PREFACE

The work presented in my thesis was performed from April 2013 to February 2014 at The University of Copenhagen Faculty Of Life Sciences, Department of Veterinary Disease Biology, The Danish Technical University Veterinarian Institute and Kwame Nkrumah University of Science and Technology, Department of Theoretical and Applied Biology, Kumasi, Ghana. Anders Dalsgaard and Heidi Huus Petersen from the University Of Copenhagen were my main and co supervisor respectively, and Heidi Larsen Enemark from The Danish Technical University was also my co-supervisor. From Kwame Nkrumah University of Science and Technology Robert Clement Abaidoo was my contact providing practical assistance.
Acknowledgements

During the nine months work of this thesis many people have been involved in my work and deserve my profound gratitude for their contributions and support to the work behind this thesis. For this I would like to thank the following:

My supervisors Anders Dalsgaard, Heidi Larsen Enemark and Heidi Huus Petersen for giving me the opportunity and chance to work under their competent supervision and having confidence in my work. Thank you for all the scientific discussions and input keeping me on the right track, helping me stay focused and providing positive and enthusiastic support when laboratory work was difficult and during times abroad. Also, thank you for supplying the project with extra funds as work progressed, securing high quality materials and data generation. Last, thank you for efficient and productive support during thesis and article writing.

From KNUST, Kumasi, Ghana Robert Clement Abaidoo for organizing transportation, housing, equipment, mediation of relevant contacts and general advice. Angelina Sampson for field and lab work assistance. All the previous was organized and provided with highest performance and collaboration.

The lab technicians at The Danish Technical University Veterinarian Institute and at The University Of Copenhagen Faculty Of Life Sciences, Department of Veterinary Disease Biology for advice and help during lab work. A special thanks to Boi-Tien Thi Pharm for performing PCR and genotyping work related to the project and Lise-Lotte Christensen for being available at inconvenient times and developing creative solutions when equipment was scarce.

Danida for funding personal expenses involved in the project work. Also, Danida funds the Safe Water For Food project (SAWAFO), which this thesis is a minor part of.
SUMMARY – ENGLISH

Protozoan parasites belonging to the genus *Cryptosporidium* are transmitted e.g. by food and water and may cause severe diarrhoea, dehydration, weight loss and malnutrition. Ingestion of 10 oocysts can lead to infection and pathogenic symptoms.

Thus, to characterize *Cryptosporidium* spp. contamination level of river water, irrigation water and lettuce, 10L of water and 16 lettuce samples were collected four times in the period of, September – October 2013, with weekly intervals from six sample sites in and around Kumasi, Ghana. Oocysts were purified from water by sedimentation for 2 x 48 hours or pulsifying of lettuce followed by immunomagnetic separation and quantification by immunofluorescence microscopy, with sensitivities of 2 and 9%, respectively. After approximately six weeks of storage at 4°C, analysis and additional storage on slides, oocysts were washed off the slides and attempts to characterize *Cryptosporidium* spp. positive samples were done by PCR amplification and sequencing of the SSU rRNA, the HSP70 and the GP60 genes after.

*Cryptosporidium* oocysts were found in 75% of the water samples and on 43% of the lettuce with concentrations of 53 – 32368 per 10 L water and 11 – 118 oocysts per 15 g of lettuce.

Positive water samples on one or more occasions were demonstrated in all water and farm sites while all farms had positive lettuce samples on all occasions. Rainfall seemingly lowered the concentration of oocysts in water but not on lettuce. Molecular characterization of *Cryptosporidium* spp. positive samples was unsuccessful, thus no conclusions can be drawn concerning sources of contamination. Nevertheless, the detection of high prevalence and concentration levels of *Cryptosporidium* oocysts on vegetables consumed raw and in water with direct contact to humans entails a potential risk of infection in humans. Implementation of preventive measures based on this study should be considered and actions taken accordingly.
Protozo parasitter fra slægten Cryptosporidium transmite af kontamineret føde eller vand og kan føre til alvorlig diarre, dehydrering, vægttab og underernæring. Indtagelse af 10 oocyster kan føre til symptomatic se infektion.

For at beskrive kontamineringsniveauet af Cryptosporidium spp. oocyste i flod og kunstvand og på salat, blev prøver af 10 L og 16 salat planter samlet fire gange i perioden fra September – Oktober 2013 med ugentlige intervaller, fra seks prøve steder i og omkring Kumasi, Ghana. Oocyster blev oprenset fra vand ved sedimentering i 2 x 48 timer eller pulsisering af salat efterfulgt af immunomagnetisk separations og kvantificeret ved fluorescerende mikroskopi, med sensitiviteter af henholdsvis 2 og 9%. Efter ca. seks ugers opbevaring ved 4°C, analyse og yderligere opbevaring på mikroskop glas, blev positive oocyst prøver vasket af glassene og forsøgt genotypet ved PCR amplifikation og sekventiering af SSU rRNA, HSP70 og GP60 genet.

Cryptosporidium spp. oocyster blev fundet i 75% af vandprøverne og på 43% af salat prøverne med koncentrationer fra 53 - 32368 / 10 L vand og 11 – 118 oocyster / 15 g salat.

En eller flere gange blev der fundet positive vandprøver fra alle vand og farm prøve steder, mens alle farme havde positive salat prøver alle gange. Regn sænkede tilsyneladende koncentrationen af oocyster i vandet, men ikke på salaten. Molekylær karakterisering af positive Cryptosporidium spp. prøver mislykkedes, og der kan derved ikke siges noget om kilden til kontamineringen. Alligevel indebære de høje prævalenser og koncentrationer af Cryptosporidium spp. oocyste på grøntsager, der indtages råt og i vand i direkte kontakt med mennesker, en potentiel risiko for infektion af mennesker. Implementering af præventive foranstaltninger i henhold til dette studie bør overvejes.
LIST OF ABRIVATIONS

AIDS – Acquired Immune Deficiency Syndrome

DTU - Danish Technical University

g – Grams

g – g-force

HCL – Hydro Chloric Acid

HIV – Humant Immundefekt-virus

H₂SO₄ – Sulfuric acid

KNUST - Kwame Nkrumah University of Science and Technology

KU – Københavns Universitet (University of Copenhagen)

KU-SUND: Københavns Universitet, Det Sundhedvidenskabelige Fakultet

L – Liter

ml – Milliliter

mm - Millimetre

MQ water – Purified water

NaOH – Sodium hydroxide

OPG – Oocysts per gram

QMRA - Quantitative microbial risk assessment

SAS – Statistical analytic software

SAWAFO – Safe Water For Food

Spp. – Multiple species

WHO – World Health Organization

WSP – Water safety plan
SAFE WATER FOR FOOD (SAWAFO)

SAWAFO is a DANIDA funded project focusing on Ghana and Tanzania involving several partner institutions including University of Copenhagen Faculty of Life Sciences (UC-LIFE), Department of Veterinary Disease Biology, Kwame Nkrumah University of Science and Technology, Department of Theoretical and Applied Biology and Sokoine University of Agriculture, Department of Medicine and Public Health.

SAWAFO aims to identify food safety and health hazards of using low quality water for food production by poor farmers in water scarce peri-urban communities in Ghana and Tanzania, and to develop and apply research-based tools to manage these risks. The low quality water will be examined for chemicals such as medical residuals, pathogenic bacteria as *E. coli* and *Campylobacter*, helminth eggs and other potential pathogenic contaminants. However, examination for the presence of important zoonotic protozoan parasites, such as *Cryptosporidium* spp. and *Giardia* spp., are lacking.

Developing and improving risk models assessing health risk by the use of low quality water in agriculture is a part of the SAWAFO project. Further, existing guidelines on the use of low quality water in agriculture are to be updated. The end goal is safe use of low quality water in agriculture, not just for the local farmer, but also for animals and end consumers of the produce.

This thesis work aims to generate lacking quantitative data on the zoonotic protozoan parasite *Cryptosporidium* spp. presence in low quality water and on vegetables. The data will further be used to improve and develop quantitative microbial risk assessment (QMRA), and help update existing guidelines on the use of low quality water in agriculture. Additionally, data on helminth eggs from the same study sites will be attempted gathered, to update existing knowledge on their presence in the area.
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1. INTRODUCTION

*Cryptosporidium* spp. are intestinal protozoan parasites belonging to the phylum Apicomplexa and family Cryptosporidiidae (see table 1 for more details). The parasite is monocellular and the infective stage (oocyst) is spherical and measures approximately 4-9 µm in diameter (Slapeta, 2006). The parasite is found in virtually all mammals, all over the world (Putignani and Menichella, 2010).

<table>
<thead>
<tr>
<th>Classification</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Domain</td>
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</tr>
<tr>
<td>Kingdom</td>
<td>Protozoa</td>
</tr>
<tr>
<td>Phylum</td>
<td>Apicomplexa</td>
</tr>
<tr>
<td>Class</td>
<td>Sporozoasida</td>
</tr>
<tr>
<td>Subclass</td>
<td>Coccidiasina</td>
</tr>
<tr>
<td>Order</td>
<td>Eucoccidiida</td>
</tr>
<tr>
<td>Suborder</td>
<td>Eimeriorina</td>
</tr>
<tr>
<td>Family</td>
<td>Cryptosporidiidae</td>
</tr>
<tr>
<td>Genus</td>
<td><em>Cryptosporidium</em></td>
</tr>
</tbody>
</table>

1.1 History of *Cryptosporidium* spp.

*Cryptosporidium* (C. muris) was first described in 1907, where it was discovered in the peptic gland of a mouse (Tyzzer, 1908), but was first acknowledged as a potential pathogen in 1955, were an association between diarrhoea and death, and *Cryptosporidium* spp. infection was seen in Turkeys (Slavin, 1955). The first described human case with a *Cryptosporidium* spp. infection was a 3-year-old girl with acute self-limited enterocolitis in the US, in 1976 (Nime et al., 1976). In the 1980ies the disease became a marker and cause of death for humans infected with AIDS and therefore started to receive more attention (Current et al., 1983).

1.2 Prevalence and occurrence

*Cryptosporidium* spp. infections in humans are reported from six continents (Putignani and Menichella, 2010), and based on reports 1983 – 1990, Current and Garcia, (1991) estimated a prevalence of 1 – 3 % in North America and Europe, 5% in Asia and 10% in Africa. The differences are supposedly due to improved sanitation, and cleaner drinking- and irrigation water in the industrialized countries. In Ghana human cases of *Cryptosporidium* spp. infections have been
confirmed on several occasions (Adjei et al., 2003; Adjei et al., 2004; Opintan et al., 2010), with Adjei et al. (2003) claiming to have detected C. parvum. However, a modified Ziehl-Neelsen staining method was used in all the studies, which to my knowledge is species unspecific.

Figure 1: Geography of worldwide occurrence of human cryptosporidiosis outbreaks and sporadic cases. A color-coded distribution of the main cases of cryptosporidiosis reported in the literature during the last decade (1998–2008). Waterborne and food-borne diseases are represented by red and grey colour, respectively. Spreading of the infection due to HIV immunological impairment is represented by green and travel-related disease by pink colour. When not applicable the definition of waterborne and food-borne disease, the term community disease has been applied to person-to-person contacts and represented by a pale blue colour. For countries characterized by two or three coexisting transmission modes, a double colour-filling effect plus thick border lines have been used, consistently with the above reported code (Putignani and Menichella, 2010).

1.3 Life cycle

Cryptosporidium spp. is transmitted via the faecal-oral route and has a direct life cycle, which means only a single host is required to complete the life cycle. Oocysts are infective immediately
after excretion and after ingestion by a host, four sporozoites excyst in the gut and primary parasitize epithelial cells of the gastrointestinal tract, but can occasionally infect other tissues such as the respiratory tract. Once inside the cells, the sporozoites develop into trophozoites and undergo merogony – an asexual multiplication resulting in several divisions of the nucleus and formation of either a type 1 or 2 meront. Type 1 meronts contain six to eight nuclei which further develop into merozoites. Merozoites leave the cell and invade new host cells, where they develop into another type 1 or 2 meront. Type 2 meronts produce four merozoits which again leave the cell and invade another cell where it differentiates into either micro- (male) or macrogamets (female). Fertilization and development of the zygote occurs in the cell occupied by the macrogamets. The zygote develops into a single oocyst and sporogony occurs, developing four sporozoites (O'Donoghue, 1995; Tzipori and Ward, 2002). Autoinfection seemingly occurs from repeated first-generation merogony and the production of thin walled oocyst from where sporozoites are released (Tyzzer, 1912).
1.4 Pathogenesis, immunity and treatment

In various mammals including humans, infection is intestinal and typical signs are watery diarrhoea associated with dehydration, weight loss, abdominal pain and discomfort (Dubey et al., 1990; Tzipori and Ward, 2002) Species and isolates of Cryptosporidium Spp. together with age and immunological status of the host determine the severity of infection. Young individuals are most...
susceptible to infection and display the most severe clinical signs. Majority of infections reported in farm animals, are found in young individuals e.g. piglets up to five weeks of age (Yin J.H. et al., 2013) and calves up to 3 weeks of age (Quilez et al., 1996). Infections in immunologically healthy humans are also seen in young individuals less than 2 years of age (Adjei et al., 2004; Gatei et al., 2006). The acute symptoms last from a few days to several weeks in immunocompetent humans and animals, whereas immunocompromised individuals can become chronically and terminal ill (Dubey et al., 1990). According to Mahin and Peletz, (2009), this is the case for many children in Sub-Saharan Africa where there is a high risk of Cryptosporidium spp. infection and 90% of all children with AIDS/HIV are found. Recent studies suggest immunocompetent hosts can become chronically infected, serving as reservoirs for extended periods, shedding low numbers of oocysts while asymptomatic (Lorenzo et al., 1993). Specific therapeutic or preventive drugs for treatment of cryptosporidiosis have yet to be discovered. Supportive treatment such as rehydration and nutritional supplementation remains the method of choice in management of clinical signs of cryptosporidiosis. The reasons behind the remarkable resilience of Cryptosporidium against antimicrobial drugs are unknown. Likely the unusual and unique occupation of the space between the cell membrane and cytoplasm, making the parasite intracellular but extracytoplasmic, contributes to its resistance against chemotherapy (Tzipori and Ward, 2002).

1.5 Zoonotic potential and host range of Cryptosporidium spp.

The Cryptosporidium genus so far consists of 30 valid species, which can infect a wide variety of animals. Some species have relatively narrow host ranges, while others can infect multiple host species and cross taxonomic class barriers (see table 2). Many Cryptosporidium species have been found to infect humans, however several of these are considered wildlife species rarely infecting humans, and are considered of minor importance to human health (Zhou et al., 2004). Other species commonly infecting both humans and animals are considering having a highly zoonotic potential and are of major importance to human health. Cryptosporidium hominis primarily infects humans, but has occasionally been isolated from sheep, goats and cattle (Ryan et al., 2005; Smith et al., 2005; Giles et al., 2009). Cryptosporidium meleagridis is a zoonotic species readily transmissible between different mammalian- and avian species including chicken, which are the source of fertilizer used in the area of this study (Slavin, 1955; Pedraza-Diaz et al., 2001; Akiyoshi et al., 2003). Cryptosporidium parvum is reported infecting 152 mammalian species including dogs, goats and cattle, all free roaming or outdoor reared in the area where this study took place (Lloyd and Smith, 1997; Fayer et al., 2000; Peng et al., 2003; Quilez et al., 2008). Cryptosporidium
*hominis* and *C. parvum* are seemingly the primary species infecting humans (Leoni et al., 2006; Bushen et al., 2007; Elwin et al., 2012; Insulander et al., 2013). In a study from the UK 14,469 human Cryptosporidium spp. infections were genotyped and more than 96% infections were caused by *C. hominis, C. parvum* and *C. meleagris* (Elwin et al., 2012).

**Table 2:** *Cryptosporidium* species. Host range: Mammal (M), Bird (B), Reptile (R), Fish (F). Extremely rare or experimental evidence is indicated by (v) and primary hosts by √. Modified table from (Slapeta, 2006).

<table>
<thead>
<tr>
<th>Species</th>
<th>Human</th>
<th>Cattle</th>
<th>Host Range</th>
<th>Public health significance</th>
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<td>v</td>
<td></td>
<td>MB</td>
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</tr>
<tr>
<td><em>C. parvum</em></td>
<td>v</td>
<td>v</td>
<td>M</td>
<td>Major</td>
</tr>
<tr>
<td><em>C. meleagris</em></td>
<td>v</td>
<td>(v)</td>
<td>MB</td>
<td>Moderate</td>
</tr>
<tr>
<td><em>C. wrairi</em></td>
<td>(v)</td>
<td>M</td>
<td>None</td>
<td></td>
</tr>
<tr>
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<td>v</td>
<td>M</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td><em>C. bovis</em></td>
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<td>M</td>
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<td></td>
</tr>
<tr>
<td><em>C. cuniculus</em></td>
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<td>M</td>
<td>Moderate</td>
<td></td>
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<tr>
<td><em>C. felis</em></td>
<td>v</td>
<td>(v)</td>
<td>M</td>
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</tr>
<tr>
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<td>(v)</td>
<td></td>
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</tr>
<tr>
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<td>M</td>
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<td><em>C. ryanae</em></td>
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</tr>
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<td><em>C. fragile</em></td>
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<tr>
<td><em>C. macropodum</em></td>
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<tr>
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<td>M</td>
<td>Moderate</td>
</tr>
<tr>
<td><em>C. scrofarum</em></td>
<td>(v)</td>
<td>(v)</td>
<td>M</td>
<td>Minor</td>
</tr>
</tbody>
</table>
1.6 *Cryptosporidium* spp. oocysts in the environment: survival and infectivity

Direct transmission occurs when an infected host faecally excretes and deposits oocysts onto a new host who ingests the oocysts, e.g. via contaminated hands after faecal depositing. Indirect transmission is via any mechanism, by which faeces contaminated material containing sufficient number of oocysts is ingested by a susceptible host (Smith et al., 2004). This process is also known as the environmental transmission cycle with water and food recognized as the two most important transmission vehicles.

Environmental contamination is contributed to wildlife, humans and production animals, with wildlife being of minor importance (Zhou et al., 2004). Humans and production animals are seemingly the most important (Tzipori and Ward, 2002), due to their high contamination potential, when waste products are used as fertilizers on fields. Further contamination by human waste via discharge of domestic wastewater is possible, where waste water infrastructure is insufficient and drainage is direct from housing to streams and rivers. Cattle, sheep, pig and fowl (Chalmers et al., 2002; Maddox-Hyttel et al., 2006; Slavin, 1955) originated waste is commonly used as fertilizer and can potentially contaminate fields with *Cryptosporidium* spp. oocysts of high zoonotic potential (see section 1.5). Fertilized fields may contain high numbers of oocysts due to the high excretion level observed e.g. in Infected calves, up to $6 \times 10^7$ oocysts per grams of faeces (OPG) (Uga et al., 2000). The potentially high number of oocysts found on fields become a major source of environmental contamination when fertilizer originated materials run off into rivers and lakes. This process can further be promoted by rainfall (Morgan et al., 1995; Meinhardt et al., 1996; Hancock et al., 1997) and may also assist oocysts in leaching through soil (Petersen et al., 2012) potentially contaminating groundwater (Hancock et al., 1998). The oocysts can remain viable in fertilized soils for more than three months (Kato et al., 2004) continuously running off fields contaminating rivers and lakes during rainfall. Once in rivers and lakes the oocysts can remain viable for more than three months in temperatures of −4 and 4°C (Robertson et al., 1992; Olson et al., 1999). When contaminated water sources are then used for irrigation and recreational purposes further contamination of raw consumed vegetables such as lettuce (Steele and Odumeru, 2004), and direct human infection from recreational water e.g. swimming pools is possible (Insulander et al., 2005). The oocysts outer layer consists of fluffy, waxy glycoproteins increasing adhesion properties (Liu et al., 2010; Jenkins et al., 2010), which may add to contamination of raw vegetables irrigated multiple times (Robertson and Gjerde, 2001), leading to further human infection after consumption (Insulander et al., 2008). The low infection dose of
down to 9 oocysts (Okhuysen et al., 1999), also contributes to success of the transmission cycle, when contaminated raw consumed vegetables or water is ingested.

Treatment of water sources are complicated by oocysts resistance to chlorine and other detergents (Current and Garcia, 1991) along with the size of the oocyst, 4 – 9 µm in diameter (Slapeta, 2006), making disinfection and filtration difficult or impossible, when dealing with large volumes of water. Ozone, UV, higher temperatures e.g. pasteurization and desiccation promotes inactivation (Anderson, 1985; Anderson, 1986; Reinoso and Becares, 2008). Freezing below – 20 °C for 24 hours has also been reported to inactivate oocysts (Fayer and Nerad, 1996). However, these methods of treatment are also difficult when dealing with large volumes of water.

1.7 Water and food-borne cryptosporidiosis

The largest known outbreak of cryptosporidiosis occurred in Milwaukee, Wisconsin, USA in 1994. An estimated 403,000 individuals were infected due to contaminated surface water supplying the municipality water system (MacKenzie et al., 1994). Several other major and small outbreaks due to contaminated water sources have been recorded since. Baldursson and Karanis, (2011) documented 120 waterborne outbreaks alone in Australia, North America and Europe from 2004 – 2010. Among these outbreaks was a fountain in New York causing 3207 cases, a public swimming pool in Australia causing 254 cases, while vegetables served in a water basin in a canteen in Denmark caused 99 cases. Cryptosporidiosis outbreaks originating from food vehicles includes parsley, apple cider, unpasteurized cow milk, and eating in cafeterias (Millard et al., 1994; Quiroz et al., 2000; Blackburn et al., 2006; Insulander et al., 2008) in Stockholm, Sweden., Ohio, USA., Maine, USA., Queensland, Australia and Washington DC, USA respectively. To my knowledge, a direct link between cryptosporidiosis outbreaks due to surface water or vegetable contamination is still to be established in Africa.
2. Wastewater irrigation

More than 10% of the world’s population consumes produce irrigated with wastewater, with considerably higher percentages found in low income and undeveloped areas. Health risks are associated with the use of wastewater in agriculture when e.g. viruses, bacteria and parasitic organisms are present. Vegetables may become contaminated with pathogens present in wastewater when it is used for irrigation. Later, the consumption of the produce e.g. raw vegetables can lead to human infection. Overall Population growth in water scarce areas is the main driving factor pressuring freshwater sources and is responsible for the competition between urban and agricultural need for freshwater. In many areas wastewater is the only irrigation source available due to lack of alternatives and proper wastewater treatment facilities. Undeveloped areas often lack the capacity to treat waste water, consequently discharging it into water bodies with little or no treatment, which are then used for irrigation. There is a growing recognition that increased population density and production of wastewater advocates for better incorporation of waste water into the overall management of water sources (WHO, 2006). This incorporation requires recognition of risk factors for a given area. This could be discharge of untreated wastewater containing pathogens in city centers into rivers and lakes, used for irrigation downstream, contaminating raw consumed vegetables. Also, the use of animal and human waste as fertilizer can potentially contain pathogens and run off into rivers and lakes. Further, quantitative data on pathogen contamination of e.g. raw consumed vegetables, is required to develop a quantitative microbial risk assessment (QMRA) model, assessing risks and contributing to development of a water safety plan (WSP).

2.1 Wastewater irrigation in Ghana

In Ghana, treatment of wastewater is rarely performed and less than 5% of the population has sewerage connections, (Data from Ghana Statistical Service, 2002), leading to rivers and lakes being used as alternative waste water infrastructure. The Subin river, which originates in the heart of Kumasi, serves as a wastewater channel for both public institutions and private housing (Obuobie et al., 2006). The consequence is potential microbial pollution by faecal contamination of both the river and connecting streams, as well as vegetables in and around Kumasi. The total faecal coliform pollution has been shown to decline with distance downstream from the city center (Obuobie et al., 2006).
Figure 2: Channel network in and around Kumasi (Obuobie et al., 2006).

Many people rely on the Subin River as well as connected streams and rivers for their domestic use and irrigation, since few have pipe-borne water. It has been estimated that about 60% of the formal irrigation is done by the use of low quality water in Ghana (Obuobie et al., 2006).

In urban and peri-urban areas, smallholders searching for irrigation water hardly find any unpolluted surface water sources and end up using drainage water for vegetable production, which raises the issue of use of low quality water and food safety (Obuobie et al., 2006).

The major health concern has been with microbiological pathogens from domestic sources, which could potentially have a negative effect on the environment and public health in general (Drechsel et al., 2008).

Studies in Norway, Costa Rica etc., have shown the presence of *Cryptosporidium* spp. oocysts on vegetables, (Monge and Chinchilla, 1995; Robertson and Gjerde, 2001) respectively. Fruit is known to act as vehicles for transmission of *Cryptosporidium* spp. infections in the form of unpasteurized apple cider in Maine, USA (Millard et al., 1994).

The link between the presence of pathogenic microorganisms in wastewater and contamination of fruit and vegetables is suggested (Steele and Odumeru, 2004), but also higher incidences of disease, in the form of diarrheal disease and protozoan infections, has been observed in populations practising irrigation with untreated low quality water compared to treated low quality, in Mexico (Camponovo et al., 2000).

Considering the observations above and the numerous human and cattle cases of Cryptosporidiosis occurring in Kumasi and Accra, Ghana (Adjei et al., 2003; Adjei et al., 2004;
it is feasible that low quality water sources and vegetables irrigated with low quality water, serve as a source of infection of Cryptosporidium spp. in Kumasi, Ghana. Despite of this, little or no examination has been done on the presence of Cryptosporidium spp. oocysts in water sources used for irrigation and on vegetables irrigated with low quality water. Confirming the presence and later applying preventive measures, could potentially lower the risk of infection and improve the public health.

2.2 Study objectives

2.2.1 Overall objective:

To assess food safety and human health risks associated with Cryptosporidium spp. oocysts contamination of vegetables irrigated with low quality water in Kumasi, Ghana, and establish preventive and control measures to reduce risks.

2.2.2 Specific objectives:

Generating data to fulfil specific objective 1 was the primary focus while planning and performing field work in Kumasi, Ghana. After returning to the University of Copenhagen I decided to describe a simplified preliminary QMRA model to fulfil specific objective 2.

1. To determine prevalence and concentration of Cryptosporidium Spp. oocysts and helminth eggs on vegetables irrigated with low quality water.

2. Develop a preliminary quantitative risk assessment model for Cryptosporidium spp. human infection associated with consumption of vegetables irrigated with low quality water.
3. MATERIALS AND METHODS

3.1 Study sites

Freshwater samples were collected from the three vegetable farms described in section 5.2.1 and from three river sites described in section 5.2.2. Water sample sites were in some cases natural formed streams and lakes, while others were partly manipulated concrete channels all displaying large amounts of sediments. The sample sites were chosen based on their demographical, vegetable production properties and their geographical location in the center or semi urban areas of Kumasi, Ghana (see figure 3).

Lettuce was sampled from three different farms using surface water as an irrigation source. The irrigation water was exposed directly or indirectly to water from a combination of private housing, public healthcare institutions and small-scale industry wastewater. The design of the farmlands were equal for the three farms, consisting of rows of approximately 50 cm high soil beds surrounded by 50 cm wide channels of water (see figure 5A and 5B).

The routines from seeding to harvest was equal for all three farms; the lettuce seeds were sowed on one bed and later transferred to harvest beds, chicken manure was applied as fertilizer once per crop life cycle just after the seedlings was transferred to harvest beds. Lettuce plants usually grow from seedlings to plants ready for harvest in six weeks. The farmers general irrigated in the morning and the evening, given no rainfall occurred the previous night. In case of rainfall, the farmers found it unnecessary to irrigate the following day. Insecticides were sprayed on the lettuce once a week, regardless of weather conditions. Water and lettuce samples were analysed for Cryptosporidium spp. oocysts and helminth eggs.
Figur 3: Map of Kumasi, Ghana, center and urban areas. Farm 1, 2, 3 and River 1, 2, 3 location are indicated (Google maps).

3.2.1 Farms

Farm 1 used a nearby stream as irrigation source seen on Figure 4(A). Collection and subsequently irrigation was performed manually with watering cans. All water samples were collected directly from the irrigation source, always from the same spot as shown on figure 4(A). The area surrounding the farm and immediate supplying the stream with runoff water was mostly green area, but upstream the river was supplied with wastewater from small scale industry, local market and private housing.

Figure 4: (A) Farm 1, Collection of irrigation water. GPS coordinates 6.688039 N,-1.557107 W–Accra road, Kumasi, Ghana. (B) Farm 1, Irrigation by watering can. GPS coordinates 6.688039 N,-1.557107 W – Accra road, Kumasi, Ghana.
At farm 2 water samples were collected directly from on-farm ponds (dugouts), which were used as irrigation source at the time of the study. Watering cans were used both for collecting water and for irrigation. The area supplying the dugouts with wastewater was mainly private housing and runoff water from a nearby green area. Goats, dogs, chicken, geese and amphibians could be found in the nearby area and on the fields.

![Figure 5](image)

**Figure 5:** (A) Farm 2, Lettuce beds and divided by runoff channels. GPS coordinates 6.64921 N, -1.57743 W. (B) Farm 2, Runoff water between lettuce beds used for irrigation. GPS coordinates 6.64921 N, -1.57743 W.

Farm 3 used a combination of river water and dugouts as irrigation sources at the time of the study. All water samples were collected from the area where the river basin met the farm and an automated pump was also placed. For large scale irrigation, river water was pumped up and distributed around the farm, using a pipe-irrigation system as seen on figure 6(A). For small scale irrigation of a single bed or two, watering cans were used. The water was collected from the dugouts at the farm, which were supplied with water from the runoff from the large scale irrigation scheme and surrounding green area. From the nearby area the River was supplied with wastewater, but seemingly not sewage, from student housing, hotels and campus hospital among others. Fish and wildlife birds were observed in and around the river supplying the irrigation water.
3.2.2 Rivers

The three river sites covered the downstream areas of the east, center and south of the city’s rivers and lakes acting as wastewater infrastructure. Furthermore, each river sampling site was supplied with wastewater from different sources upstream. River samples were collected directly from the bank, using a 3.5 L plastic container to fill up a 10 L container. Both fast running and still water was sampled when possible.

River 1 was located east of the city center, receiving wastewater from private housing, public institutions (primary school, police station etc.) and local markets. The sampling area of the river was currently being used for both irrigation and recreational purposes.
River 2 was downstream of the largest hospital in Kumasi, Komfo Anoyke Teaching Hospital, hotels, private housing and the city center, all supplying this sample site with wastewater. The river was not used for agricultural purposes where the samples were taken.

River 3 was located south of the city center, receiving wastewater from the central market commonly recognised as the largest market in West Africa, free roaming cattle, chicken, goats small scale industry, and private housing. The river was currently being used for irrigation, fish ponds and small scale industry where the water samples were taken.
River 4 was located in the center of campus, south-east of the city center. The river site was currently being used for irrigation, fishing and for recreational purposes.

3.3 Sampling

All samples were collected from all six sites described above the 25th September, 3rd October, 11th October and the 18th October. The water samples were each collected in a 10 L plastic container for Cryptosporidium spp. oocyst analysis and in three 3.5 L plastic containers for helminth analysis per site. Other studies used sample sizes of e.g. 100 ml and 20 L (Amoros et al., 2010; Koompapong and Sukthana, 2012), we rationalised 10 L as a fairly large representative and manageable sample size. Lettuce samples were collected from 1 or 2 beds containing the lettuce plants closest to harvest. All together 16 randomly chosen lettuce plants were collected per farm per sampling round, by either the farmer, driver or by randomly throwing a disc. The collection of samples by the farmer and driver was based on no instructions besides from the choosing of lettuce bed. The soil-contaminated root and outer leaves were removed and thrown away, while the rest of the lettuce plant was placed in a plastic bag. After collection, all samples were brought back to the laboratory for analysis and storage (4 °C) at KNUST. Oocysts purified from the samples were later returned to DTU and KU-SUND in Copenhagen, Denmark in a cooling bag for further analysis and enumeration.
Table 3: Number of samples collected.

<table>
<thead>
<tr>
<th>Sample sites</th>
<th>Water samples</th>
<th>Lettuce samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Cryptosporidium</em> spp. oocysts</td>
<td><em>Helminth egg</em></td>
</tr>
<tr>
<td></td>
<td>(10L)</td>
<td>(10L)</td>
</tr>
<tr>
<td>River 1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>River 2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>River 3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Farm 1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Farm 2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Farm 3</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

*Sampled as 1 times 10L
Sampled as 3 times 3.5L

3.4 Detection of *Cryptosporidium* spp. oocyst and helminth eggs in water

3.4.1 *Cryptosporidium* spp. oocysts – recovery in the lab

Validation and recovery rates of the method developed for oocysts detection and enumeration required a batch with a known number of oocyst. The batch was obtained as follows.

Faeces were collected rectally from 1- to 3-week-old naturally infected Holstein calves from Gjorslev Gods, Holtug, Denmark. Faeces were transported in a cooling box and *Cryptosporidium*-positive samples were identified by modified Ziehl-Neelsen technique (Henriksen and Pohlenz, 1981) upon arrival at the laboratory, by the following procedure. A glass slide smear was made per faeces sample and dried at room temperature. The sample was fixed in 1.1% HCL 97% methanol for 3 min and dried at room temperature, followed by staining with concentrated carbol fuchsin for 20 min and rinsing in tap water. Sample slide differentiation twice in 10% H₂SO₄ for 10 sec and rinsed in tap water, followed by counterstaining with 5% malachite green for 5 min, rinsing of slide in tap water and drying at room temperature. A drop of oil was placed on the sample slide and a cover glass was placed on the slide just before positive samples was identified by microscopy.

Approximately 1 ml of faeces from each oocyst-positive sample were suspended in 7 ml 0.01% Tween MQ water in a 50 ml tube (1). Solution was homogenized by vortexing, filtered through 4 layers of gauze into a new 50 ml tube (2). Tube 1 was added 3.5 ml 0.01% Tween 20 MQ water and vortexed to clean the tube and added threw the gauze to tube 2. Faeces solution was underlayed with 7 ml flotation fluid (1:1 MQ water and flotation fluid). The solution was centrifuged at 53 g for 3 min, supernatant was transferred (containing oocysts) to tube 1 and
filled up with MQ water and centrifuged at 1540 g for 10 min. Supernatant was removed down to 2 ml, vortexed, filled up with MQ water and mixed (first wash), followed by centrifugation at 1540 g for 10 min. Supernatant was removed down to 2 ml, vortexed, filled up with MQ water and mixed (second wash), followed by centrifugation at 1540 g for 10 min, supernatant was removed down to 2 ml and functioned as stock solution, which was later enumerated by fluorescent microscopy, as described in section 3.7.1.

The recovery rate for oocysts in river water was examined in six replicate water samples collected from river site 4. All together 60 L of water was collected and placed in a 60 L container, continuously stirred, distributed into six 10 L plastic containers (the same containers as used in the experiment) and spiked with a 1 ml solution containing 1000 oocyst each. The oocysts were purified and enumerated as described in 3.4.2, 3.6 and 3.7.1.

The recovery rate of oocysts on lettuce was examined by adding a 1ml solution containing 1000 oocyst to six 15 g (wet weight) (Scale: Mettler Toledo PB 5001) inner lettuce leaves from a single plant. Although 30 g was previously used (Cook et al., 2006), the stomacher bags used in this study promoted 15 g. To enhance adhesion and simulate the interactions between Cryptosporidium spp. oocysts and lettuce from farm to consumer, samples were left to dry for 24 hours, followed by purification as described in section 3.5.1, 3.6 and 3.7.1 respectively. Cook et al., (2006), left lettuce samples to dry for 2 hours before purification, but this did not correspond with our rational above or our protocol where lettuce samples were collected one day and processed the following.

3.4.2 Detection of Cryptosporidium spp. oocysts in water samples

To determine the contamination level by naturally occurring Cryptosporidium spp. oocyst in sample site waters, samples were collected in 10 L containers at the sample sites and brought to the lab as described in section 3.3. The containers were left on the table for 48 hours, promoting sedimentation from top to bottom (21.5 cm) of the containers, as Cryptosporidium spp. oocysts theoretical settling time is 60.48 cm / 48 hours, according to stokes law (Medema et al., 1998). The supernatants were removed by pump-suction system (homemade, see figure 10 (A)), leaving approximately 0.75 L water in the containers. The remaining solutions were transferred to 3 L containers, followed by a 3 x 150 ml tap water cleaning cycle (manual voretexing) of the 10 L containers also transferred. The 3 L containers were placed at a 30° angle and left on the table to sediment for another 48 hours. Subsequently the supernatants were removed, leaving
approximately 90 ml in the containers. The remaining solutions were transferred to 50 ml tubes, followed by a 3 x 20 ml tap water cleanup cycle of the 3 L containers also transferred. The 50 ml tubes were centrifuged at 1583 g for 10 min (Homef LG-30) and the supernatant removed leaving approximately 5 ml. Pellets were collected in one of the 50 ml tubes followed by a 3 x 5 ml cleanup cycle with 0.01% tween 20 in distilled water also transferred. The 50 ml tube containing the pellet and cleanup solution was centrifuged at 1585 g for 10 min and the supernatant removed leaving approximately 5 - 10 ml. The samples were now ready for further analysis, see section 3.6. (Robertson and Gjerde, 2000), also used 50 ml tubes, but centrifuged at a 1000 g for 10 min, we choose to use DTU standards.

Figure 10: Laboratory facility at KNUST Kumasi, Ghana. (A) 10 L oocysts sample containers and homemade pump suction system. (B) 3.5 L helminthe sample containers.

3.4.3 Detection of Helminths eggs in water samples

To determine the contamination level by naturally occurring helminth eggs in sample site waters, samples of 3.3 L were collected in 3 x 3.5 L containers (10 L in total per site). All 10 L water samples were filtered through a 20 µm sieve followed by a 3 x tap water cleanup of the 3 x 3.5 L containers. The clean-up water was likewise filtered through the 20-µm sieve. The content from the sieve was poured into a 250 ml beaker followed by a tap water cleaning of the sieve. All materials from the sieve were likewise collected in the beaker. The content of the beaker was distributed into 50 ml tubes and centrifuged at 291 g for 7 min and the supernatant was removed, leaving only the pellet, modified from Roepstorff and Nansen, (1998). The samples were now ready for further analysis, see section 3.7.2.
3.5 Detection of Cryptosporidium spp. oocyst and helminth eggs on lettuce

3.5.1 Detection of Cryptosporidium spp. oocysts on lettuce

The natural contamination level by Cryptosporidium spp. oocysts on lettuce was examined by pairing plants 2 by 2 and collecting 15 g of inner leaves (wet weight) from every lettuce. Each 15 g was divided in two 7.5 g and pooled with its respective pair to provide a fast positive/negative analysis (of all samples) and later a plant specific enumeration analysis. The pooled 15 g of lettuce was placed in a stomacher bag, covered by 100 ml 0.01% Tween water and pulsified for 1 min (Microgen bioproducts PUL 100 E). The solution from the stomacher bag was immediately distributed into 2 x 50 ml tubes, leaving the lettuce in the bag. The lettuce was again covered with 100 ml 0.01% Tween, pulsified, and solution distributed into another 2 x 50 ml tubes. The tubes were centrifuged at 1585 g for 10 min, removing the supernatant to approximately 5 ml and collecting all pellets in one 50 ml tube, filtering through a single layer of gauze to hold back lettuce pieces. The three remaining 50 ml tubes were subjected to a 3 x 2 ml cleanup cycle with 0.01% Tween water and transferring it to the 50 ml tube containing the pellets, while rinsing the gauze. The 50 ml tube containing pellet and cleanup solution (water used for cleaning 50 ml tubes), was centrifuged at 1585 g for 10 min and the supernatant was removed to approximately 1 ml. The pellet was transferred to an eppendorf tube and the 50 ml tube was cleaned with 0.5 ml tap water and transferred to the eppendorf tube. The samples were now ready for further analysis, see section 3.6 and 3.7.1 respectively.

3.5.2 Detection of Helminth on lettuce

Same purification method was used as in section 3.5.1 until the last step as follows. The remaining 50 ml tube containing pellet and cleanup solution (water used for cleaning 50 ml tubes), was centrifuged at 291 g for 7 min and the supernatant was removed leaving only the pellet, modified from Roepstorff and Nansen, (1998). The samples were now ready for further analysis, see section 3.7.2.

3.6 IMS protocol

Pellets remaining from water and lettuce sample procedures were transferred to Dynal L10 Tubes, adding 1 ml of 10× SL buffer A, 1 ml of 10× SL buffer B and 100 μl of anti-Cryptosporidium bead conjugates (Life Technologies). Samples were rotated for 1 h at room temperature. The magnetic beads (with attached Cryptosporidium spp. oocysts) were recovered from the Dynal L10 Tube.
using a magnetic particle concentrator. The recovered bead-bound parasites were resuspended in 1 ml of 1× SL buffer A, transferred into a 1.5-ml eppendorf tube, and further separated using a magnetic particle concentrator. The oocyst complexes were dissociated from beads using 50 μl of 0.1 N HCl and the magnetic particle concentrator. Dissociated oocysts were transferred to a new 1.5-ml eppendorf tube and the pH was neutralized with 5 μl of 1 N NaOH. The magnetic beads were subsequently washed with 45μl 0.1% tween 20 (Merc milipore®), which also were added to the eppendorf tube. The samples were now ready for enumeration, see section 5.7.1.

3.7 Enumeration

3.7.1 Oocyst

After IMS, samples (100 μl) were transferred to 3-well slides (3 samples per slide) and left to dry, followed by fixation with azetone (20 μl). Anti-Cryptosporidium spp. fluorescein isothiocyanate (FITC)-labelled antibody mix (Crypto-Cell IF test, CellLabs, Australia) was added to each well (25 μl), the slide was placed in a humidity chamber and incubated for 45 min. at 37 °C. FITS was removed and wells were washed twice with a 100 μl of MiliQ water per wash. 12 μl of Mounting fluid (Crypto-Cell IF test, CellLabs, Australia) plus a cover was added to the slide and the oocyst were counted by epifluorescence at 200 magnification microscopy (495 nm excitation, 519 nm emission wavelength).

3.7.2 Helminth

The size of pellet was different for each sample and varied between 2 and 30 ml. The pellet was suspended with flotation fluid until the solution was clear enough for microscopy (50 g NaCl, 75 g of glucose in a saturated 1 L solution containing distilled water, yielding a specific gravity of 1.2737 ± 0.0162 g/ml) and 4 x 0.30 ml of the sample, was added to a McMaster chamber. Two slides of each sample were counted at 10 x magnification. A McMaster slide holds 0.30 ml and if the whole sample was 4 ml the calculation would look as follows; 4ml / 0.6ml = 6.66 x number of helminth eggs, modified from Roepstorff and Nansen, (1998).

3.8 Genotyping

After sample collection, processing and storage at KNUST, Kumasi, Ghana for a duration of approximately one month at 4°C, samples were brought back to KU and DTU, Copenhagen, Denmark for further purification. Final analysis required transfer to slides, staining, desiccation
and additional storage for approximately two weeks. Hereafter, oocysts were washed off the slides and a lab technician from DTU attempted to characterize Cryptosporidium spp. positive samples by PCR amplification and sequencing of the SSU rRNA, the HSP70 and the GP60 genes.

3.9 Data processing

Simple data processing and correlations were done in excel, while data transformation to log-scale and regression modelling was done in SAS.
4. Results

4.1 Oocysts in water

In total 24 water samples were collected either from rivers or from irrigation water on lettuce farms four times (rounds) with weekly intervals, and examined for the presence of *Cryptosporidium* spp. oocysts. Based on microscopic examination, 18 (75%) water samples had detectable numbers of oocysts, with seven of the irrigation water samples from the farm sites being positive (58.3%), and 11 of the 12 river samples (91.7%) were positive. Oocyst were detected in both river water and irrigation water in all four sampling rounds, although only two of the irrigation water samples were positive at sample round 3 and 4. The oocysts recovered ranged 53 – 32368 oocysts per 10 L water (see table 4) with a mean of 1513 ± 6575. The mean number of oocysts in irrigation water was 2811.4 ± 9310.1, while a mean of 214.9 ± 214.6 was found in river water. Recovery efficiency and precision of the method used to purify oocysts from water samples (see section 3.4.2, 3.6 and 3.7.1) were calculated to 1.9% and 19 ± 14.9, respectively. The data in table 4 classifies the waters examined in this study between “heavy and grossly polluted” waters (10 - 100 oocysts / L, respectively) according to WHO (2006) standards.

Overall no difference was observed in between the numbers of oocysts found on each water sampling site (P = 0.0771), the same was true for round effects (P = 0.2182). Individually effects were seen between river 1 and all other river and farm sites ranging from P = 0.006 – 0.051. These effects were due to the small number of oocysts recovered from farm 1.

**Table 4: Oocyst water recovery data.**

<table>
<thead>
<tr>
<th>Oocysts/10 L</th>
<th>Sampling round</th>
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<tr>
<td></td>
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<td>&lt;53</td>
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<td>Farm 2</td>
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<tr>
<td>Farm 3</td>
<td>632</td>
<td>32368</td>
<td>53</td>
<td>&lt;53</td>
</tr>
<tr>
<td>River 1</td>
<td>&lt;53</td>
<td>53</td>
<td>105</td>
<td>105</td>
</tr>
<tr>
<td>River 2</td>
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</tr>
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<td>River 3</td>
<td>53</td>
<td>105</td>
<td>684</td>
<td>263</td>
</tr>
</tbody>
</table>

4.2. Oocysts on lettuce

A total of 96 lettuce samples were collected from three farms four times with weekly intervals and examined by microscopy for the presence of *Cryptosporidium* spp. oocysts. Based on
microscopic analysis 41 (43%) lettuce samples had detectable numbers of oocysts, with 75% (24/32) oocyst-positive lettuce collected from farm 1, 47% (15/32) oocyst-positive lettuce sampled from farm 2 and 50% (16/32) oocyst-positive lettuce sampled from farm 3. All farms had lettuce samples with detectable numbers of oocysts in all 4 rounds. Farm 3 only had two positive samples (25%) in sampling round 4, while farm 1 had eight positive samples (100%) in sampling round 3, representing a minimum and maximum within farm prevalence, respectively. The number of oocysts recovered ranged 11 – 118 oocysts per 15 g lettuce (see figure 11) with a mean of 19 ± 24.8. Individual means for farm 1, 2 and 3 were 28 ± 28.6, 15 ± 15.4 and 13 ± 20.8, respectively.

Recovery efficiency and precision of the method used to purify oocysts from the lettuce samples (see section 3.5.1, 3.6 and 3.7.1) were calculated, and were 9.3% and 93 ± 34.3, respectively. There was a significant effect for farms (p = 0.0061) and for sample rounds (p = 0.0092), while no significant effect was seen between farms and sample rounds (0.4313). The farm effects were seen for farm 1 and farm 2 and 3 (p = 0.0080 and 0.0074 respectively), these effects were due to the higher number of oocysts recovered from farm 1. The round effects were seen between round 3 and 1, 2 and 4 (P = 0.0079, 0.0064 and 0.0170 respectively), these differences were due to the higher number of oocysts recovered from round 3.

![Figure 11: Oocysts recovered from lettuce samples.](image-url)
4.3 Helminth eggs

A total of 24 water samples and 96 lettuce samples were collected either from river or farm sites over four sampling times with weekly intervals and examined by microscopy for the presence of helminth eggs. Based on microscopic examination *Strongile* and *Toxocara* resembling eggs were found in irrigation water and river water samples collected in round 4, and from lettuce samples from farm 2 collected in round 3. However, species are not verified since the sources of the eggs are unknown lacking making species determination difficult. Concentrations were not calculated due to condition of the sample material and the few results they yielded, for further details see section 7.

4.4 Turbidity and pH

Turbidity and pH was measured for all water samples and ranged 1.6 – 229.0 NTU and 7.0 – 8.5, respectively (see table 5). There was no significant correlation between either pH or turbidity and the number of oocysts found in the water samples (R = -0.1 and R = 0.009 respectively).

<table>
<thead>
<tr>
<th>Round</th>
<th>Turbidity (NTU)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Farm 1</td>
<td>206.7</td>
<td>7.1</td>
</tr>
<tr>
<td>Farm 2</td>
<td>49.5</td>
<td>52.8</td>
</tr>
<tr>
<td>Farm 3</td>
<td>41.7</td>
<td>49.3</td>
</tr>
<tr>
<td>River 1</td>
<td>53.1</td>
<td>1.6</td>
</tr>
<tr>
<td>River 2</td>
<td>22.0</td>
<td>77.5</td>
</tr>
<tr>
<td>River 3</td>
<td>40.8</td>
<td>75.8</td>
</tr>
</tbody>
</table>

4.5 Rainfall – water correlation

Specific rainfall data was unavailable and therefore based on personal observations, further ranked into levels; none, light, medium and heavy (see table 6). Recoveries of oocysts in water samples were ranked into intervals; low, medium and high (see table 6). There was no obvious association between precipitation and number of oocysts found on either lettuce samples or in water samples during the study period.
Table 6: Rainfall during the week leading up to sampling and oocysts recovered. Intervals of oocysts recovered: Low (<200) Medium (201 – 400) High (>401)

<table>
<thead>
<tr>
<th></th>
<th>Round 1</th>
<th>Round 2</th>
<th>Round 3</th>
<th>Round 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rainfall</td>
<td>Heavy</td>
<td>None</td>
<td>Light</td>
<td>Medium</td>
</tr>
<tr>
<td>Farm 1</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Farm 2</td>
<td>Medium</td>
<td>Medium</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Farm 3</td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>River 1</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>River 2</td>
<td>High</td>
<td>Low</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>River 3</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
<td>Medium</td>
</tr>
</tbody>
</table>

4.6 Water – Lettuce correlation

There was no correlation between the number of Cryptosporidium spp. oocysts in water and on lettuce samples (R= -0.01). Alternatively transformation of the data into contamination levels (see table 7) could reveal a different picture, but was not the case. The data of water and lettuce from farm site 1 illustrates a relationship; with lettuce being more contaminated than water (see table 7).

Table 7: Comparison of contamination levels between water and lettuce samples from farm sites. Water, Intervals of oocysts recovered from: Low (<200) Medium (201 – 400) High (>401). Lettuce, Intervals of oocysts recovered from: Low (<12) Medium (12 – 30) High (>30).

<table>
<thead>
<tr>
<th></th>
<th>Round 1</th>
<th>Round 2</th>
<th>Round 3</th>
<th>Round 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lettuce</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farm 1</td>
<td>Medium</td>
<td>Low</td>
<td>High</td>
<td>Medium</td>
</tr>
<tr>
<td>Farm 2</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Farm 3</td>
<td>Low</td>
<td>Medium</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farm 1</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Farm 2</td>
<td>Medium</td>
<td>Medium</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Farm 3</td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
</tr>
</tbody>
</table>

4.7 Genotyping

Amplification of DNA by PCR was successful for a positive water sample and further sequencing is pending, while PCR of positive lettuce samples was unsuccessful.
4.8 Quantitative Microbial Risk Assessment

A simplified QMRA model based on the quantitative lettuce Cryptosporidium spp. oocyst data from this study, 1.25 oocysts / g lettuce, and the following data from Obuobie et al., (2006) and WHO, (2006) could be conceived. An estimated 130,000 – 150,000 street food meals containing lettuce are consumed daily in Ghana, each serving contains an average of 12 g of lettuce, which is usually isolated in a plastic container. Of all lettuce consumed 83% is provided by the street food industry while 15 and 2% is provided by hotels, restaurants, canteens and private households respectively (see figure 12). WHO estimates a person consumes a 100 g of lettuce every second day, this could be the case for the 17% lettuce consumed in households, restaurant, hotels, etc. (see figure 12), amounting to 9,828 salad meals each containing a 100 g of lettuce consumed daily.

![Flow chart distribution of lettuce in Kumasi, Ghana (Obuobie et al., 2006).](image)

Based on the above and assuming optimal infection parameters, a simple QMRA model would show a daily infection rate of all 149,828 individuals consuming either street food or salad meals in Ghana.
5. DISKUSSION

The contamination level by *Cryptosporidium* spp. oocysts and helminth eggs in low quality water sources and on lettuce was examined weekly over a period of four weeks. The prevalence found was 75% in water samples and 43% on lettuce with means of 1513 ± 6575 and 19 ± 24.8 oocysts, respectively. Examination of helminth eggs provided insufficient data for processing. The *Cryptosporidium* spp. oocyst data generated will later be used to develop a QMRA model.

Our results are in accordance with studies from several countries, where *Cryptosporidium* spp. oocysts was found on lettuce irrigated with surface waters. A Norwegian study found *Cryptosporidium* spp. oocysts on 26% of the lettuce, with a recovery efficiency of approximately 42%. However, oocysts were undetectable in the irrigation water used for the lettuce sample, but was detected in 16% of all irrigation waters examined with recovery efficiency of 43% (Robertson and Gjerde, 2001). A study in Valencia, Spain had prevalence of 63% from lettuce with a recovery efficiency of 25% ± 4%, and irrigation water had a mean of 48 *Cryptosporidium* spp. oocysts/L ranging 10 – 70 oocysts/L from the irrigation water channel receiving wastewater (Amoros et al., 2010). In Costa Rica 2.5% of lettuce was positive for *Cryptosporidium* spp. oocysts (Monge and Chinchilla, 1995), while a market study from Nigeria showed the presence of *Cryptosporidium* spp. oocysts on several raw consumed vegetables, including 48% of lettuce samples (Maikai et al., 2013). In Central America 36% of irrigation waters were tested positive for *Cryptosporidium* spp. oocysts, with a mean of 227 oocysts / hundred L (Thurston-Enriquez et al., 2002) and in Thailand a PCR detection study showed a prevalence of 11% *Cryptosporidium* spp. oocyst from 72 water samples taken along a river used for irrigation purposes. *Cryptosporidium parvum*, *C. meleagridis* and *C. serpentis* was detected (Koompapong and Sukthana, 2012), which shows the potential for contaminating vegetables is present, even though they were not tested. Different vegetables, root vegetables and fruit have been shown to carry and act as vehicles for *Cryptosporidium* spp. oocysts on several occasions (Monge and Chinchilla, 1995; Robertson and Gjerde, 2001; Amoros et al., 2010; Maikai et al., 2013). The presence of *Cryptosporidium* spp. oocysts in the environment and on vegetables have yet to be studied in Ghana, but human and livestock infections have previously been confirmed. Infections (Adjei et al., 2003; Adjei et al., 2004; Opintan et al., 2010; Squire et al., 2013). Humans and cattle are considered the most important sources of environmental contamination (Tzipori and Ward, 2002) hence the presence of infection in these hosts, together with our findings suggests the parasites appearance in the environment is nothing recent. The lack of data on *Cryptosporidium* spp. oocysts in the environment in Ghana,
encumbers interpretation of the data from this study in terms of low, average or high contamination levels, compared to other periods and regions. The difference in prevalence and concentration between all the studies mentioned above, including this, are from different geographical areas, which are further associated with different income levels and agricultural practices, probably making up for differences seen in prevalences and concentrations. Another factor is differences in the oocysts purification methods used both for water and lettuce, making any true comparison of the results difficult. This advocates for a “Golden standard method” for purification of oocysts in water and on vegetables, as seen for other parasites like helminthes (Roepstorff and Nansen, 1998), making studies comparable. The costly materials needed for purification of oocysts from environmental samples becomes a limitation in many studies, leading to small sample sizes or simply a lack of environmental studies. Alternatively, methods like Ziehl-neelsen staining (see section 3.4.1) and filtration can be used but require samples contain very little material other than oocysts or very small sample sizes with high concentrations of oocysts, which can be difficult to find in the environment. This study and the above, confirms *Cryptosporidium* spp. oocysts are found in surface irrigation water and on lettuce in many parts of the world. Similar scenarios are probably common any place where surface water is used for irrigation.

The simplified QMRA model outcome described in section 4.7 is obviously incorrect, due to the assumption of optimal infection parameters. The model should account for viability of oocysts, zoonotic potential of cryptosporidium spp., individual dose – response, etc. before any conclusions can be based on the outcome of the model. This type of data is next to prevalence and concentration data essential, since they alone may determine the outcome of the QMRA model. Low viability can result in Low risk and vice versa, even if oocysts prevalence and concentration is high, since the oocyst viability reflects the parasites ability to establish in a host. The same goes for genotyping, as wildlife species are considered of little risk to human health (Zhou et al., 2004), whereas species commonly seen in production animals and humans are considered high risk to human health. These types of data are not only missing from this study, but also from other environmental studies of *Cryptosporidium* spp. Considering the lack of sewage, use of chicken waste as fertilizer, free roaming goats and outdoor reared cattle, one could expect to find *C. hominis, C. parvum,* and *C. meleagridis* as the most dominant species in the environment. Relatively high humidity, rainy seasons and daily irrigation of vegetables promote high viability. While approximately 12 hours of sunlight all year round in Ghana advocates inactivation of the oocysts. A study from Spain showed a 40% reduction in viability of *C. parvum*
oocysts exposed to sunlight for four days, in top waters of a pond system (Reinoso and Becares, 2008). Considering the periods Cryptosporidium spp. oocysts are exposed to sunlight in Ghana, both in water and on lettuce one could speculate a relative low viability of the oocysts found in the environment.

PCR was unsuccessful for lettuce samples due to the environmental exposure, sample processing, low concentrations of oocysts in samples and long term storage, all advocating low amounts of intact DNA material remaining for PCR analysis. Successful PCR of a single water sample was due to the high concentration of oocysts in the sample, yielding higher amounts of DNA material for PCR analysis. Further, PCR of environmental oocyst samples are difficult to perform since the source of origin is unknown, advocating the use of species unspecific primers, with higher risk of disturbances from DNA of other origin than Cryptosporidium spp..

Runoff and leaching from agricultural fields caused by rainfall can induce surface water and groundwater Cryptosporidium spp. oocysts contamination and are linked to outbreaks (Millard et al., 1994; Morgan et al., 1995; Hancock et al., 1998; Patel et al., 1998; Aksoy et al., 2007; Pelly et al., 2007). Heavy rainfall can lead to higher water flows in rivers and lakes, consequently resuspending and reentering sediment pathogens into water sources and transmission cycles (Sengupta et al., 2012). Coherence between Rainfall and the number of Cryptosporidium spp. oocysts found in surface waters was apparently absent in this study (see table 6). E.g. round one and two show almost the same levels of contamination, while the rainfall was heavy and absent days in between, respectively. This might simply be coured by the overall design of this study, time of sampling, inadequate sample replicates and missing data on mm rainfall. Alternatively there is no correlation between rainfall and level of contamination of surface waters in this area, and the difference has a geographical explanation. The small scale of the fields surrounding the surface waters examined, lesser amounts of animal fertilizer used on the fields (based on personal observations), than seen in industrialized countries, where the studies mentioned above took place. Further, in industrialized countries animal fertilizer is mostly derived from cattle and pigs sources, which can excrete up to $6 \times 10^7$ and $10 \times 10^5$ OPG (Uga et al., 2000; Maddox-Hyttel et al., 2006) respectively. Whereas chickens are shown to excrete $5 \times 10^6$ oocysts/animal for the entire period of oocyst excretion (Akiyoshi et al., 2003). This could potentially lead to very different numbers of oocysts found in the cattle and pig fertilizers, used in industrialized countries, and to the chicken fertilizers used in the area where this study took place. Consequently, a lower contamination level of water sources by Cryptosporidium spp. oocysts...
during precipitation and runoff is feasible where chicken fertilizer is used. A factor advocating higher level of surface water contamination in Ghana compared to the cases mentioned above (Ireland, U.S.A, United Kingdom, and Turkey), is the lack of sewage, potentially washing human faeces into rivers and streams during rainfall in the area of this study. Sewage connected to treatment plants is only available for approximately 5% of the population in Ghana (Data from Ghana Statistical Service, 2002), but public toilets are available to some extent, which could to some degree compensate for the 95% of population without proper sewage. The considerations above and the outcome of this study calls for further examination, of the correlation between rainfall and contamination by Cryptosporidium spp. oocysts in the surface waters of this area.

Oocysts in irrigation water and on vegetables irrigated with the same water, is to my knowledge being studied here for the first time. However, correlation between oocysts in water and on lettuce samples was absent (R= -0.01). Single water samples and low recovery efficiency of the methods making false negative results possible (see section 4.1 and 4.2 respectively), could potentially cause this. Alternatively, transformation of the data into contamination levels (see table 7) could make up for the false negative results and yield a different picture, but this was not the case. The data of water and lettuce from farm site 1 illustrates an unexpected inverted relationship, with lettuce being more contaminated than water (see table 7). Further, this is supported by farm site 1 having the lowest number of oocysts in water samples, while having the highest number of oocysts on lettuce samples, both showing significant differences with all other water and lettuce sampling sites (see section 4.1 and 4.2). The above indicates the prime contaminating source of lettuce is found elsewhere than irrigation water. The chicken waste used as fertilizer could contaminate the lettuce during rainfall via splash, which was the case for pathogenic Salmonella bacteria onto tomato plants (Cevallos-Cevallos et al., 2012). The zoonotic species C. meleagridis infecting chicken (Chapel et al., 2011), could potentially be found in the fertilizer in the area where this study took place, and should be considered a risk factor contaminating the lettuce via splash during rainfall.

Turbidity or pH and the number of oocysts found in the water samples in this study (R= -0.01 and R= 0.1 respectively) was uncorrelated. Yet, (House, 2011) stated that high concentrations of oocysts are usually found in high turbidity waters, simply because the oocysts are picked up with solids during rainfall and washed into surface water sources. Meaning the relationship between oocysts and turbidity concentrations can vary with geography, highly dependent on large scale
agricultural industry or a lack of sewage, where many oocysts can potentially be washed into surface waters as runoff during rainfall.

Helminth eggs examination yielded few *Strongile* and *Toxocara* resembling eggs. The results correspond well with free roaming dogs and goats as potential hosts. However, environmental samples make identification difficult, since the source of the eggs is unknown and free living nematode eggs and pollen can resemble parasitic helminth eggs. Sedimentation of helminth eggs (0.0612, 0.1487 and 0.1262 mm x s\(^{-1}\), *Ascaris suum*, *Trichuis suis* and *Oesephagostomum spp.* respectively) (Sengupta et al., 2011) and sampling of top waters could explain the small amount of data generated. A study on helminth eggs found on vegetables from Ghanaian markets including lettuce, showed a mean of 1.14 helminth eggs per gram, including *Ascaris lumbricoidis*, hookworm, *Trichostrongylus*, *Schistosoma heamatobium* and *Trichuis trichiura* (Obuobie et al., 2006). The study by Obuobie et al., (2006) confirms the presence of several helminth species on lettuce and in relatively high numbers compared to the findings of this study.

### 6. CONCLUSION AND FURTHER STUDIES

#### 6.1 Conclusion

The high prevalence and concentrations of *Cryptosporidium* spp. oocysts found in water with direct contact to humans and on vegetables consumed raw, entails a potential risk of infection in humans. The outcome of the simplified QMRA model is obviously incorrect, but illustrates the potential risk of infection and the need for further development of the model. Implementation of preventive measures based on the outcome of the improved model should be considered and actions taken accordingly.

Water and lettuce contamination was apparently incoherent in this area, alternatively other sources contaminating lettuce should be considered in regards to prevention. Further, incoherence between rainfall and water contamination was observed, which might be specific for this and areas with similar agricultural habits.
6.2 Further studies

Examination of viability and genotypes of oocysts found in both water and on lettuce samples would be a major step in improving the general knowledge on Cryptosporidium spp. in the area and in development of an improved QMRA model. Also, knowledge on the presence of Cryptosporidium spp. oocysts on other raw consumed vegetables would improve the QMRA model greatly.

Development of golden standard methods for purification of Cryptosporidium spp. oocysts from environmental samples, based on cheap and available materials, would be a major improvement for future environmental studies and make accurate comparison between studies possible.
7. REFERENCES


Tyzzer, E.E., 1912. *Cryptosporidium parvum* (sp. nov.), a coccidium found in the small intestine of the common mouse. Archives Für Protiskunde 26, 394-412.


