by PI between the 100 ppm chlorine treatment and the 200 ppm chlorine treatment ($p = 0.11$) (Figure 5). Figure 6 shows the PI peak obtained with the flow cytometer after analysis of a sample treated with 100 ppm chlorine.

Table 6: p values for t-test analysis of % inactivated oocysts after 100 ppm chlorine and 200 ppm chlorine treatments compared to the control

Treatment	p-value
100 ppm chlorine	0.032
200 ppm chlorine	0.387



Error bars on columns represent standard deviations

Average values with different superscripts letters differ significantly ($p < 0.01$)

Figure 4: Effects of different levels of chlorine on the survival of *Cryptosporidium parvum* oocysts inoculated on green pepper (n=6).

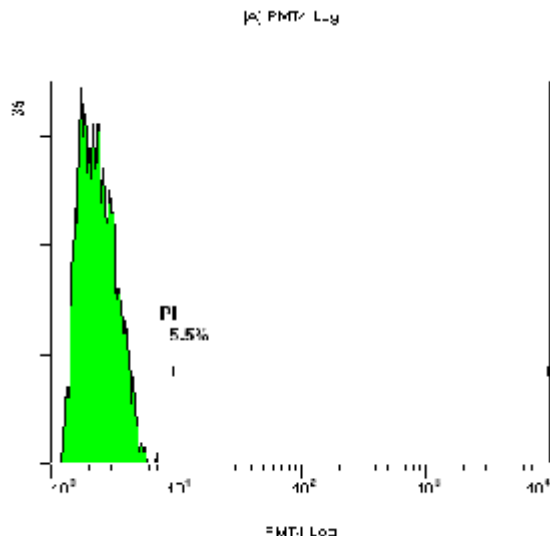


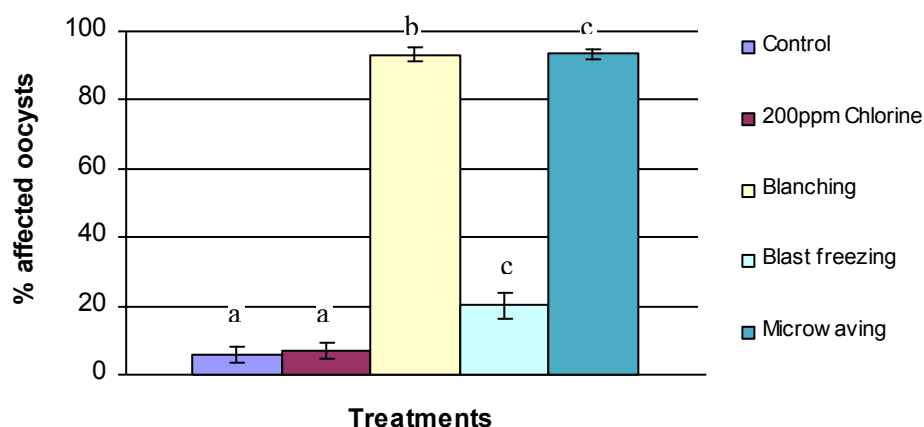
Figure 5: Percentage *Cryptosporidium parvum* oocysts positive for PI fluorescence after treatment with 100 ppm chlorine.

3.2.3.2 EFFECTS OF INDIVIDUAL TREATMENTS ON THE VIABILITY OF *CRYPTOSPORIDIUM PARVUM* OOCYSTS INOCULATED ON GREEN PEPPER

5.7 % oocysts included PI in the control samples. 7 % of the oocysts chlorinated with 200 ppm included PI and had thus been affected by the treatment (Figure 7). The difference between the control samples and 200 ppm chlorine treatment samples was not significant ($p=0.39$) (Table 8). Blanching and microwave heating inactivated 93.2% and 93.5% of oocysts respectively (Figure 7). Both blanching and microwave heating were significantly more effective ($p<0.01$) than blast freezing to inactivate *C. parvum* oocysts inoculated on green pepper when compared to the control (Table 8). A significant percentage ($p<0.01$) of oocysts were affected by the blast freezing (20.1%) treatment compared to the control group (Figure 7). Figures 8 and 9 show the PI peaks obtained after analysis of each sample with the flow cytometer.

Table 7: p values for t-test analysis of % inactivated oocysts after chlorine, blanching, blast freezing and microwave heating treatments compared to the control group

Treatment	p-value
Chlorination with 200 ppm	0.387
Blanching	<0.001
Blast freezing	<0.001
Microwaving	<0.001



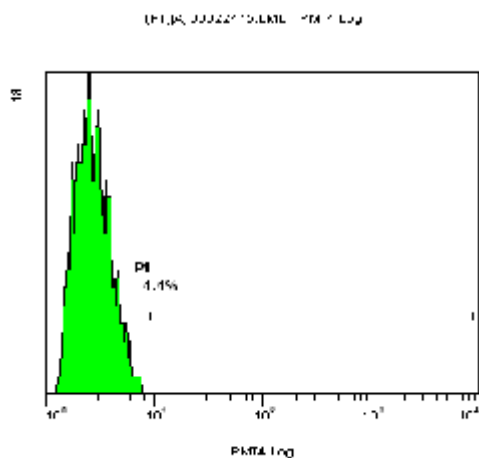
Error bars on columns represent standard deviations

Average values with different superscripts letters differ significantly ($p < 0.01$)

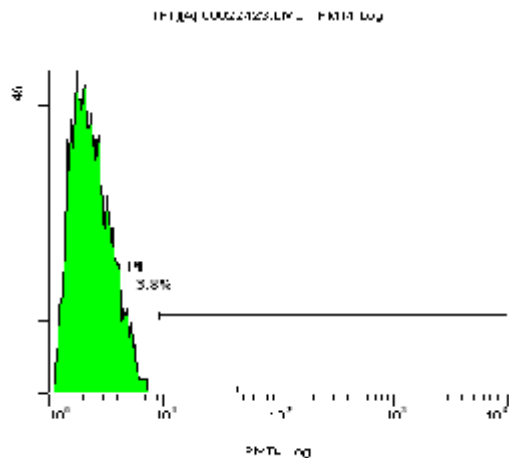
Figure 6: Effect of chlorination, blanching, microwaving and blast freezing treatments on the survival of *Cryptosporidium parvum* oocysts inoculated on green pepper (n=6).



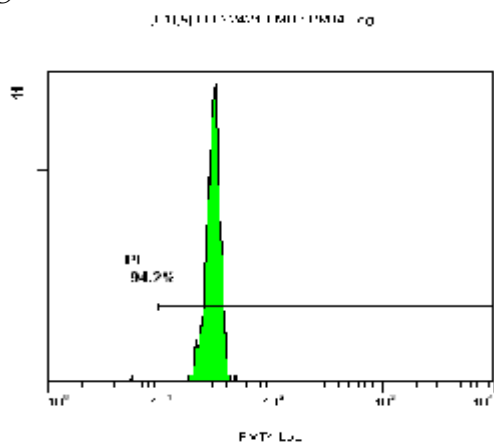
A



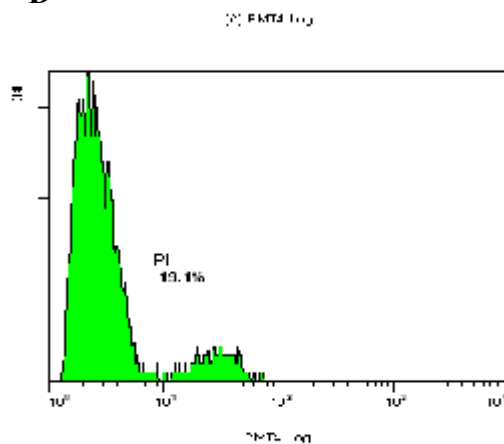
B



C



D



E

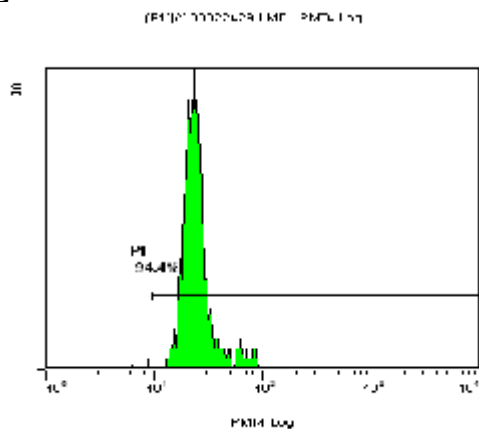
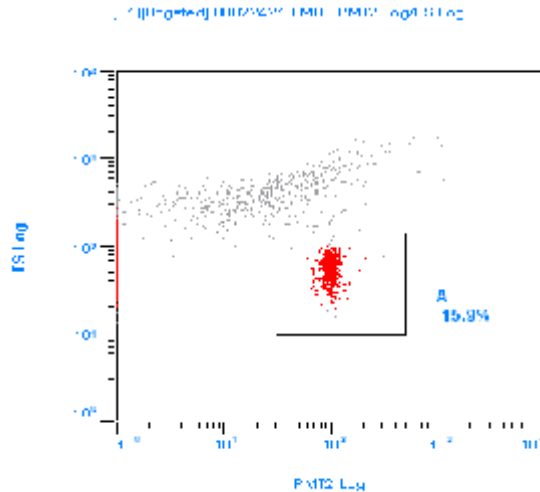


Figure 7: Percentages *Cryptosporidium parvum* oocysts positive for PI fluorescence after no treatment (Control) (A), after treatment with 200ppm chlorine (B), after blanching (C), after blast freezing (D) and after microwave treatment (E).

(1)



(2)

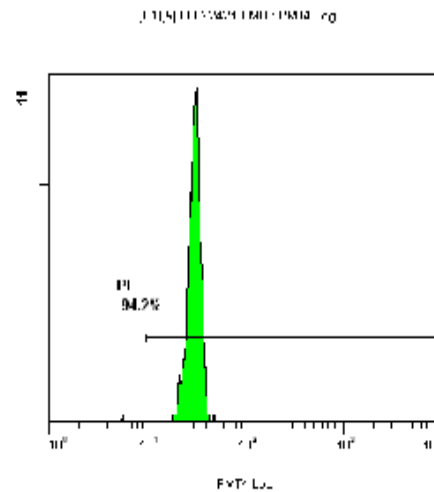


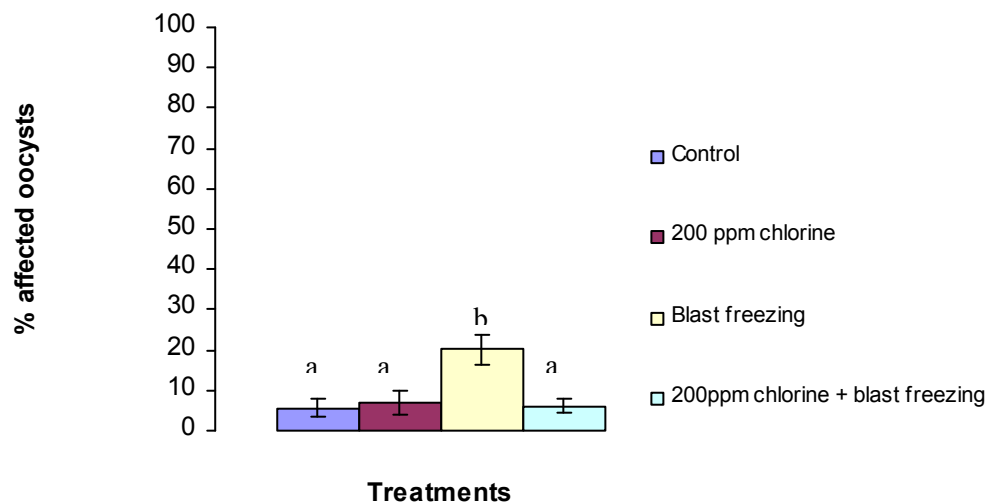
Figure 8: (1) analysis with flow cytometer of *Cryptosporidium parvum* on bivariate dotplots of size (FS) versus green fluorescence (pmt2). The gate (a) was defined to include all FITC fluorescent cells. (2) Percentage *Cryptosporidium* oocysts positive for PI fluorescence after blanching.

3.2.3.2 HURDLE EFFECT OF COMBINED CHLORINATION AND FREEZING TREATMENTS ON THE VIABILITY OF *CRYPTOSPORIDIUM PARVUM* OOCYSTS INOCULATED ON GREEN PEPPER

The combined treatments with 200 ppm chlorine followed by blast freezing did not affect the integrity of the *C. parvum* oocysts more than the individual chlorine treatment (Figure 10). Blast freezing alone was more effective than the combined treatments as there was no significant difference in PI inclusion ($p=0.77$) between the control samples and the samples that had been treated with 200 ppm chlorine and then placed in a blast freezer (Table 9). Figure 11 shows the PI peak obtained with the flow cytometer after analysis of a sample treated with chlorine and blast freezing.

Table 8: p values for t-test analysis of % inactivated oocysts after individual and combined chlorine and blast freezing treatments compared to the control group

Treatment	p-value
Chlorination at 200ppm	0.387
Blast freezing	<0.001
Chlorination + blast freezing	0.766



Error bars on columns represent standard deviations

Average values with different superscripts letters differ significantly ($p < 0.01$)

Figure 9: Effect of individual chlorination and blast freezing treatment or combination of both treatments on the survival of *Cryptosporidium parvum* oocysts inoculated on green pepper (n=6).



(F1) 0022-1-0-1-0 PMT Log

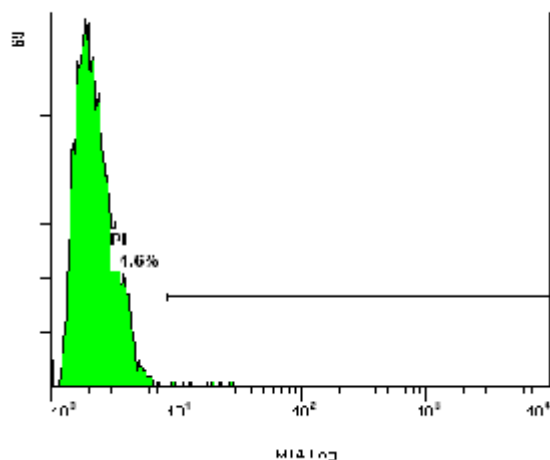


Figure 10: Percentage *Cryptosporidium parvum* oocysts positive for PI fluorescence after combined treatments with 200 ppm chlorine and blast freezing.

3.2.3.3 HURDLE EFFECT OF COMBINED CHLORINATION AND MICROWAVE HEATING ON THE VIABILITY OF *CRYPTOSPORIDIUM PARVUM* OOCYSTS INOCULATED ON GREEN PEPPER

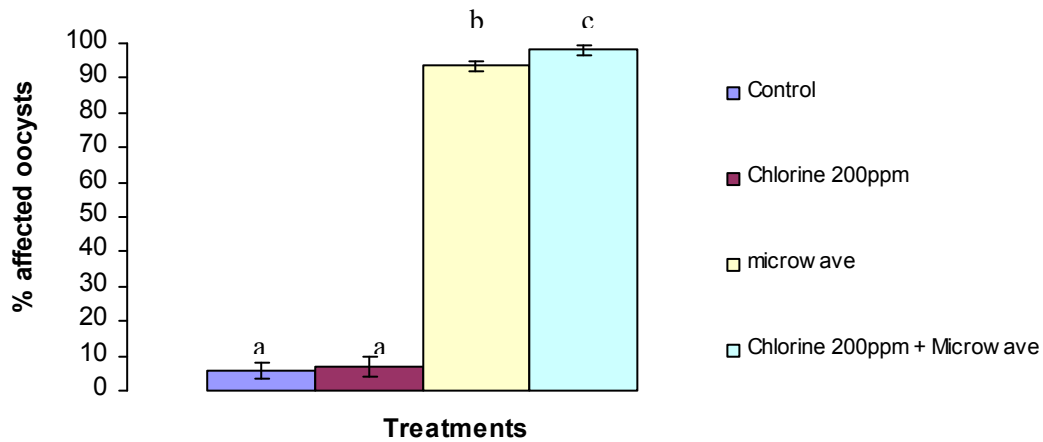
The combination of chlorination and microwave heating treatments was significantly ($p < 0.01$) more effective than either chlorination or microwave heating alone at inactivating *C. parvum* oocysts (Table 10). Almost all oocysts (98.1%) included PI after the combination of the two treatments (Figure 12). Figure 13 shows the PI peak obtained with the flow cytometer after analysis of a sample treated with chlorine and microwave heating.

Table 9: p values for t-test analysis of % inactivated oocysts after individual and combined chlorine and microwave heating treatments compared to the control group

Treatment	p-value
Chlorination at 200 ppm	0.387
Microwaving	<0.001



Chlorination + microwaving	<0.001
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Error bars on columns represent standard deviations

Average values with different superscripts letters differ significantly ($p < 0.01$)

Figure 11: Effects of individual chlorination and microwave heating treatment or combination of both treatments on the survival *Cryptosporidium parvum* oocysts inoculated on green pepper (n=6).

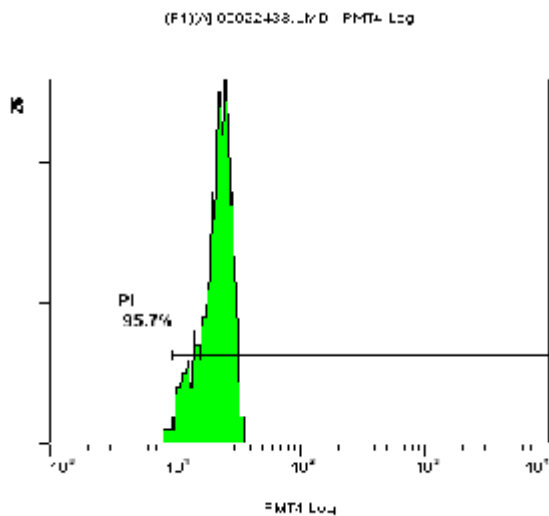


Figure 12: Percentage *Cryptosporidium parvum* oocysts positive for PI fluorescence after combined treatments with 200 ppm chlorine and microwave heating.

3.2.4 DISCUSSION

This study investigated the individual effects of chlorine, heat treatments (hot water and microwave) and blast freezing on *Cryptosporidium parvum* oocysts inoculated on the surface of green peppers. The combined effects of chlorine and blast freezing treatments as well as the combined effects of chlorine and microwave heating were also investigated. The aim of the study was to determine whether these treatments were able to render the oocysts non infectious and whether or not they could be used to reduce the risk associated with the presence of *C. parvum* on vegetables. The green peppers were inoculated with oocysts at a concentration exceeding levels that would normally be found on the surface of vegetables. This was done to maximize recovery and detection of oocysts after the treatments in order to determine how effective the treatments were.

Inclusion of propidium iodide (PI) into an oocyst only occurs if the integrity and permeability of the cell wall have been compromised. A strong correlation between inclusion of PI and loss of infectivity as defined by loss of in vitro excystation has been described by Campbell *et al.* (1992). The inclusion of PI was thus used as an indication of loss of viability in this study. The low PI inclusion in the control samples indicates that most *C. parvum* oocysts were viable prior to the treatments. Inclusion of PI in a small percentage of the control samples can be explained by the fact that even though the oocysts were certified viable upon delivery, some oocysts might have been damaged during transit from the United States of America, during storage or during analysis. The percentage of PI inclusion in the control samples was used to compare the killing effect of the other treatments.

Less oocyst were affected by the 200 ppm chlorine treatment than by the 100 ppm chlorine treatment. This could be attributed to the increase in pH caused by higher concentration of chlorine (Sanz *et al.*, 2002). Increase in pH has been found to nullify the antimicrobial effect of chlorine (Sanz *et al.*, 2002). It was found by Sanz, *et al.* (2002) that if the pH was not controlled, chlorine's antimicrobial action only increased with

increase in concentration up to a 50 mg/l chlorine. Further addition of chlorine up to a concentration of 200 mg/l to the washing bath did not further decrease microbial load and was actually less effective than 50 mg/L. The insignificant difference between the control and the chlorine treatments showed that the addition of chlorine to washing baths during the preparation of fresh produce would not be expected to inactivate oocysts present on the surface of vegetables. *Cryptosporidium*'s resistance to chlorine has also been reported in other studies (Korich *et al.*, 1990; Fayer, 1994). Furthermore, as *Cryptosporidium* oocysts have been shown to be internalised through the stomata and resists hours of washing, the risk of not eliminating *Cryptosporidium* oocysts from contaminated vegetables through the use of chlorinated washing bath is even greater (Macarisin *et al.*, 2010; Ortega, Roxas, Gilman, Miller, Cabrera, Taquiri and Sterling, 1997). These findings have implications for the safety of minimally processed and ready-to-eat vegetables as these products are assumed to be safe and free of pathogens by the consumers who do not further wash the products before consumption.

Blanching of the green peppers in hot water was sufficient to kill most *C. parvum* oocysts present on the inoculated green peppers. Blanching of vegetables in hot water during the manufacture of frozen vegetables should thus be sufficient to kill *Cryptosporidium* oocysts present on the surface of vegetables. At home cooking of vegetables in hot water should also be sufficient to kill *Cryptosporidium* oocysts. The killing effect of heat on *Cryptosporidium* oocysts has also been reported in previous studies (Harp *et al.*, 1996; Moriarty *et al.*, 2005).

Blast freezing of inoculated green pepper only affected a small percentage of the oocysts. Pre-treatment of inoculated green pepper with 200 ppm chlorine did not increase the susceptibility of the oocysts to freezing. This could be due to the methodology used which created different environments which affected the survival of the oocysts. Indeed, the oocysts on green peppers undergoing solely blast freezing were dry while the oocysts on green peppers that had been treated with chlorine were in solution. It has in fact been observed by Kato, Jenkins, Fogarty and Bowman (2002) that *Cryptosporidium* oocysts in dry soil are more resistant to freezing than oocysts in moist or wet soil. The influence of

moisture on the effect of freezing on *C. parvum* oocysts viability might thus have been observed here.

These results showed that initial blast freezing of green pepper with or without prior chlorine treatment, are not sufficient to kill *C. parvum* oocysts present on their surface. Frozen vegetables that are washed or not in chlorinated water and then frozen without any blanching steps could thus still carry live *Cryptosporidium* oocysts. These findings are supported by a previous study by Fayer and Nerad (1996) who found that freezing of oocysts in water at -20°C for one hour was not sufficient to render *Cryptosporidium* oocysts non infective and that freezing of oocysts for 24 h at -20°C was necessary to kill all oocysts. The effect of the initial blast freezing of vegetables on *C. parvum* viability was investigated in this study and not the effect of long term frozen storage. It could therefore be that the viability of oocysts would be affected during storage of frozen vegetables but that is beyond the scope of this study.

Microwave heating of the inoculated green pepper inactivated most *C. parvum* oocysts. These results correlate with the finding of Ortega and Liao (2006) who found that oocysts were inactivated after exposure to microwaves at 1 100 W for 20 s or to 700 W for 45 s. Pre-treatment of inoculated green peppers with 200 ppm chlorine for a contact time of 40 s significantly increased the efficacy of the microwave heating against *C. parvum* oocysts by 4.6%. The mechanisms of action of chlorine on *Cryptosporidium* oocysts is poorly documented but the spores of *Bacillus subtilis* have been used as a surrogate to measure the effect of disinfectant on *Cryptosporidium* oocysts (Radziminski, Ballantyne, Hodson, Creason, Andrews and Chauret, 2002). It has been observed that chlorine decreases heat resistance in *B. subtilis* spores by removing proteins from the spore coat (Wyatt and Waites, 1975). Exposure of *Cryptosporidium* oocysts to chlorine could thus enhance the killing effect of microwave heating by damaging protein in the oocysts' cell wall. It could also be that the presence of moisture rendered the oocysts more sensitive to heat seeing as the oocysts on green peppers undergoing solely microwaving were dry while the oocysts on green pepper that had been treated with chlorine were still in solution. It has indeed

been observed that microorganisms are more sensitive to wet heat than to dry heat (Jay, 2000).

These results show that microwave heating can be used to kill *C. parvum* oocysts present on vegetables. Microwave blanching of vegetables during the manufacture of frozen vegetables would eliminate the risk associated with the presence of oocysts on the produce. Similarly, at home microwave heating of vegetables should also be sufficient to kill oocysts present on the surface of the produce.

3.2.5 CONCLUSIONS

The use of chlorine in washing water baths does not inactivate *Cryptosporidium parvum* oocysts inoculated on green pepper. Therefore, chlorination alone cannot be relied on to ensure the safety of minimally processed green peppers. Other hurdles must be used during the manufacture of minimally processed vegetables to ensure the elimination of *C. parvum* oocysts. Heating of vegetables in hot water or in a microwave is effective against *C. parvum* oocysts inoculated on green peppers. Products that undergo these steps during processing should thus not carry live *C. parvum* oocysts after processing. Blast freezing only has a slight effect on the viability of *C. parvum* oocysts and cannot be relied upon to kill oocysts present on fresh produce. If vegetables do not undergo any heat treatment new hurdles must be specifically designed to target *Cryptosporidium* during vegetable processing to ensure the elimination of *Cryptosporidium* oocysts. On the other hand if the vegetables are blanched during manufacture or are cooked prior to consumption, the safety risk associated with the presence of *Cryptosporidium* on the vegetable is low since the oocysts are susceptible to heat and will be killed by the heat treatment.

CHAPTER 4: GENERAL DISCUSSION

4.1 CRITICAL REVIEW OF METHODOLOGY AND EXPERIMENTAL DESIGN

The method used in this study for the isolation of *Cryptosporidium* and *Giardia* from raw water was the method approved by the Environment Protection Agency (EPA) which is used internationally and also locally by the Rand Water laboratories in Vereeniging. One limitation of this method is the amount of water that can be filtered through the filter capsule. In the official method, 10 litres are to be filtered but in this study 50 litres were filtered to increase *Cryptosporidium* and *Giardia* recovery as no sample had tested positive during a preliminary study in 2008 where 10 litres of water from three rivers had been filtered and analysed (Duhain, Minnaar and Buys, 2008). While sampling larger volume of water increased the chance of isolating the protozoa, filtering a larger volume of turbid water caused the filter to clog up with sediment. The high amount of sediment in some of the water samples interfered with the Immuno-Magnetic Separation (IMS) of the oocysts and cysts and might have resulted in lower affinity of the magnetic beads with the cysts and oocysts. Water samples with high content of sediment should have been filtered first with a larger pore filter in order to remove larger particles and to facilitate further analysis.

The recorded recovery rate of the method was lower than expected but different recovery efficiencies were observed with ColorSeeds and EasySeeds. ColorSeeds and EasySeeds are irradiated oocysts used to calculate the recovery efficiency of the detection method. Higher recovery rate was observed when using EasySeeds compare to when using ColorSeeds. The oocysts and cysts from the EasySeeds were more distinct and fluoresced more strongly while ColorSeeds were difficult to distinguish from the background under fluorescence microscope. The lower recovery could have been due to a bad batch of ColorSeeds and the exact reason is still unknown. But this affected the recorded average

recovery rate of the method which could have been higher if EasySeeds had been used since the beginning of the study.

Another limitation of the method 1623 is the high cost of the consumables. The cost of the method limited the amount of samples that could be analysed during the duration of the study. The study would have been more representative of the actual incidence of *Cryptosporidium* and *Giardia* in irrigation water in South Africa if more samples from more locations had been taken.

Cryptosporidium oocysts DNA was extracted from the microscope slides and an attempt was made to replicate the extracted DNA to identify the *Cryptosporidium* species present on the slides using real time Polymerase Chain Reaction (PCR) and melting curves analysis. More than 15 species of *Cryptosporidium* have been identified, each associated with a particular human or animal host, the identification of the exact species of *Cryptosporidium* present in the water analysed would therefore have been of interest to find out the source of contamination of the rivers (Fayer, 2004). *C. parvum* and *C. hominis* are the two species most associated with human. Zoonotic infections, when species from animal hosts are transferred to humans and are able to cause infections in human, have however also been reported. Identification of species would have been useful to determine whether the source of contamination of the river with *Cryptosporidium* oocysts was from human source e.g. informal settlements or animal source and if so from which animal e.g. ruminant, bird, reptile, dog, cat, etc. This information could be used to source the problem and develop strategies to limit such contamination in the future. This could also indicate variation in types of contamination sources between rivers or in the contrary widespread contamination from mainly one source, for example, mainly human faeces e.g. run-off from informal settlements. The identification of the species isolated from the water could however not have been used to determine whether the oocysts present in the water posed a risk of human infection or not as all species have the potential to cause infections in human (Fayer, 2004).

Unfortunately the presence of inhibitors and low concentration of template on the microscope slides did not allow for successful real time PCR identification. Conventional PCR was thus used to confirm the presence of *Cryptosporidium* oocysts on the microscope slides.

E. coli serotyping was performed using the GeneDisc system (Pall GeneSystem, Bruz, France) which is an automated real time PCR system which allows for the detection of *E.coli* O157:H7 DNA, shigatoxic *Escherichia coli* and entero-hemorrhagic *E. coli* O26, O103, O145 and H7 DNA. A drawback of this method is that it does not allow for detection of other *E. coli* apart from those listed above. *E.coli* spp. isolated from the rivers were selected on selective *E. coli*/coliform chromogenic medium (Oxoid LTD, Basingstoke, Hampshire, England). Selection on this chromogenic medium is based on the production of purple colonies by *E. coli* producing the enzyme B-glucuronidase. However *E. coli* O157:H7 does not produce this enzyme (Abbott, Hanson, Felland, Connell, Shum and Janda, 1994). This means that *E. coli* O157:H7 colonies would appear pink on this chromogenic media and would be mistaken for being a coliform but not *E. coli*. Some samples might thus have been incorrectly identified as not containing *E. coli* after confirmation on chromogenic medium and thus not serotyped any further. This might have led to false negatives and non detection of *E. coli* O157:H7 in actually positive samples and therefore underestimation of *E. coli* positive samples. A different media should have been used for the isolation of all *E. coli* including *E. coli* O157:H7. *E.coli* eae virulence factor was isolated from the water but the sample did not test positive for *E. coli* O157:H7 so the virulence factor must have originated from another *E. coli* spp. Further serotyping could be done on the *E. coli* isolate to determine the exact serotypes and pathogenicity of the *E. coli* present in the irrigation water.

Propidium iodide (PI) is a DNA stain that is used to determine the membrane integrity of cells and cells with damaged membranes are considered as non viable. Considerable correlation has been found between PI staining and loss of viability of *Cryptosporidium* (Campbell *et al.* 1994). However more recent studies have found discrepancy between the results obtained with PI and infectivity studies (Bukhari, Marshall, Korich, Fricker,

Smith, Rosen and Clancy, 2000). The results obtained in this study can be used as an indication of the effect of treatments on the viability of *Cryptosporidium* but cannot replace infectivity studies.

During the preliminary studies, green peppers were inoculated with 50 000 *Cryptosporidium* oocysts and the oocysts were then recovered from the green peppers by stomaching in elution solution and then centrifugation as would be used when testing for the presence of oocysts on vegetables. Not enough oocysts were recovered with this method to allow enumeration of the oocysts with a flow cytometer because the flow cytometer used can only detect and enumerate cells if the samples contain a high concentration of cells of above 5000 oocysts/ ml. This showed that isolation of oocysts from vegetables is difficult to achieve even when high numbers of oocysts are present on the vegetables. This could explain why no field samples of broccoli or lettuce tested positive for *Cryptosporidium* or *Giardia* while high incidence of the two protozoa had been recorded in the irrigation water.

Green pepper was chosen as a model vegetable for the challenge tests because of its smooth firm outer surface which does not disintegrate upon mixing in solution and therefore facilitates the recovery of oocysts from the vegetable after the treatments. Green peppers are sold raw as minimally processed vegetables and also frozen e.g. in stir-fry. Green peppers undergo washing in chlorinated water and blast freezing during the manufacture of frozen vegetables. Green pepper was thus an acceptable model vegetable for the chlorine and freezing treatments. However, green peppers are one of the few vegetables that are not always blanched before freezing and were therefore not the ideal model vegetable for the blanching treatment. Frozen green peppers can however be cooked at home in hot water or in a microwave and could therefore still be used as model vegetable for the heat treatments. Trials had been done using broccoli as a model vegetable since broccoli undergoes blanching during the manufacture of frozen broccoli. But the granular structure of broccoli interfered with the recovery of oocysts after the treatments.

Oocysts can only reproduce *in vivo* or in a tissue culture and cannot replicate on commonly used microbiological media. The number of treatments that could be investigated in this challenge study was therefore limited by the number of oocysts that were bought from Waterborn Inc. The selection of treatments was made to simulate the production of minimally processed vegetables, the manufacture of frozen vegetables and the cooking of vegetables at home. The literature shows that *Cryptosporidium* oocysts are resistant to chlorine but susceptible to heat treatments. It was therefore hypothesised that the oocysts would not be affected by chlorine treatments alone but would be affected by the hot water blanching treatments. Few studies have been done on the effect of microwave heating on the viability of oocysts and few studies have look at the effect of short term blast freezing on the viability of oocysts. The microwave heating and blast freezing treatments of inoculated green peppers were not expected to fully inactivate all oocysts inoculated on the green peppers. The effect of combined treatments with chlorine and microwave heating and the effect of combined treatments with chlorine and blast freezing were therefore investigated. However, since it happened that the microwave heating had the same effect on *C. parvum* oocysts than the hot water blanching, the effect of combined treatments with chlorine and hot water blanching should also have been investigated. There were however not enough oocysts available to test the effect of these treatments on *C. parvum* viability.

During the hot water blanching treatment, the tubes containing the inoculated green pepper pieces were placed in hot water but the green pepper pieces did not come in direct contact with the hot water. This was done to avoid losing oocysts in the water and to be able to recover sufficient amount of oocysts for enumeration with the flow cytometer. The temperature reached inside the tube was lower (88°C) than the water temperature (96°). The inactivation effect observed in this study was thus conservative and higher inactivation rate could be expected during hot water blanching of vegetables when the vegetables come in direct contact with the water.

4.2 CONSEQUENCES OF THE PRESENCE OF *CRYPTOSPORIDIUM* IN IRRIGATION WATER AND EFFECT OF TREATMENTS ON *C. PARVUM* VIABILITY

Cryptosporidium oocysts and *Giardia* were found on several occasions in the three rivers tested during the duration of the field survey. This means that these protozoa are present in water used for irrigation purposes and have the potential to contaminate vegetables in the field and thus cause human infections. *Cryptosporidium* oocysts and *Giardia* cysts were found in water samples containing both high and low levels of faecal coliforms or *E. coli*. Some water samples had faecal coliform counts below the WHO standard for irrigation water and could thus be classified as acceptable in term of microbiological quality but yet tested positive for *Cryptosporidium* and *Giardia*. No statistical correlation was found between faecal coliform or *E. coli* counts and the incidence of *Cryptosporidium* or *Giardia*. It can thus be concluded that the use of faecal coliform or *E. coli* as indicator organism to predict the presence of human pathogens in river water is not foolproof. Other organisms, like *Clostridium perfringens*, have been suggested to reliably predict the presence of *Cryptosporidium* or *Giardia* in river water (Payment and Franco, 1993). *C. perfringens* is a gram positive spore-former that is typically found in soil and in the intestine of animals (Griffin *et al.*, 2001). *C. perfringens* can survive for a longer period of time than *E. coli* in the environment and is resistant to environmental stresses such as heat, desiccation and ultraviolet light (Griffin *et al.*, 2001). Its survival in the environment is thus more similar to protozoa's.

The concentration of *Cryptosporidium* oocysts in the water samples analysed was not determined and only the presence of the parasite in water was detected. If we assume that only one oocysts was detected in 50 litres of water, taking the 13.5% average recovery efficiency of the method into account, this mean 7 oocysts were actually present in 50 litres of water. This number could very well be much higher considering the fact that the recovery efficiency was lower at times and that all oocysts on microscope slides were not counted. Many litres of water are sprayed onto the surface of vegetables during irrigation

and this is done repeatedly throughout the growing period. *Cryptosporidium* oocysts can thus accumulate on vegetables and survive until harvest and possibly consumption.

Although many studies have confirmed the presence of *Cryptosporidium* oocysts on fresh vegetables in both developed and developing countries, few studies have determined the concentration of oocysts on the fresh produce (Monge and Chinchilla, 1995; Ortega *et al.*, 1997; Al-Binali, Bello, El-Shewy and Abdulla, 2006). In a field study in Spain, Amoros, Alonso and Cuesta (2010), have reported an incidence of between 4 and 15 *Cryptosporidium* oocysts/50 g of lettuce. In Norway, Robertson and Gjerde (2001b) analysed various vegetables for contamination with *Cryptosporidium* oocyst and detected between 1 and 8 oocysts/100 g of samples. Knowing the concentration of oocysts present on contaminated vegetables would be useful to assess the extent of the problem. However the mere presence of viable *Cryptosporidium* oocysts on vegetables should be seen as a potential health risk due to the low infectious dose of this pathogen (Okhuysen *et al.*, 1999).

The results of the challenge test indicate that *C. parvum* oocysts are resistant to chlorine and can thus survive the washing step during the preparation of ready-to-eat minimally processed vegetables. *Cryptosporidium* oocysts' infection dose varies between species and individual and as little as 1 oocyst is suspected to be able to cause infections in human. Hoornstra and Hartog (2003) investigated the risk of infection associated with ingestion of *Cryptosporidium* oocysts. They determined that there is a 5% probability of developing cryptosporidiosis infection if consuming 10 oocysts.

The percentage of inactivated oocysts after the blast freezing treatment was 20%. However, since 5.7% of the oocysts in the control samples were also stained by PI, it can be deduced that the blast freezing treatment actually inactivated about 14% of the oocysts. This means that to reach a final load of 10 or less oocysts on the fresh produce with blast freezing being the only hurdle aimed at reducing the viability of *Cryptosporidium* oocysts, the initial load on the produce should not be more than 12 oocysts (Figure 15).

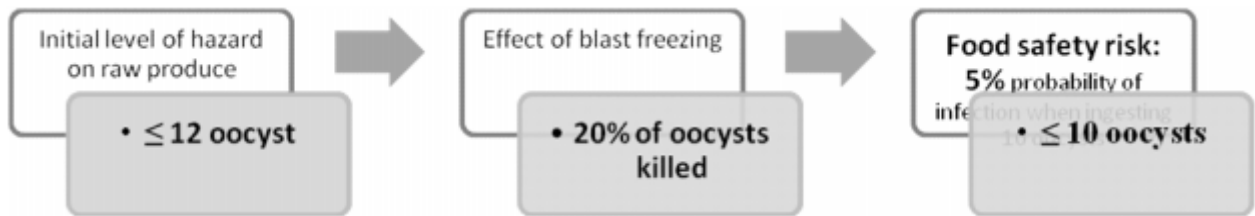


Figure 1: Model indicating the relationship between initial level of hazard on raw product, effect of blast freezing, and food safety risk of final product

Blanching and microwave heating treatments were more effective than chlorine and freezing. 93% of oocysts were stained by PI after these heat treatments. By subtracting the percentage of oocysts that were stained by PI in the control samples we can see that the blanching and microwave treatments actually inactivated 87% of the oocysts. This means that out of the 50 000 oocysts inoculated on the green pepper 6350 were still viable after microwave and blanching treatments. Therefore to reach a final load of 10 or less viable oocysts on fresh produce after hot water blanching or microwave heating the initial load on the produce should not be more than 79 *Cryptosporidium* oocysts (Figure 16).

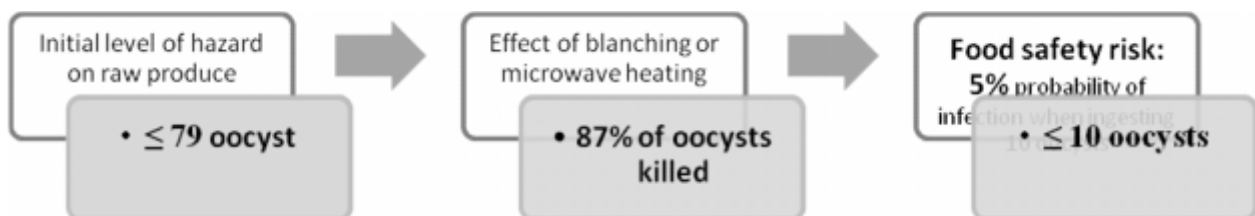


Figure 2: Model indicating the relationship between initial level of hazard on raw product, effect of microwave heating or blanching, and food safety risk of final product

During the manufacture of frozen vegetables, the vegetables are usually washed in chlorinated water, blanched and then blast frozen before being stored under frozen condition. This study did not determine the combined effect of all three treatments but let's assume that the vegetables are washed in chlorinated water, blanched in a microwave oven and then subjected to blast freezing. We can use the results of the combined chlorine and microwave heating treatments and then assume that the blast freezing treatment has a cumulative lethal effect on the viability of the oocysts. The combined chlorine and microwave heating treatment affected 98% of the oocysts. When taking into account the percentage of oocysts stained by PI in the control sample we found that 92.4% of the oocysts were actually affected by the treatments. So if 92% of the oocysts present on the surface of the fresh produce are affected by the combined chlorine and microwave heating treatment and a further 14% of the surviving oocysts are affected by the blast freezing treatment, then 153 oocysts is the maximum amount of oocysts that can be present on the vegetable before processing that can still be reduced to 10 or less oocysts after processing (Figure 17).

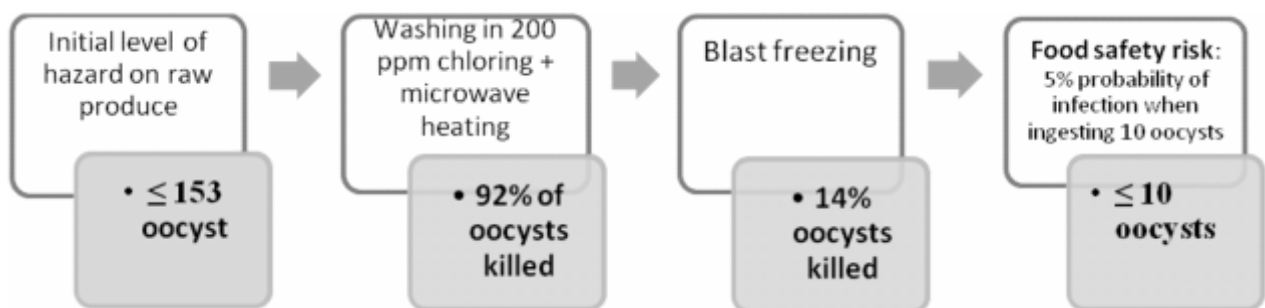


Figure 3: Model indicating the relationship between initial level of hazard on the raw product, effect of chlorine, microwave heating and blast freezing, and food safety risk of final product

This study was part of a bigger national research project investigating the microbiological quality of irrigation water all over South Africa. The combined results of the various studies are alarming as high levels of faecal contamination and human pathogens have

been found in most water tested (Britz *et al.*, 2008). The presence of *Cryptosporidium* in rivers used for irrigation purposes is of even higher concern due to oocysts' resistance to chlorine. *Cryptosporidium* oocysts can be transferred from irrigation water to fresh produce and then survive the washing step in chlorinated water. This would lead to the presence of viable *Cryptosporidium* oocysts on the finished product. This is of particular concerns for vegetables eaten raw. Ready-to-eat minimally processed vegetable manufacturers must be aware of the risk and implement strategies targeting specifically *Cryptosporidium* oocysts to ensure the safety of the finished product. The presence of *Cryptosporidium* oocysts on vegetables that are cooked or where blanching is used as a pre-treatment is not of as much concern as *Cryptosporidium* oocysts are susceptible to heat and boiling of vegetables or microwave heating on full power will kill the oocysts. As little information is available on the occurrence of *Cryptosporidium* oocysts and *Giardia* cysts on vegetables in South Africa, more testing of vegetables from the field to the retailers' shelf is require to determine the extent of the contamination and evaluate the potential health risk to the consumers.

Due to the high cost and complexity of the method for the isolation of *Cryptosporidium* and *Giardia*, water samples in South Africa are seldom tested for their presence. Indicator organisms are used to indicate contamination with faecal matter and possible presence of human pathogens. However as this and other studies have shown, little correlation has been found between levels of faecal coliforms or *E. coli* and the presence of *Cryptosporidium* and *Giardia*. Therefore, more research needs to be done to find suitable indicator organisms to predict the presence of *Cryptosporidium* and *Giardia* in surface water and on fresh produce.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

High levels of faecal coliforms and *E. coli* were observed in the three rivers monitored during this study. The levels of faecal coliforms in most of the samples were above the WHO standard for irrigation water. These alarming results indicate heavy contamination of the rivers with faecal matter.

Cryptosporidium oocysts were isolated from 43% of the water samples and *Giardia* cysts were found in 23% of the samples. These results show a widespread presence of *Cryptosporidium* and *Giardia* in surface water used for irrigation purposes. This is of public health concern as these protozoa can be transferred to vegetables during irrigation and survive on the fresh produce (Macarisin *et al.*, 2010).

No significant correlation was established between levels of faecal coliforms or *E. coli* in the water and presence of *Cryptosporidium* oocysts and *Giardia* cysts. Faecal coliforms and *E. coli* are thus not good predictors of the presence of *Cryptosporidium* and *Giardia* in water. The first hypothesis must therefore be rejected and faecal coliforms or *E. coli* should not be used as the sole indicators of water quality. The link between the presence of *Cryptosporidium* and *Giardia* and other indicator organisms, such as *Clostridium perfringens*, must be investigated to establish a suitable indicator for the presence of *Cryptosporidium* and *Giardia* in water.

The second hypothesis can be accepted. *Cryptosporidium* oocysts are resistant to chlorine at levels used in the industry for the washing and disinfection of ready-to-eat minimally processed vegetables. This is a serious concern since oocysts can thus survive on vegetables during processing and still be viable on the finished product. Blast freezing affects the viability of oocysts only slightly and cannot be relied upon to render a contaminated product safe. More research is required to determine the survival of *Cryptosporidium* during frozen storage. *Cryptosporidium* oocysts are killed by blanching. There is therefore little risk of infection from consuming frozen vegetables that have been

blanched during manufacture or that are cooked in boiling water prior to consumption. Microwave heating is also effective against *Cryptosporidium* oocysts. Cooking of fresh or frozen vegetables in a microwave should thus be sufficient to inactivate oocysts present on the vegetable.

In conclusion, the results of this study indicate the presence of *Cryptosporidium* oocysts and *Giardia* cysts in irrigation water in South Africa and confirmed the resistance of *Cryptosporidium* oocysts to chlorine. The potential therefore exists for acquisition of *Cryptosporidium* oocysts and/or *Giardia* cysts through the consumption of contaminated fresh or minimally processed vegetables. These findings indicate possible risk for the food safety of South African fresh and minimally processed produce and indicate the need for further research in this area.

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