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# Waterborne transmission of *Toxoplasma gondii:* novel approaches to development of analytical techniques

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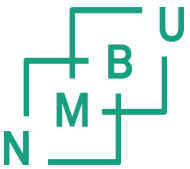
Adamstuen (2017)

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“I like friends who have independent minds because they tend to make you see problems from all angles” Nelson Mandela (1918-2013)

To my parents: the foundation of all these events!

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# Acknowledgements

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# List of specialist abbreviations

| | |
|---|---|
| AP | Acidified pepsin |
| BSA | Bovine serum albumin |
| $C_{24}H_{40}O_4$ | Deoxycholate |
| CIAC | Competitive internal amplification control |
| DABCO | 1, 4-diazabicyclo [2.2.2] octane |
| DIC | Differential interference contrast |
| DPBS | Dulbecco's phosphate-buffered saline |
| FAM | Fluorescent dye attached to oligonucleotides |
| FITC | Fluorescein isothiocyanate |
| GlcNAc | N-acetyl, D-glucosamine |
| IMS/ IFAT | Immunomagnetic separation / Immunofluorescent antibody test |
| $K_2Cr_2O_7$ | Potassium dichromate |
| kd | Dissociation constant |
| LAMP | Loop Mediated Isothermal Amplification |
| LMS/NLMS | Lectin-Magnetic separation/non-Lectin-Magnetic separation |
| LOC | Lab-on-a-chip |
| mAb | Monoclonal antibody |
| MPC/MPS | Magnetic particle concentrator/ Magnetic particle separator |
| NaClO | Sodium hypochlorite |
| NASBA | Nucleic acid sequence based amplification |
| NMBU | Norwegian University of Life Sciences |
| PhHV | Phocine Herpes virus |
| SDS | Sodium dodecyl sulfate |
| SELEX | Systematic evolution of ligands by exponential enrichment |
| TWIST | DNA detection using fluorescent probes with TwistAMP® kit and probe |
| WGA | Wheat germ agglutinin |

# List of papers

**Paper I**

Surface binding properties of aged and fresh (recently excreted) *Toxoplasma gondii* oocysts.

Authors: Harito, J.B., Campbell, A.T., Prestrud, K.W., Dubey, J.P., Robertson, L. J.

Published: *Experimental Parasitology* 165 (2016): 88-94

**Paper II**

Lectin-magnetic separation (LMS) for isolation of *Toxoplasma gondii* oocysts from concentrated water samples prior to detection by microscopy or qPCR.

Authors: Harito, J.B., Campbell, A.T., Tysnes, K. R., Dubey, J.P., Robertson, L. J.

Published: *Water Research* 114 (2017): 228-236

**Paper III**

Use of Lectin-Magnetic separation (LMS) for detecting *Toxoplasma gondii* oocysts in environmental water samples.

Authors: Harito, J.B., Campbell, A.T., Tysnes, K. R., Robertson, L. J.

Manuscript: submitted to *Water Research* (under review)

# Summary

Access to safe drinking water is essential for healthy life. Drinking water contaminated with waterborne pathogens is one of the most significant environmental contributors to human disease burden. Protozoan parasites that cause waterborne diseases generally have very robust transmission stages and are able to survive the most commonly practiced physical and chemical water treatment processes.

Although *Cryptosporidium* and *Giardia* are the most widely known and researched protozoan parasites that may be transmitted by water, consumption of water contaminated by sporulated oocysts of *Toxoplasma gondii* is emerging as a major concern and several waterborne outbreaks have been documented. This protozoan parasite, which may infect all warm-blooded animals, usually causes only mild symptoms in the immunocompetent host. However, in the immunocompromised host it may result in severe, possibly fatal, disease. Furthermore, congenital transmission can result in serious fetal damage should the mother acquire her primary infection during pregnancy. However, the extent of contamination of different waters with this successful protozoan parasite has not been adequately investigated due to the lack of effective and appropriate detection methods. Although methods for the analysis of water for *Cryptosporidium* oocysts and *Giardia* cysts have long been established, methods of analysis of water for contamination with *T. gondii* oocysts remain elusive.

This PhD study had aimed at investigating different novel approaches for analysis of drinking water for contamination with *T. gondii* oocysts. Various methods were investigated in this work. These include the use of a microfluidic chip, the development of aptamers for detection, the investigation of oocyst wall binding to monoclonal antibodies and lectins, and the use of both microscopy and qPCR for detection. Of the methods investigated, surface treatment of oocysts to enable binding of the lectin wheat germ agglutinin was found to be most promising for developing an LMS approach, and was applicable to both fresh and aged oocysts. Parameters investigated using spiked samples included different bead types and different water turbidities. Proof of principle for the technique having been established, final investigations addressed bringing the procedure from the research lab to the water analysis laboratory, and how to

address the associated challenges. The studies were completed by a pilot study on some “real life” environmental samples, and my initial results indicate that contamination of drinking water sources with *Toxoplasma* oocysts may occur in Norway, paving the way for further large-scale analysis.

# Sammendrag (Norwegian summary)

Tilgang til rent drikkevann er essensielt for et sunt liv. Drikkevann kontaminert med vannbårne patogener er en av de viktigste miljøfaktorene som påvirker menneskers sykdomsbyrde. Parasittiske protozoer som overføres med vann er ofte meget hardføre og har robuste overføringsstadier som kan overleve de vanligste fysiske og kjemiske vannrensningsmetodene. Blant parasittiske protozoer som kan smitte via vann er *Cryptosporidium* og *Giardia* de mest kjente og studerte, men andre protozoer, blant annet sporulerte oocyster av *Toxoplasma gondii,* har vist seg å være et økende problem, med flere registrerte vannbårne utbrudd. Alle varmblodige dyr kan infiseres med *T. gondii*. Hos individer med velfungerende immunsystem vil det sjeldent gi noe mer enn milde symptomer, mens hos immunkompromiterte kan en infeksjon være svært alvorlig og ha døden til følge. Dersom gravide kvinner eller drektige dyr infiseres med *T. gondii* for første gang under graviditet kan dette resultere i alvorlige fosterskader da *T. gondii* kan overføres fra mor til fosteret via placenta.

I motsetning til *Cryptosporidium* og *Giardia* har derimot ikke omfanget av forurensning av forskjellige vannkilder med *T. gondii* blitt tilstrekkelig undersøkt, mye på grunn av mangel på effektive og hensiktsmessige deteksjonsmetoder. Selv om metoder for analyse av vann for *Cryptosporidium* oocyster og *Giardia* cyster har vært etablert i lang tid, har analysemetoder for vann kontaminert med *T. gondii* oocyster vært fraværende. Dette doktorgradsprosjektet hadde til hensikt å undersøke ulike nye tilnærminger for å analysere drikkevann forurenset med *T. gondii* oocyster.
Ulike metoder ble undersøkt i dette arbeidet, disse inkluderer: bruk av mikrofluidiske brikker, utvikling av aptamerer for deteksjon, undersøkelse av oocystens vegg, og monoklonale antistoffer og lektiners evne til å binde til veggen, samt bruk av både mikroskopi og qPCR for deteksjon.

Av metodene som ble undersøkt, ble overflatebehandling av oocystene for å muliggjøre binding av lektinen hvetekimagglutinin funnet å være den mest lovende for å utvikle en LMS-tilnærming, og var anvendelig for både nylig utskilte og eldre oocyster. Parametere som ble

undersøkt ved hjelp av kunstig positive prøver, inkluderte forskjellige magnetkuletyper og ulike vannturbiditeter. Når prinsippet bak teknikken kunne bevises eksperimentelt og metoden var etablert arbeidet vi videre for å bringe prosedyren fra forskingslaboratoriet til vannanalyselaboratoriet, og hvordan man skal takle de utfordringene som er forbundet med dette. Resultatene med kunstig positive prøver var tilfredsstillende og metoden ble også prøvd ut på reelle drikkevannsprøver i en pilotstudie av norske drikkevannskilder. Resultatene fra pilotstudien tyder på at *Toxoplasma* oocyster forekommer i disse drikkevannskildene, og dette bør legges til grunn for vurdering av videre storskala studier for å kartlegge hvor stort dette problemet er på nasjonal basis.

# 1. Introduction

## 1.1. Background

Water is one of the major natural resources that is vital for all known forms of life. Safe drinking water is essential to sustain life; it is the basis for human health, survival, growth and development. According to World Health Organization (WHO), access to safe drinking water is a basic human right. Recognition of this right contributes to the survival of human beings and disease prevention, since water is not only used for drinking but also for many other purposes such as hygiene, food production, agriculture, cooking and industry. Globally, a lack of access to safe drinking water sources, coupled with inadequate sanitation and hygiene, remains one of the most critical public health challenges. Despite the significant achievements by the end of the Millennium Development Goal (MDG) era, an estimated 663 million people still lack access to an "improved" source of drinking water and 159 million of these people rely on untreated surface water, which poses even greater health risks than other water sources. Many more still lack access to "safe" drinking water, with at least 1.9 billion people relying on an unimproved source or an improved source that is fecal contaminated (WHO 2016). Unsafe water, in combination with inadequate sanitation and hygiene, still contributes to the deaths of some 842 000 people every year, representing 58% of deaths caused by diarrhea. About 361 000 of these deaths occur in children aged under 5 years (WHO 2014). Hence, despite tremendous efforts and advances in water treatment technologies, waterborne disease outbreaks continue to occur in both developed and developing countries as a result of factors that jeopardize the quality and safety of drinking water (WHO 2016).

This creates a major public health concern worldwide as waterborne pathogens and related diseases can cause not only morbidity and mortality, but also there is a high cost that represents their prevention and treatment (Ramirez-Castillo et al. 2015). In addition, substantial impacts from waterborne pathogens include loss of productivity, missed days of education and work, and lack of achievement of potential.

Pathogens that can be transmitted via water can be divided into three main categories: viruses, bacteria, and parasites, the latter of which are comprised of protozoa and helminths. Such pathogens often reach water sources when infected hosts shed different forms of microbes in excreta (e.g. feces, urine, or exudate). The sewage composed of the aforementioned excreta released untreated, undertreated or by accidental leakage, allows entry of pathogens to water sources (Figure 1). An alternative mechanism is through runoff to source water or permeation into ground water from animal feces or sewage utilized as fertilizer. It should also be noted that many waterborne pathogens are zoonotic, i.e. capable of infecting both humans and animals (Bridle 2014). Consequently, protozoan pathogens originating from animal and human waste have been recorded from water sources throughout the world. Rain and surface water may transport infective stages (e.g. oocysts, cysts) into drinking water supplies, recreational sites, including fresh and marine waters, and irrigation waters, which, in turn, can contaminate the food supply through the agricultural and food industry or household practices, from the farm to the consumer (Petersen et al. 2010).

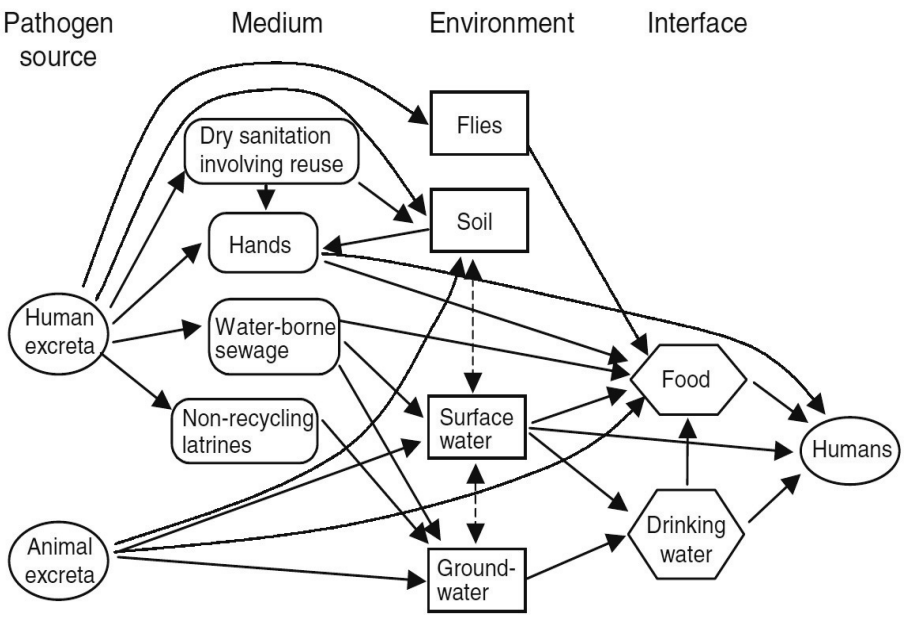



Figure 1. Fecal-oral transmission pathway of pathogens (Prüss-Üstün et al. 2002)

*Giardia*, *Cryptosporidium* and *Toxoplasma* are among the most frequently reported zoonotic parasites; widely dispersed and associated with outbreaks of infection resulting from drinking contaminated surface water (Fayer et al. 2004). Although waterborne transmission of *Toxoplasma gondii* was considered uncommon until recent decades, a large human outbreak linked to contamination of a municipal water reservoir in Canada by oocysts from wild felids and the widespread infection of marine mammals in the USA have forced us to recognize the importance and impact of waterborne *Toxoplasma* (Jones & Dubey 2010). Likewise, a number of waterborne outbreaks have been documented from several countries (Shapiro et al. 2010a) particularly from Brazil, possibly because the more virulent strains enable such outbreaks to be more readily recognized (Vaudaux et al. 2010). It is also probable that waterborne infections due to *T. gondii* do occur in Europe, but are not identified, as most of the infections are asymptomatic due to strain variation (Robert-Gangneux et al. 2015). Under current situations of increased globalization of the food chain, tourism and lack of controls for *Toxoplasma* in imported food, it seems unlikely to expect that the more virulent strains remain isolated in South America (Robertson et al. 2014). Moreover, circumstantial evidence suggests that oocyst-induced infections in humans are clinically more severe than tissue cyst-acquired infections (Jones & Dubey 2010).

Hence, it requires a proper assessment of pathogens on water and water quality monitoring as key factors for decision-making regarding to water distribution systems, the choice of best water treatment and prevention of waterborne outbreaks (Ramirez-Castillo et al. 2015). In view of that, standardized methods for analyzing water samples for contamination with other protozoan parasites, such as *Giardia* and *Cryptosporidium* species, have been developed and are used globally in research, following up of outbreaks, and for determining the effects of measures to reduce contamination. However, no such optimized and standardized methods available for the concentration and detection of *Toxoplasma gondii* oocysts in water supplies. Lack of such methods for detection of *T. gondii* oocysts in water also limits research on the dissemination and destiny of this pathogen through aquatic habitats. This prompts the necessity to develop and/or adapt methods for analyzing water samples for *T. gondii* oocysts contamination. Therefore, the main goal of this PhD research was to investigate different novel

approaches for developing a robust, reproducible, and, preferably, user-friendly method for the detection of *T. gondii* in water samples such that we have the necessary tool for assessing contamination with this parasite in the aquatic environment.

## 1.2. Description of *Toxoplasma gondii*

*Toxoplasma gondii* is a coccidian parasite, with the domestic cat and other felids as its definitive hosts and a wide range of birds and mammals as intermediate hosts (Petersen & Dubey 2001). It was first discovered in 1908 by two separate research teams on two different continents. While Nicolle and Manceaux described the organism in the tissues of a hamster-like rodent, the *gundi* (*Ctenodactylus gundi*) in Tunisia, Splendore detected the same in the tissues of a rabbit in Brazil. The name *Toxoplasma gondii* (toxo: arc or bow; plasma: life) was suggested by Nicolle and Manceaux in 1909 (Dubey 2010). It belongs to Phylum Apicomplexa (Levine, 1970), Class Sporozoasida (Leukart, 1879), Subclass Coccidiasina (Leukart, 1879), Order Eimeriorina (Leger, 1911), and Family Toxoplasmatidae (Biocca, 1956) (Dubey 2010; Hill et al. 2005). There is only one species, *T. gondii*; one of the most successful parasitic organisms estimated to infect one-third of the world's human population (Innes 2010; Weiss & Dubey 2009). However, its complete life cycle was finally understood only in the late 1960s (Dubey & Frenkel 1972; Ferguson et al. 1974; Frenkel et al. 1970; Hutchison et al. 1969), with the discovery of the central role of the cat as a definitive host harboring the sexual developmental stages within the small intestine and spreading oocysts through feces.

## 1.3. Life cycle and transmission

The life cycle of the parasite (Figure 2) consists of asexual reproduction in the intermediate hosts and sexual reproduction in the intestinal mucosa of the definitive host. Domestic cats and other members of the carnivore family Felidae are the only known definitive hosts shedding millions of oocysts in their feces (Dubey et al. 1970a; Dubey et al. 1970b; Frenkel et al. 1970). There are three infectious stages of the parasite for all hosts: the tachyzoites (the rapidly dividing form) in

tissues, the bradyzoites (the slowly dividing form) inside cysts in tissues, and the sporozoites in the oocyst (Jones & Dubey 2010; Petersen & Dubey 2001).

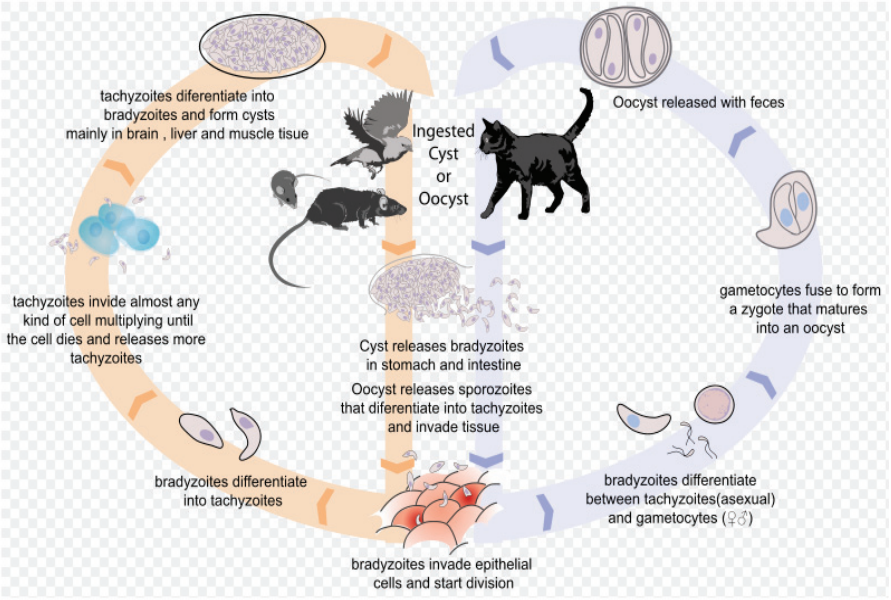



Figure 2. Life cycle of *Toxoplasma gondii*

(Source: https://commons.wikimedia.org/wiki/File:Toxoplasmosis_life_cycle_en.svg accessed on 09 June 2017).

Intermediate hosts (including humans) can acquire *T. gondii* either by ingesting food, water or soil contaminated by sporulated oocysts (Figure 3), ingestion of tissues of infected animals harboring viable cysts, or by transplacental transmission (Dubey 2010; Jones & Dubey 2010). After oocyst ingestion, sporozoites are released and penetrate the intestinal epithelium. Then, they transform to tachyzoites; and become surrounded by a parasitophorous vacuole that protects from host defense mechanisms. The tachyzoite multiplies asexually by repeated endodygeny and they spread first to mesenteric lymph nodes and then to distant organs by invasion of lymphatics and blood (Hill et al. 2005; Jones & Dubey 2010). After a few multiplication cycles, tachyzoites give rise to bradyzoites in a variety of tissues. Because of the conversion from tachyzoite to bradyzoite, tissue cysts arise as early as 7 to 10 days post infection and may remain throughout life in most hosts, predominantly in the brain or musculature (Dubey 2010; Jones & Dubey 2010; Robert-Gangneux & Dardé 2012). Infection from tissue cysts

may occur by consuming raw or undercooked meat (Dubey 2010). Upon the ingestion of these tissue cysts by an intermediate host, cysts are ruptured as they pass through the digestive tract, causing the release of bradyzoites. The bradyzoites infect the intestinal epithelium of the new host and differentiate back into the rapidly dividing tachyzoite stage for dissemination throughout the body (Robert-Gangneux & Dardé 2012). A primary *T. gondii* infection during pregnancy can lead to infection of the fetus since the parasite (tachyzoites) can cross the placenta (congenital transmission). Congenital toxoplasmosis in humans, sheep, and goats can kill the fetus (Dubey 2010). Organ transplant recipients can also develop toxoplasmosis due to transmission of the parasite with the transplanted organ from a *Toxoplasma*-seropositive donor to a *Toxoplasma*-seronegative recipient. It is also possible (but rarely) that parasite transmission could occur as the result of blood transfusion, hematopoietic stem cell or bone marrow transplantation. Infections in laboratory personnel have arisen by contact with contaminated needles and glassware or infected animals (Montoya & Liesenfeld 2004).

Cats become infected after consuming intermediate hosts (such as a mouse or bird) harboring tissue cysts or directly by ingestion of sporulated oocysts (Robert-Gangneux et al. 2015). Upon ingestion of a tissue cyst, proteolytic enzymes in the stomach and small intestine digest the walls of the cyst and bradyzoites released. The bradyzoites penetrate the epithelial cells of the small intestine and initiate development of numerous generations of asexual and sexual cycles of *T. gondii* (Dubey & Frenkel 1972). *T. gondii* multiplies profusely in intestinal epithelial cells of cats (enteroepithelial cycle) and these asexual stages are known as schizonts. Organisms (merozoites) released from schizonts transform to sexual stages of male and female gametes. The male gamete (microgametocyte) has two flagella and it swims to and enters the female gamete (macrogametocyte). After the female gamete is fertilized by the male gamete leading to the production of zygote, oocyst wall formation begins around the zygote. The zygotes differentiate into unsporulated oocysts and when mature, oocysts are discharged into the intestinal lumen by the rupture of intestinal epithelial cells and are shed in the feces of the definitive host (Dubey 2010; Hill et al. 2005; Jones & Dubey 2010).

Prepatent periods and frequency of oocyst shedding vary according to the stage of *T. gondii* ingested. Prepatent periods are 3 to 10 days after ingesting tissue cysts, and more than 18 days

after ingesting oocysts, irrespective of the dose. The prepatent period after ingesting tachyzoites may vary. While less than 50% of cats shed oocysts after ingesting tachyzoites or oocysts, nearly all cats shed oocysts after ingesting tissue cysts. *T. gondii* is adapted to be transmitted biologically by carnivorism in cats and transmission by the oocysts is more efficient in non-feline hosts; bradyzoites are more infective to cats and oocysts are more infective to mice (Dubey 2010). In freshly passed feces, oocysts are unsporulated (noninfectious), subspherical to spherical in shape and are 10 µm x 12 µm in diameter. Sporulation (become infectious) occurs outside the cat within 1-5 days depending upon aeration and temperature. Sporulated oocysts are subspherical to ellipsoidal and are 11 µm x 13 µm in diameter. They contain two ellipsoidal sporocysts each containing four sporozoites. The sporozoites are 2 µm x 6 µm to 2 µm x 8 µm in size (Dubey et al. 1998; Hill et al. 2005).

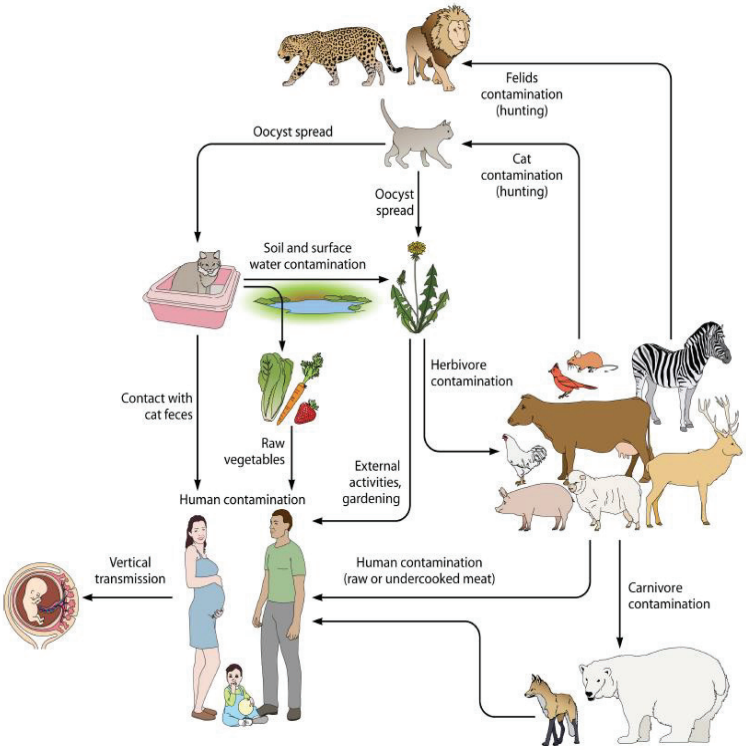



Figure 3. Modes of transmission of *T. gondii* and various sources of infections in humans (Robert-Gangneux & Dardé 2012)

## 1.4. Pathophysiology and significance of toxoplasmosis

The pathophysiology of toxoplasmosis results from the dissemination of tachyzoites throughout the body. After trans-epithelial passage across the intestinal barrier, tachyzoites rapidly invade monocytes and gain access to the blood flow, and from there virtually to all organs (Robert-Gangneux & Dardé 2012). In acute toxoplasmosis, a host may die due to necrosis (caused by intracellular growth of tachyzoites) of the intestine and mesenteric lymph nodes before severe damage to other organs. Focal areas of necrosis may develop in many organs; the clinical picture is determined by the extent of injury to these organs, especially vital organs such as the eyes, heart, and adrenals (Dubey 2010). If the host survives, the invasive stages (tachyzoites) convert into latent form (bradyzoites) within cells and persist as cysts lifelong, mostly in muscles, retina, and brain, following the onset of an efficient immune response (Robert-Gangneux et al. 2015).

In humans, *T. gondii* infection is widespread although its prevalence varies widely from place to place (Hill et al. 2005). It is widely believed that postnatally acquired toxoplasmosis is frequently mild or asymptomatic in immunocompetent individuals, but may cause mild and non-specific symptoms including fever, asthenia and lymphadenopathy in about 20 % of patients (Robert-Gangneux et al. 2015). Reports suggest that oocyst-induced infections are more severe than those induced by tissue cysts and bradyzoites by the natural oral route, irrespective of the dose (Dubey 2010). Among the manifestations, lymphadenopathy is the most frequently observed clinical form of toxoplasmosis in humans and may be associated with fatigue, muscle pain, sore throat, and headache (Aramini et al. 1998; 1999). However, toxoplasmosis is life threatening in immunocompromised individuals since factors severely impairing cellular immune response (such as HIV infection and immunosuppressive therapies) can lead to severe disease associated with either primary acquired infection or the reactivation of latent infection. Certainly, profound immunosuppression can favor cyst rupture and tachyzoite multiplication and dissemination (Robert-Gangneux et al. 2015). As a result, toxoplasmosis ranks high on the list of diseases that lead to death in patients with AIDS; approximately 10% of AIDS patients in USA and up to 30% in Europe died of toxoplasmosis in 1980s (Dubey 2010; Luft & Remington 1992). Cyst reactivation is mostly localized to the brain and the retina, but can occur in other tissues,

as tachyzoites can invade all organs that can be subsequent potential sites for cyst reactivation (Patrat-Delon et al. 2010). The risk for disseminated infection is closely related to the duration and degree of immunosuppression, with hematopoietic stem cell transplant (HSCT) patients being most at risk (Derouin & Pelloux 2008), whereas focal disease, such as cerebral toxoplasmosis or retinochoroiditis, is more commonly observed in HIV-infected patients (Robert-Gangneux et al. 2015). Most AIDS patients suffering from toxoplasmosis have bilateral, severe, and persistent headache, which responds poorly to analgesics (Renold et al. 1992). Moreover, patients may have disorientation, drowsiness, hemiparesis, reflex changes, and convulsions, and many become comatose (Hill et al. 2005). The predominant lesion in the brain is necrosis, especially of the thalamus (Renold et al. 1992). Encephalitis is the most important manifestation of toxoplasmosis in immunosuppressed patients as it causes the most severe damage to the patient (Dubey 2010; Luft & Remington 1992).

Fetal infection can occur when a woman or female animal acquire infection for the first time during pregnancy by transplacental transfer (congenital infection) of tachyzoites to the fetus either during blood flow dissemination or at a later stage (Robert-Gangneux et al. 2011). In humans, the frequency of congenital infection and the severity of fetal damage depends on the stage of pregnancy when maternal infection occurs. Fetal infection occurs in less than 10 % of cases during the first trimester, but increases to 30 % of cases in the second trimester and 60–70 % in third trimester and even more close to delivery (Dunn et al. 1999). Regarding the severity of fetal infection, in more than 80 % of the cases, neonates infected during the third trimester of gestation are usually asymptomatic (Desmonts & Couvreur 1974; Remington et al. 1995). Conversely, when transplacental transmission occurs during the first trimester of pregnancy, the consequences on fetal development are severe, often leading to gross abnormalities involving the brain and eye tissues, or to abortion (Robert-Gangneux et al. 2015). Mild disease may consist of slightly diminished vision, whereas severely diseased children may have the full tetrad of lesions: retinochoroiditis (inflammation of the inner layers of the eye), hydrocephalus (big head), convulsions and intracerebral calcification. Of these, hydrocephalus is the least common but most significant lesion of toxoplasmosis. So far, the most common sequela of congenital toxoplasmosis is ocular disease (Desmonts & Couvreur 1974; Remington et al. 1995).

Additionally, *T. gondii* is capable of causing infection and severe disease in many animals used for food, including sheep, pigs, goats, and rabbits (Hill et al. 2005). Toxoplasmosis causes great losses in sheep and goats, and may cause embryonic death and resorption, fetal death and mummification, abortion, stillbirth, and neonatal death in these animals (Dubey 2010). Although reports indicate outbreaks of toxoplasmosis in pigs from several countries, mortality is more common in young pigs than in adults. Pneumonia, myocarditis, encephalitis, and placental necrosis were the lesions in infected pigs. Sporadic and widespread outbreaks of toxoplasmosis occur in rabbits, mink, birds, and other domesticated and wild animals. Animals that survive infection may harbor tissue cysts and can therefore transmit *T. gondii* infection to human consumers (Hill et al. 2005). While various tissues of infected pigs, sheep and goats, most frequently harbor tissue cysts, it is less frequent in the tissues of infected poultry, rabbits, dogs and horses. On the other hand, tissue cysts are found only rarely in skeletal muscles of cattle or buffaloes (Tenter et al. 2000).

Despite one single species (*T. gondii*) responsible for toxoplasmosis in humans and animals, three different genotypes I, II, and III have been described (Howe & Sibley 1995) since the 1990s from Europe and USA which are equivalent to clonal lineages (Ajzenberg et al. 2002a; Darde et al. 1992; Howe & Sibley 1995). However, new genotyping tools such as multilocus sequence typing were applied more recently to field studies in other continents. These studies revealed a much more complex population structure with a greater genetic diversity, likely reflecting frequent exchanges of strains between hosts as well as recombination of isolates during sexual life cycle within the definitive hosts (Ajzenberg et al. 2004). This led to the generation of recombinant isolates (I/II, I/III or II/III), but also to new clonal haplogroups, and in some areas (Table 1), particularly in South America, to atypical genotypes with many unique polymorphisms (Robert-Gangneux et al. 2015). Type I is highly virulent in laboratory animals, whereas types II and III are non-virulent. In humans, Type II predominates in AIDS and congenital infections and isolated in about 75–80% of AIDS and non-AIDS immunocompromised patients (Ortega 2006) in Europe (Ajzenberg et al. 2002b; Aubert et al. 2010) and North America (Howe & Sibley 1995).

Table 1. Genetic diversity, virulence and global distribution of *T. gondii*

| Genotype | Virulence | Distribution | | | | |
|---|---|---|---|---|---|---|
| | | Europe | North America | South America | Africa | Asia |
| I | High | Rarely | | | | |
| II | Low | Dominant | Dominant | Rarely | Common | |
| III | Medium | Sporadic | | | Common | Occasional |
| Atypical (IV) | Very high | | High | Dominant | | |
| Halogroup 12 | | | Common | | | |
| Halogroup 1-3 | | | | | Common | |

Moreover, observations of a higher prevalence of atypical strains as well as occurrences of a clonal haplogroup (haplogroup 12) has been recently identified (Khan et al. 2011) in North America. However, atypical genotypes largely predominate in South America while type II are rare (Pena et al. 2008). Reports indicate that strains from South America have shown to be of particularly high virulence (de-la-Torre et al. 2013). Infection with atypical strains displayed a severe and even lethal toxoplasmosis in immunocompetent individuals from French Guiana suggesting a high degree of virulence to humans (Carme et al. 2002; Carme et al. 2009; Darde et al. 1998). In Africa, clonal lineages known as Africa 1-3 haplogroups coexist with type II and III lineages (Mercier et al. 2010).

## 1.5. Diagnosis in humans and animals

As clinical signs of toxoplasmosis are non-specific and are not sufficiently characteristic for a definite diagnosis, its detection depends mainly on biological, serological, or histological methods or the combination of some of these methods (Hill et al. 2005). Available serologic procedures for the detection of *T. gondii* humoral antibodies include; the Sabin-Feldman dye test (DT), the modified agglutination test (MAT), the indirect hemagglutination test (IHAT,) the indirect fluorescent antibody assay (IFA), the direct agglutination test (DAT), the latex agglutination test (LAT), the enzyme-linked immunosorbent assay (ELISA), and the immunosorbent agglutination assay test (IAAT). The IFA, IAAT, and ELISA tests have been

modified to detect IgM antibodies (Remington et al. 1995). The IgM antibodies appear sooner after infection than the IgG antibodies and disappear faster than IgG antibodies after recovery (Remington et al. 1995). The methylene blue dye test for the detection of antibodies, introduced in 1948 by Sabin and Feldman, is maintained as a gold standard for serology tests by reference laboratories, but is labor-intensive and requires a continual supply of live organisms. Since IgG can persist for decades, IgM, which typically persists for 6–9 months, is used as a marker of recent infection, although IgM antibodies have been detected for up to 18 months (Wilson and McAuley, 1999). Diagnosis in critical clinical cases (pregnant women, HIV/AIDS patients, neonates etc.) requires specialist testing including enhanced IgA/IgM detection, measurement of IgG avidity and direct detection by PCR, undertaken by reference laboratories (Dubey 1998a; Jauregui et al. 2001). Recently, progress has been made in the diagnosis of human infection with *Toxoplasma* using PCR. Infection has been diagnosed using nested, stage-specific primers and cerebrospinal fluid from AIDs patients with suspected toxoplasmic encephalitis (Contini et al. 2002; Joseph et al. 2002); in immunocompromised patients undergoing hematopoietic stem cell transplantation (Lewis et al. 2002); and in suspected cases of fetal toxoplasmosis using amniotic fluid (Antsaklis et al. 2002; Desmonts et al. 1985; Foulon et al. 1999). Similarly, the detection of tissue cysts in meat animals is difficult since the numbers present are low and may be as few as 1/100 g meat. Digestion of the sample to rupture the cyst wall and release hundreds of bradyzoites prior to bioassay in mice or application of PCR to detect DNA has been used to assess *T. gondii* in meat samples (Dubey 1998a; Jauregui et al. 2001). Although cats may shed high numbers of oocysts for a limited period, concentration methods using high-density sucrose solution may be required and oocysts should be definitively recognized following sporulation and bioassay in mice (Dubey 2010). For epidemiological surveys, however, oocyst detection is impractical and serological prevalence is a better marker of exposure to *T. gondii*.

## 1.6. Treatment and control

Treatment of toxoplasmosis generally involves any sulfonamide that diffuses across the host cell membrane. Most commonly prescribed sulfonamides include; sulfadiazine, sulfamethazine,

and sulfamerazine, are effective against toxoplasmosis and widely used in combination with pyrimethamine (Daraprim). The two drugs (in combination) act synergistically by blocking the metabolic pathway involving *p*-aminobenzoic acid and the folic-folinic acid cycle, respectively (Dubey 2010). Although these drugs have beneficial action when given in the acute stage of the disease process (active multiplication of the parasite), they will not usually eradicate infection (Dubey 2010; Hill et al. 2005). Certain other drugs including spiramycin, piritrexin, roxithromycin, clindamycin, cyclosporin A, atovaquone, ponazuril, a novel triazine, and others have been found effective in experimentally induced *T. gondii* infection in animals or cell cultures. Spiramycin produces high tissue concentrations, particularly in the placenta, without crossing the placental barrier although it has inferior anti-*T. gondii* properties compared with sulfadiazine and pyrimethamine (Dubey 2010). Nevertheless, drugs including diaminodiphenylsulfone, atovaquone, spiramycin, and clindamycin (Hill et al. 2005) have been used to treat toxoplasmosis in difficult cases of which atovaquone was reported to kill tissue cysts and Clindamycin giving good results despite causing ulcerative colitis (Dubey 2010).

Thorough washing of hands with soap and water following meat handling could prevent *T. gondii* infection in humans. In addition, any kitchen materials (cutting boards, sink tops, knives and others) that had contact with uncooked meat should be washed with soap and water (Dubey 2010; Lopez et al. 2000). Meat of any animal should be cooked until internal temperature has reached 67°C (Dubey et al. 1990) before human or animal consumption, and tasting meat while cooking or seasoning homemade sausages should be avoided. Freezing meat to an internal temperature of -13°C (Kotula et al. 1991) is effective in killing tissue cysts; freezing meat overnight in a household freezer is effective in killing most tissue cysts. Salting, curing, smoking, and the addition of products to meat to enhance color and taste (enhancing solutions) can have deleterious effect on the viability of *T. gondii* in meat, but there is too much variability in standards for these procedures to make a safety recommendation. Pregnant women, especially, should avoid contact with cats, soil, and raw meat(Dubey 2010). Pet cats should be fed only dry, canned, or cooked food. The cat litter should be emptied every day (to prevent sporulation of oocysts), preferably not by a pregnant woman. Gloves should be worn while

gardening, while changing cat litter, and while handling soil potentially contaminated with cat feces. Owners may also be advised to keep dogs away from the cat litter box to prevent ingestion of and passage through of oocysts. Vegetables should be washed thoroughly before eating, because they may have been contaminated with cat feces (Dubey 2010). Irradiation at 50 krads (Dubey & Thayer 1994) or high-pressure processing at 400 MPa are effective in killing tissue cysts as well as oocysts (Dubey 2010). Pregnant women should be aware of the dangers of toxoplasmosis (Foulon et al. 1994; Foulon et al. 2000).

Feeding cats with uncooked meat, viscera, or bones should be strictly avoided and efforts be made to keep cats indoors to prevent hunting. Access to trashcans by scavenging cats should be prevented, and, in addition, measures to control the stray cat population should be implemented. A prompt removal of the bodies of dead pigs and other animals is highly recommended to prevent cannibalism by pigs and scavenging by cats. Sheep that have aborted due to toxoplasmosis usually do not have recurrent toxoplasmic abortions, and thus can be saved for future breeding. However, fetal membranes and dead fetuses should be handled safely using gloves and buried or incinerated to prevent infection of felids and other animals on the farm. Pregnant sheep and goats as well as grain stores should be prevented from access by cats to avoid potential oocyst contamination (Dubey 2010).

To prevent infection of zoo animals with *T. gondii*, cats, including all wild felids, should be housed in a building separate from other animals, particularly marsupials and New World monkeys. As a rule, cats should not be fed uncooked meat, but where this is inevitable, frozen thawed meat is preferable, as tissue cysts are less infectious post-freezing. Dissemination of *T. gondii* oocysts in the zoo should be prevented because of potential exposure of children. Brooms, shovels, and other equipment used to clean cat cages and cat enclosures should be autoclaved or heated to 70°C for at least 10 min. While cleaning cages, animal caretakers should wear masks and protective clothing. Feline feces should be removed daily to prevent sporulation of oocysts (Dubey 2010).

At present, there is no vaccine to prevent toxoplasmosis in humans (Hill et al. 2005) but in sheep, a vaccine against toxoplasmosis intended to protect against *Toxoplasma*-induced abortion is

commercially available (Innes 2010). It contains live attenuated tachyzoites derived from Strain 48 originally developed in New Zealand although vaccination did not prevent fetal or placental infection (O'Connell et al. 1988). The vaccine is available as TOXOVAX® (MSD Animal Health New Zealand). Nevertheless, recent reports reveal that immunization with live-attenuated whole organisms of non-reverting mutants has been shown to induce remarkably potent immune responses associated with control of acute and chronic toxoplasmosis. In view of that, the non-cyst-forming mutants are promising new tools for the development of veterinary vaccines against *T. gondii* infection (Zhang et al. 2015).

## 1.7. Oocyst survival and dissemination

Oocyst number and viability are important (Torrey & Yolken 2013) since these factors enhance the probability of transmission to intermediate hosts such as birds, rodents, and humans (Robert-Gangneux et al. 2015). Study reports suggest that approximately 1% of cats are shedding oocysts at any given time and continue to excrete oocysts for a median of 8 days (1-3 weeks following primary infection) with a total of up to 55 million oocysts per day (Dabritz & Conrad 2010; Dubey 2010; Jones & Dubey 2010). The total number of oocysts shed by a single cat varies widely from 3 to 810 million (Dabritz & Conrad 2010). Contamination of the environment by oocysts is widespread as domestic cats and other members of the Felidae shed oocysts (Hill & Dubey 2002). The oocysts are remarkably stable, especially if deposited in shady, moist, and temperate conditions. According to Yilmaz and Hopkins (1972), oocysts remained viable during a 13-month experiment under outdoor shaded conditions with a mean air temperature of 19.5°C. Sporulated oocysts buried in moist soils maintained infectivity for up to 18 months (Frenkel et al. 1975). Oocysts maintained experimentally at 4°C in seawater or freshwater remained infective to mice for 24 and 54 months, respectively (Dubey 1998b; Lindsay & Dubey 2009). Dubey (1998b) further reported that the duration of infectivity decreases with increasing temperatures. While sporulated oocysts maintained infectivity for at least 200 days in the temperature range of 10-25°C, the infectivity persisted only for 1 month at 35°C, for 1 day at 45°C and non-infective after 1 minute at 60°C (Dubey 1998b). Oocysts also survived for over a year in vials of 2% sulfuric acid at 4°C (Frenkel & Dubey 1972). Since almost

all of these studies were terminated while at least some of the oocysts were still viable, the outer limit of viability for *T. gondii* oocysts deposited in various environmental conditions is currently unknown (Torrey & Yolken 2013).

Studies using chemical exposure experiments indicate that neither sodium hypochlorite nor ozone effectively inactivated *T. gondii* oocysts, even when used at high concentrations (Wainwright et al. 2007b). Moreover, a minimum UV exposure dose of 1,000 mJ/cm$^2$ may be required for either Pulsed UV or Continuous UV treatment of water to increase the probability of consistent and complete oocyst inactivation (Wainwright et al. 2007a). Although, radio frequency induced thermal inactivation of ≥ 60°C for 1 minute was able to inactivate oocysts in an initial experiment, subsequent experiments conducted under similar conditions failed to attain the same results (Wainwright et al. 2010). Altogether, these findings suggest that most public water treatment processes involving chemical or physical methods do not seem to disinfect *T. gondii* oocysts from water supplied for consumption.

## 1.8. Impact of waterborne transmission

The waterborne outbreak that occurred in British Columbia (Canada) in 1995, resulted in 110 cases of human acute *Toxoplasma* infection following which, a case-control study conducted in the area have estimated that between 2894 and 7718 individuals in Greater Victoria were infected (Bowie et al. 1997). The source was municipal drinking water, probably contaminated by cougar and/or domestic cat feces (Aramini et al. 1998; 1999). However, this was not the first time ever that toxoplasmosis has emerged as a waterborne outbreak since earlier documents were available.

The first recorded toxoplasmosis outbreak occurred in Panama in 1979 involving 31 military troops from USA (Benenson et al. 1982), and associated with creek water contaminated by oocysts excreted by jungle cats as a source. Another outbreak where 290 human cases reported in Brazil, involved an unfiltered water reservoir (Keenihan et al. 2002). Additionally, Bahia-Oliveira et al. (2003) found a high *T. gondii* prevalence in a Brazilian community, related to drinking unfiltered water. Likewise, de Moura et al. (2006) reported an investigation of toxoplasmosis outbreak in Santa Isabel do Ivai, Parana´ state, Brazil, where unfiltered,

municipally treated water was the epidemiologically implicated source of *T. gondii* dissemination. This outbreak involved at least 426 people. Two outbreaks of acquired ocular toxoplasmosis involving 248 people (Balasundaram et al. 2010) and 182 seropositive cases (Palanisamy et al. 2006) have occurred in India in which municipal drinking water was suspected as a source for infection in both occasions. Moreover, oocysts in chemically disinfected river water were associated with cases of severe toxoplasmosis observed in immunocompetent military personnel returning from French Guyana (Darde et al. 1998). Likewise, Hall et al. (1999) identified drinking water as the probable source of infection among Jains, a community based on Hinduism in India (strict vegetarians, so infection via undercooked meat is highly improbably). A serological study conducted among pregnant women in Turkey had revealed that prevalence was strongly associated with drinking water (Ertug et al. 2005). In Poland, *T. gondii* was found in water from shallow wells located on farms of poor hygienic state than those from deep pump-operated wells, thus highlighting the potential risk of waterborne toxoplasmosis in rural environments (Sroka et al. 2006). Some studies have also identified the presence of other risk factors than water suggesting the importance of oocysts in the transmission of *Toxoplasma* infections to humans. According to Cook et al. (2000), contact with soil was identified as a strong risk factor in a European multicenter case-control study, accounting for 6-17% of primary infections in humans. Likewise, a case-control study in Norway indicated that eating unwashed raw vegetables or fruits was associated with an increased risk of primary infection during pregnancy (Kapperud et al. 1996).

## 1.9. Detection of *Toxoplasma* oocysts in environmental samples

### 1.9.1. Conventional techniques

Direct detection of *Toxoplasma* oocysts in environmental samples is possible but is hampered by the low density of oocysts within the sample and the large amount of other material that hinders detection (Dumetre & Darde 2003). Conventionally, detection of protozoa such as *Cryptosporidium* and *Giardia* in water requires their concentration from large volume of water samples by filtration or centrifugation, isolation from concentrated particulates using immunomagnetic separation (IMS) or other methods, and detection by immunofluorescence

microscopy, infection of cultured cells, biochemistry, bioassays, molecular techniques, or combinations of these (Dumetre & Darde 2003; Zarlenga & Trout 2004). However, there is no rapid detection method for *T. gondii* oocysts in water or other environmental samples since there are no commercially available IMS techniques, no commercially available immunofluorescent staining reagents, and no standardized protocols (Dumetre & Darde 2003; Jones & Dubey 2010).

Identification of oocysts from environmental samples has included differential flotation and mouse inoculation (Isaac-Renton et al. 1998), filtration using membrane filters, and bioassays (de Moura et al. 2006), and microscopy or molecular methods have been used for detection. Attempts to develop efficient IMS techniques for the isolation of *T. gondii* oocysts and sporocysts in water have been made recently (Dumetre & Darde 2005; 2007). However, both the oocyst and sporocyst IMS assays displayed poor specificity due to cross reactions of antibodies with background debris and the sporocyst walls of *Hammondia hammondi*, *H. heydorni*, and *Neospora caninum* (Dumetre & Darde 2007). Moreover, Hohweyer et al. (2016) developed an IMS Toxo tool coupled to qPCR assay, which is for the detection and quantification of *T. gondii* oocysts in environmental samples. Although some IMS results appeared promising in clean samples (in a range from 32 to 56%), the recovery efficiency decreased substantially to below 2% when used with samples containing debris (Hohweyer et al. 2016).

Methods involving sucrose flotation or flocculation were evaluated for isolation of *T. gondii* oocysts in water samples prior to DNA extraction (Kourenti & Karanis 2004; 2006; Sroka et al. 2006; Villena et al. 2004), both methods resulted in oocyst losses leading to poor recovery rates (Jones & Dubey 2010). Alternatively, PCR is becoming a favored technique for detection of *T. gondii* oocysts in water (Jones et al. 2000; Kourenti & Karanis 2004; 2006; Schwab & McDevitt 2003; Sroka et al. 2006; Villena et al. 2004) over the conventional mouse bioassay (Isaac-Renton et al. 1998; Villena et al. 2004), as it reduces the detection time from weeks to 1-2 days. PCR assays targeting different genes have been developed for the detection of *T. gondii* in biological samples. Of these target genes, the 35-copy B1 gene (Burg et al. 1989), the 200 to 300-fold repetitive 529-bp DNA fragment (Homan et al. 2000), and the 110-copy internal transcribed spacer (ITS-1) or 18S rDNA gene sequences (Payne & Ellis 1996; Tenter et al. 1994) are often

used. The application of qPCR that targets the repetitive DNA sequences has shown high sensitivity (detecting about 1 parasite genome equivalent) according to recent results (Calderaro et al. 2006; Contini et al. 2005; Edvinsson et al. 2006; Jauregui et al. 2001; Reischl et al. 2003). Likewise, qPCR approach has gained popularity not only for detecting, but also quantifying *T. gondii* in biological samples (Bell & Ranford-Cartwright 2002; Contini et al. 2005) since its sensitivity is superior to nested-PCR assays (Contini et al. 2005) detecting over a range of 6-7 orders of magnitude (Contini et al. 2005; Jauregui et al. 2001; Lin et al. 2000). Nevertheless, there are several unresolved issues regarding the effectiveness of PCR detection of *T. gondii* oocysts in water. Although they have been developed for detection of *T. gondii* in clinical specimens (Switaj et al. 2005), use of PCR for detection of oocysts in water samples is challenging, possibly because of expected high concentrations of PCR inhibitors and low numbers of *T. gondii* oocysts in environmental samples (Villena et al. 2004). In view of the effects of PCR inhibitors, different pre-PCR processing strategies to circumvent PCR inhibition to allow accurate and precise DNA amplification have been described (Rådström et al. 2004) . Furthermore, a competitive internal amplification control (CIAC) was developed to enable identification of false negative PCR results (Hoorfar et al. 2004a; Hoorfar et al. 2004b) and has been effectively used in studies involving detection of *T. gondii* DNA in tissue and water samples (Opsteegh et al. 2010; Wells et al. 2015). According to Wells et al. (2015), the inclusion of CIAC to monitor the PCR assay process facilitated the detection of false-negative results due to PCR inhibitors that was solved by reducing the template volume afterwards.

The use of Loop Mediated Isothermal Amplification (LAMP) for detection of *Toxoplasma* in water has also been explored by some research groups with apparently successful results (Gallas-Lindemann et al. 2013; Mahmoudi et al. 2015; Sotiriadou & Karanis 2008) and is described as “sensitive, specific, rapid and cost effective”. However, known problems with the use of LAMP for detection of contamination of *Cryptosporidium* and *Giardia* in water samples, led us to using a method in which positive findings can be checked by sequencing.

Table 2. Some selected commonly used oocyst/cyst concentration and detection methods

| Method (parasites applied to) | Approximate efficiency | Comments | References |
|---|---|---|---|
| Concentration methods | | | |
| Chemical flocculation and centrifugation (*Toxoplasma*) | > 80% | Applied only in non-turbid water | Kourenti et al. (2003) |
| Chemical flocculation (*Cryptosporidium* and *Giardia*) | Alum (59%)<br><br>Calcium carbonate (65-77%) | | Vesey et al. (1993)<br><br>Campbell et al. (1994); Shepherd and Wyn-Jones (1996) |
| Frontal filtration with cellulose acetate membranes or polycarbonate membranes (include parasites used on here) | 49% | Useful in small volume application and turbid water | Nieminski et al. (1995) |
| Cartridge filtration (include parasites used on here) | 12% | Oocyst elution time consuming; costly | Nieminski et al. (1995) |
| Capsule filtration using Gelman Envirochek standard (*Giardia*) | 50% | Useful for non-turbid water, but reduced to <1% for high turbid; costly | Simmons III et al. (2001) DiGiorgio et al. (2002) |
| Capsule filtration using IDEXX Filta-Max system (*Giardia*) | Up to 70% | Large volume, tap water, seeded matrix, but costly | McCuin and Clancy (2003) |
| Continuous separation channel centrifugation (*Cyclospora* and *Toxoplasma*) | 73-99.5% (tap water); 68.5-100% (turbid water) | A secondary method of separation or purification required to concentrate some pathogens | Borchardt et al. (2009) |
| Membrane filtration using Envirocheck HV filters (*Toxoplasma*) | 2-25% | Not checked for repeatability and robustness | Shapiro et al. (2010b) |
| Hollow fiber ultrafiltration (*Toxoplasma* ) | 2-30% | Efficient for size-based separation (from viruses to protozoa) but no recovery in turbid water even with PCR detection | Morales-Morales et al. (2003); Shapiro et al. (2010b) |

| | | | |
|---|---|---|---|
| Immunomagnetic separation (IMS) (*Toxoplasma*) | 32-56% in clean samples | Displaying poor specificity and cross reactivity;<br>Poor recovery (<2%) in samples with debris | Dumetre and Darde (2005);<br>Dumetre and Darde (2007);<br>Hohweyer et al. (2016) |
| IMS (*Cryptosporidium* and *Giardia*) | Up to 100% | Seeded environmental waters | Bukhari et al. (1998);<br>Rochelle et al. (1999) |
| Detection approaches | | | |
| Molecular methods (*Toxoplasma*-related coccidian oocysts) | Detection limit of 10 oocysts | Problems with PCR inhibitors | Dumetre and Darde (2003) |
| Centrifugation and qPCR detection (*Toxoplasma*) | | Problems with PCR inhibitors | Wells et al. (2015) |
| IMS-Toxo with qPCR (*Toxoplasma*) | | | Hohweyer et al. (2016) |
| qPCR and mouse bioassay (*Toxoplasma*) | QPCR (8%);<br>Mouse bioassay (0%) | High sensitivity and specificity of qPCR | Villena et al. (2004) |
| Loop-mediated isothermal amplification (LAMP) (*Toxoplasma*) and nested PCR | LAMP (48%);<br>nested PCR (13.5%) | Isothermal (no temperature cycling), rapid. | Sotiriadou and Karanis (2008) |

In addition, development of a quantitative reverse transcription-PCR method to differentiate between viable and nonviable oocysts detected in environmental samples has been suggested over the mouse bioassay that takes too long for public health sentinel purposes (Villena et al. 2004). In addition, there has been more progress as new primers were developed to distinguish between *H. hammondi* and *T. gondii* in samples containing both parasites (Schares et al. 2008; Sreekumar et al. 2005).

### 1.9.2. Novel techniques used in this research

#### *1.9.2.1. Microfluidic chips for separation of waterborne parasites*

Microfluidics refers to the science and technology of systems that process or operate small volumes of fluids that vary from microliters ($10^{-6}$ liters) to femtoliters ($10^{-15}$ liters) in networks of channels with dimensions of ~5-500 µm (Weibel & Whitesides 2006; Whitesides 2006). The technology itself has evolved over the past few decades from a molecular analysis aimed at enhancing separation performance through reduced dimensions, into a diverse field influencing an ever-expanding range of disciplines(Tian & Finehout 2009). As a result, microfluidic-based research has made significant advances and become very much a 'hot topic' recently (Yi et al. 2006) having features that have attracted users in biology, chemistry, engineering and medicine (Weibel & Whitesides 2006). In particular, microfluidic chip-based systems for biological cell studies have attracted significant attention owing to their advantages including low reagent and power consumption, short reaction time, portability for in situ use, low cost, versatility in design, and potentials for parallel operation and for integration with other miniaturized devices (Yi et al. 2006). Microfluidic biochips-based technologies make the development of fast, sensitive and portable diagnostic tools possible, thus promising rapid and accurate detection of a variety of pathogens. Moreover, they offer real alternatives capable of filling the technological gap compared to conventional identification methods, which are tedious, cost intensive and time consuming in nature (Mairhofer et al. 2009).

Recently, the number of applications of microfluidics in biology, analytical biochemistry, and chemistry has grown as a range of new components and techniques have been developed and

implemented for introducing, mixing, pumping, and storing fluids in microfluidic channels. Furthermore, microfluidics offers structures with length scales that are comparable to the intrinsic dimensions of prokaryotic and eukaryotic cells, collections of cells, organelles, and the length scale of diffusion of oxygen and carbon dioxide in tissues (every cell, *in vivo*, is no more than ~100 µm from a capillary) (Weibel & Whitesides 2006). These characteristics make microfluidics particularly useful in studying biology and biomedicine (Sia & Whitesides 2003).

The significance of applying miniaturized (microfluidic) devices or so-called 'laboratory on a chip' (LOC) as a tool for a rapid monitoring of microbial pathogens in the environment has been described by (Liu & Zhu 2005). The obvious advantage of using one simple and rapid approach to integrate sample trapping has supported this and concentration steps with whole-cell immunological assay in a silicon-based LOC consisting of micro-chambers, micro-channels and filter weirs (Zhu et al. 2004). Through the filter weirs, microbial cells (i.e. *Cryptosporidium parvum* and *Giardia lamblia*) which are larger in size than the weir gap (1-2 µm) are predicted to be effectively trapped, concentrated and labeled during sample injection together with staining solution containing fluorescently labeled antibodies (Liu & Zhu 2005).

Bridle et al. (2012) reviewed the available standardized technologies that use a wide range of miniaturized systems capable of detection of *Cryptosporidium* oocysts demonstrated by proposed methods. In order to ease the detection process of waterborne pathogens, the potential of microfluidics was investigated using spiral channels designed for separating particles, in a single device and without any external forces or additional buffer, depending on their size at high flow rates (Jimenez et al. 2017). The study initially focused on the impact of the channel length, flow rate, particle concentration and size on the separation efficiency of polystyrene beads of relevant sizes (~4-7µm). The system was then tested with viable and non-viable oocysts of *Cryptosporidium parvum* with an average size around 4-5µm and efficiently separated and concentrated them.

Bridle et al. (2014) reported recent advances in microfluidics based systems for the monitoring of waterborne pathogens with emphasis on existing, commercial and state-of-the-art systems and research activities in laboratories worldwide. In general, the authors recommend integration of optical and electrical detection components as essential to realize a fully

automated system for wider exploitation of microfluidic devices. Furthermore, multiplexed detection systems capable of classifying a range of pathogens from the different kingdoms (virus, bacteria, and protozoa) ideally beyond the species level would be highly desirable and is perhaps the ultimate goal of any detection system for waterborne pathogens (Bridle et al. 2014).

Additionally, Ganz et al. (2015) recently demonstrated the efficiency of inertial separation protocol using microfluidic device in concentrating *Giardia duodenalis* cysts and eliminating nonspecific particles. The microfluidic chip was successfully integrated into a detection method for *G. duodenalis* cysts on lettuce samples and was shown to separate the parasites from food particles. The reduction in background particles resulted in increased speed and accuracy in sample analysis. Nevertheless, despite the obvious promise of these LOC or microfluidic devices, they are not widely used in the field. This seems largely to be because although they work successfully at the lab bench scale, and proof of principle has been established, efforts to bring the techniques into the environmental analysis lab or the field have been relatively unsuccessful with problems with contamination, blockage, and inhibitors. Thus, despite there clearly being tremendous advantages and potentials for detecting waterborne pathogens using microfluidic platforms, they have not yet been applied to separating oocysts of *T. gondii* from water and currently no reports on the use of such devices for analyzing samples for these parasites is currently available.

The Trilobite® technology, developed as a three-layered microfluidic chip with microstructures, is intended for separation and concentration of particles in different kinds of fluids. It has been successfully used for a range of applications, mostly at the lab scale, including dewatering of algae (Hønsvall et al. 2016). This microfluidic chip (Figure 4) can be scaled up and down depending on the size of the particles to be separated. It is a kind of cross-flow (tangential) filtration device, which avoids accumulation of particles on the filter surface unlike in dead-end filtration. The chip is designed to work based on a hydrodynamic principle and a cross-flow effect, in which suspended particles are continuously concentrated and/or separated as the liquid flow runs through it. The fluid that contains particles enters the inlet at one end of the chip (blue arrow in Figure 4), and exits at two outlets at the other end of the chip. At one outlet,

referred to as the *concentrate*, concentrated particles are collected (red arrow in Figure 4); and at the other outlet, referred to as the *permeate*; a purified fraction is collected (green arrow in Figure 4). The purified fraction contains no, or only small particles, depending on the gap size of the structures within the chip (Hønsvall 2017).

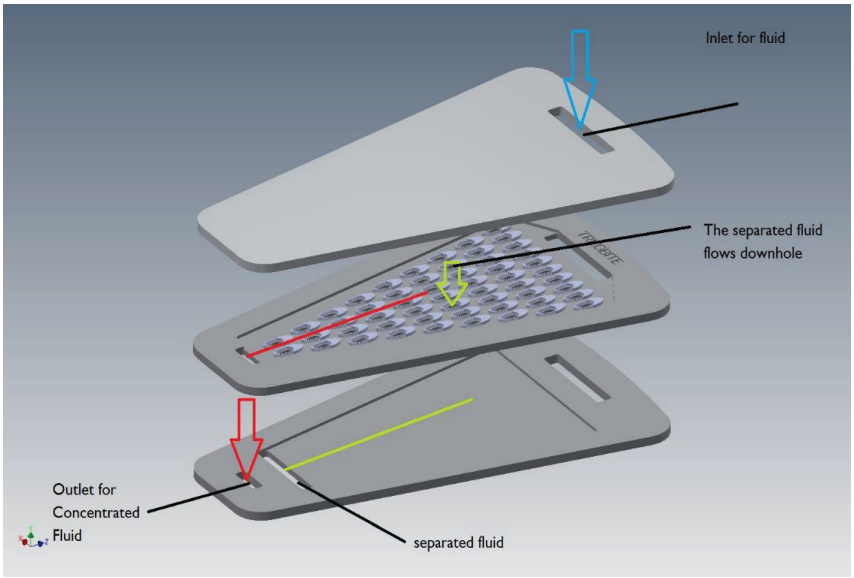
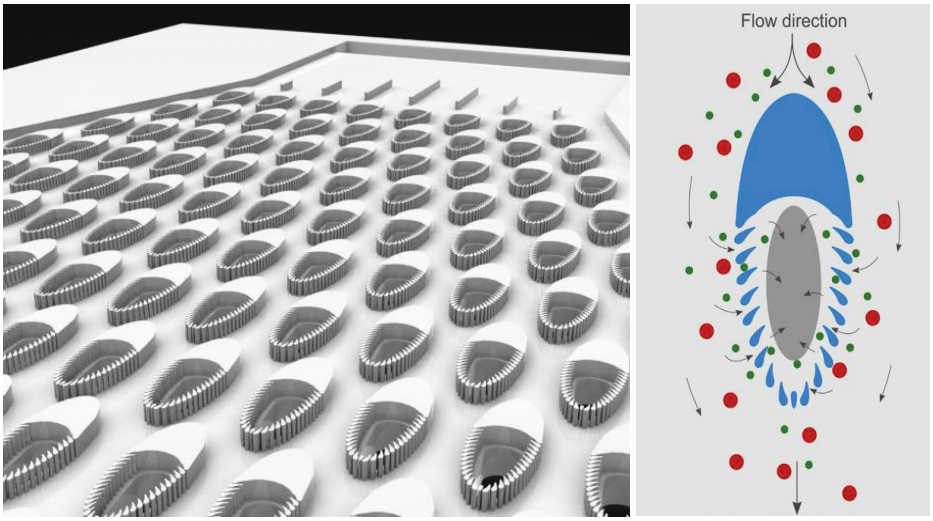



Figure 4. The Trilobite® chip (picture acknowledgement, Erik Bentzen, Trilobite AS)




Figures 5. Inside the Trilobite® chip (picture acknowledgement, Erik Bentzen, Trilobite AS)

Within the upper layer of the chip is a field of microstructure-units (Figure 5, right) that play a key role for particle separation. The chip 'trilobite' is derived from the extinct trilobite marine animal that had structural resemblance to the micro-units of the chip. Each unit consists of a solid "head", and several turbine blades, or "feet", along the rest of the body (Figure 5, left). Inside the trilobite unit, a hole leads down to the underlying layer. The flow through the chip is unidirectional, and meets the "head" of the trilobite unit first, and then flows along the body. Some fluid then flows between the blades ("feet") and into the hole (as in a crossflow), while the remainder will flow past the unit, and forward through the field of the upper chip layer. The gap size between the blades of the trilobite unit defines the size of the particles that are separated out. Particles smaller than the gap size; can pass through the gaps and into the layer underneath. Particles larger than the gap cannot enter between the blades, but flow past outside the units. The Trilobite® chip allows for continuous treatment of fluid by separating and concentrating particles without requiring any replacement, as there is no dead-end that accumulate particles within the chip (Hønsvall 2017).

The ability of Trilobite® chips to concentrate and separate particles is of clear application for many biological samples. Often when screening environmental samples for contamination, the situation is that there are relatively few particles, often biological, that are suspended in large volumes of water or other media and should be detected or quantified. In sample preparation, the sample volume usually need to be reduced prior to analysis to enable detection, but the method should ensure that the target particles are not lost in the process. When water samples are being analyzed for unwanted or harmful pathogens, such as cysts or oocysts of waterborne protozoa, the samples need to be concentrated before they are analyzed. The nature of the chip makes it suitable for continuous sample collection, for example by incorporating the system in inlet- and outlet pipes in a water treatment plant for continuous water sampling, to concentrate any possible particles. Furthermore, the chip can be assembled into larger complexes, containing multiple trilobites in multiple layers, and up-scaled to handle large volumes (Hønsvall 2017).

### *1.9.2.2. Nucleic acid aptamers for detection of parasites*

Antibodies, one of the most popular biomaterials for molecular recognition, have been widely used for more than three decades in various fields, especially in medical diagnostics and therapeutics (Jayasena 1999; Kim & Gu 2014). However, the recent awareness that nucleic acids can assume stable secondary structures and that they can be easily synthesized and functionalized, has led to the idea of selecting new nucleic acid ligands designated as aptamers (Tombelli et al. 2007). The term aptamer is derived from the Latin word ''*aptus*''- which means 'fitting' (Ellington & Szostak 1990) and the Greek word ''*meros*'' meaning 'particle' (Stoltenburg et al. 2007) for these nucleic acid-based ligands. This new class of oligonucleotide-based molecular recognition elements has more recently emerged as a rival of antibody-based methods (Kim & Gu 2014). Aptamers are short single-stranded nucleic acid oligomers (ssDNA or RNA) or peptide molecules that can bind to their targets with high affinity and specificity due to their specific three-dimensional structures (Song et al. 2012) characterized by stems, loops, bulges, hairpins, pseudoknots, triplexes, or quadruplexes (Patel 1997; Stoltenburg et al. 2007).

The idea of using aptamers as affinity molecules was first described in 1990 when two laboratories independently developed the technique of selection. While the Gold laboratory used SELEX (Systematic Evolution of Ligands by Exponential enrichment) for the process of selecting RNA ligands against T4 DNA polymerase (Tuerk & Gold 1990), the Szostak laboratory, coining the term in vitro selection, reported selecting of RNA ligands against various organic dyes (Ellington & Szostak 1990).

Since its discovery, aptamer technology has received considerable attention in scientific communities and widely adopted as a tool for the development of research reagents, and appears promising for the generation of diagnostic and therapeutic agents (Lee et al. 2004; Song et al. 2008). As a result, DNA and RNA aptamers have been identified as binding tightly to a broad range of target molecules such as drugs, proteins or other organic or inorganic molecules with high affinity and specificity (Clark & Remcho 2002; Jayasena 1999; Luzi et al. 2003; Patel & Suri 2000; You et al. 2003). Likewise, aptamers have also been shown to bind to whole cells or

microorganisms such as bacteria (Stoltenburg et al. 2007; Tombelli et al. 2007), especially with the development of rapid, automated, selection technologies (Song et al. 2008).

Aptamers often possess high affinity for their targets, which is derived from their capability of folding upon binding with their target molecule: they either can incorporate small molecules into their nucleic acid structure or be integrated into the structure of macromolecules such as proteins. The molecular recognition of aptamers results from intermolecular interactions such as the stacking of aromatic rings, electrostatic and van der Waals interactions, or hydrogen bonding with a target compound. In addition, the specific interaction between an aptamer and its target is complemented through an induced fit mechanism, which requires the aptamer to adopt a unique folded structure to its target (Hermann & Patel 2000a; Hermann & Patel 2000b; Patel 1997). Aptamers are also recognized for their remarkable specificity as they can discriminate targets based on inherent structural differences such as the presence or absence of a methyl or a hydroxyl group and the d- vs l-enantiomer of the target (Jayasena 1999).

Based on these properties of aptamers, the screening method (SELEX process), has been modified or evolutionarily changed over the years to develop aptamers with higher affinity and selectivity with more efficient, less time-consuming, or automatic ways (Kim & Gu 2014). The process starts with the synthesis of a single-stranded library of oligonucleotides; each oligonucleotide comprises a central region of random sequence flanked by a 5' and a 3' region of defined sequences. Generally, the starting round contains around $10^{15}$ individual sequences, a very large number that permits to generate a high probability of selecting an aptamer specific for the target of interest (Kim & Gu 2014; Song et al. 2012; Tombelli et al. 2005). The higher order structure of oligonucleotides is accomplished by changing intramolecular base pairing. This means that a random library is actually a library of three-dimensional (3D) structural DNA or RNA (Kim & Gu 2014; Patel 1997).

The SELEX procedure (Figure 6) involves the reiteration of successive steps consisting of selection (binding, partition, and elution), amplification and conditioning (Stoltenburg et al. 2007).

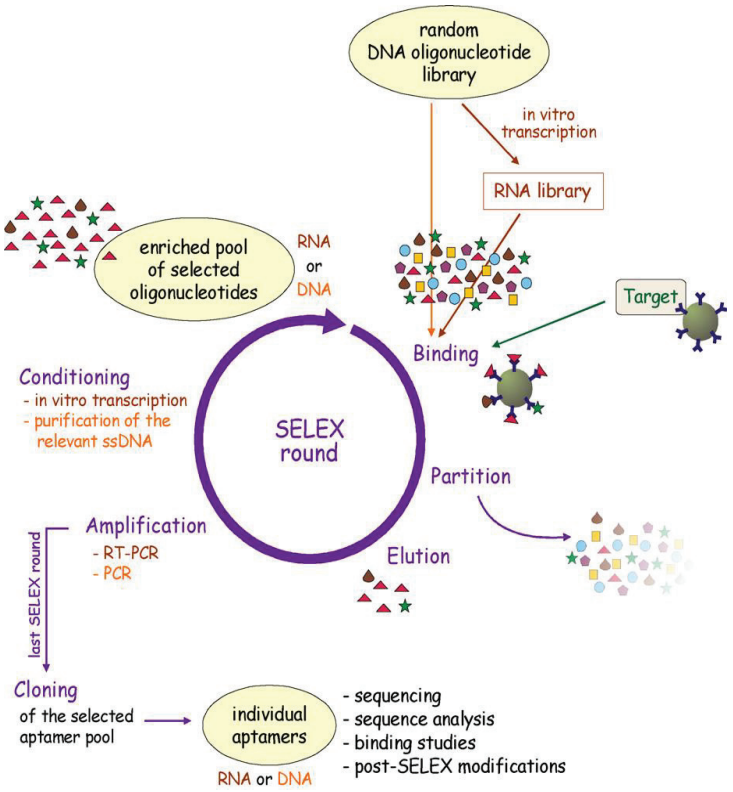



Figure 6. *In vitro* selection of target-specific aptamers using SELEX technology (Stoltenburg et al. 2007)

Therefore, a random sequence oligonucleotide library is incubated with a target of interest in a buffer of choice at a given temperature. During this step, a very small fraction of individual sequences tends to interact with the target, and these sequences are separated from the rest of the library by means physical separation technique (Jayasena 1999; Kim & Gu 2014; Stoltenburg et al. 2007). Typically, nitrocellulose filter partitioning (for protein target) or affinity chromatography (normally for small molecular target) are used (Kim & Gu 2014; Song et al. 2012; Tombelli et al. 2005).

Following partition, the target-bound oligonucleotides are eluted and subsequently amplified by PCR or RT-PCR. A new enriched pool of selected oligonucleotides is generated by preparation of the relevant ssDNA from the PCR products (DNA libraries) or by in vitro transcription (RNA

libraries). This selected oligonucleotide pool is then used for the next selection round (Stoltenburg et al. 2007; Tombelli et al. 2005). The enrichment of the high-affinity sequences at the expense of the low-affinity binders requires several iterations of the selection process carried out under increasing stringent conditions. The progress of the enrichment of high-affinity binders can be determined by carrying out binding analysis of enriching populations against the target (Jayasena 1999). To achieve high degree of aptamer specificity during selection, the population of aptamers bound to the target is subjected to affinity elution with structural analogs and the sequences eluted are discarded. This process called "counter-SELEX" also referred as 'negative selection' often applied to eliminate ligands that have ability to bind the target as well as closely related structural analogs of the target (Tombelli et al. 2005) and retain only target specific ligands. Moreover, once affinity saturation is achieved after several rounds of selection and amplification, the enriched library is cloned and sequenced to obtain the sequence information of each member. Individual sequences are further characterized on the basis of their ability to bind to the target. Usually, the majority of individual sequences, >90%, in an enriched library are "winners", aptamers that bind to the target used for selection (Jayasena 1999).

The number of cycles required for aptamer identification is usually dependent on the degree of stringency imposed at each round as well as on the affinity of interaction between the target and the aptamers (Jayasena 1999; Tombelli et al. 2005). Generally, around 8-15 cycles of affinity selection and amplification are needed before selecting an oligonucleotide population that is dominated by those sequences which bind the target best (Tombelli et al. 2005). Once the sequence is identified, an aptamer is produced by chemical synthesis. In general, a researcher could be able to accomplish one cycle of SELEX every 2 days. Including cloning and sequencing, a typical SELEX experiment may take approximately 2-3 months (Jayasena 1999). However, automation of the methods involving RNA selection enabled to shorten the route from target to novel reagent within only a few days (Cox et al. 1998). The automated platform carries out the iterative SELEX process around the clock with little or no human intervention and intuition (Jenison et al. 1994) and has parallel processing capabilities to handle multiple SELEX experiments run on microtiter plates, allowing fast and high-throughput discovery of aptamers.

Nevertheless, the whole process in either of case is faster than the amount of time typically spent in generating a cell-line to produce a specific monoclonal antibody and purification of the antibody (Jayasena 1999).

Aptamers that come out of a SELEX experiment are full-length sequences containing the fixed sequences that were included to aid the amplification process. These full-length aptamers are generally 70-80 nucleotides long and can be truncated to eliminate nucleotide stretches that are not important for direct interaction with the target or for folding into the structure, which facilitates target binding. The identification of truncated aptamers restricted to the minimal target-binding domain requires some effort, but has been successfully carried out to obtain functional aptamers less than 40 nucleotides long. In the majority of cases, the fixed sequence regions used for primer binding are unimportant for aptamer function and can be eliminated. Technological advances have already been made to eliminate the requirement for the fixed regions in random sequence libraries used for the SELEX process, thereby producing short aptamer sequences (Jayasena 1999).

Aptamers can offer advantages over antibodies that make them very promising for analytical applications, in addition to their very important aspect of having an unlimited source of identical affinity recognition molecules (Kedzierski et al. 2013; Luzi et al. 2003; O'Sullivan 2002; You et al. 2003). The main advantage is the overcoming of the use of animals or cell lines for the production of the molecules. Although most antibody production starts in biological systems by inducing an immune response to the target molecule (Birch & Racher 2006), the resulting immune response can fail when the target molecule, i.e. protein, has a structure similar to endogenous proteins and/or when the antigen consists of toxic compounds. In contrast, aptamers are isolated by in vitro methods that are independent of animals and an in vitro combinatorial library can be generated against any target (Jayasena 1999). In addition, generation of antibodies in vivo depends on the animal immune system that selects the sites on the target protein to which the antibodies bind. The in vivo parameters can limit the identification properties of antibodies to recognition of targets only under physiological conditions. These results in the extent to which antibodies can be functionalized and applied (Tombelli et al. 2005; 2007).

Moreover, in the aptamer selection process, manipulations can be made to obtain aptamers that bind a specific region of the target and with specific binding properties in different binding conditions (Jayasena 1999). Owing to this property, it is possible to modify aptamers or link them to labeling molecules (dyes) to immobilize them on the surface of beads or substrates for different applications (Kim & Gu 2014). This provides a tremendous advantage for diagnostic and biosensor applications, because the uniform alignment and immobilization of receptors are very important in analytical systems. Labeling with signal-generating molecules is a common method for signal production or amplification in biosensors (Jayasena 1999; Kim & Gu 2014). Likewise, modifications introduced in the aptamer during chemical synthesis can enhance the stability, affinity, and specificity of the molecules (Ferreira & Missailidis 2007). Often the kinetic parameters of aptamer-target complex can be changed for higher affinity or specificity (Jayasena 1999). The dissociation constant (Kd) of aptamers to their targets is typically from the micromolar to picomolar range, which is comparable to, or sometimes even better than, the affinity of antibodies to their antigens (Kim & Gu 2014).

As mentioned above, in vitro chemical synthesis makes it possible for the production of purified aptamers with low cost and without batch-to-batch variation commonly encountered when using antibodies. In vitro selection is also appropriate for development of a high-throughput or automated system for aptamer isolation (Kim & Gu 2014). An additional advantage over antibodies can be seen in the higher temperature stability of aptamers; in fact, antibodies are large proteins sensitive to temperature and they can undergo irreversible denaturation. In contrast, aptamers are very stable and can recover their native active conformation after denaturation (Kim & Gu 2014; Mascini 2008; Tombelli et al. 2005) and hence maintain a much longer shelf life. This excellent flexibility of the aptamer structure is useful in developing new types of sensing methods. Additionally, immunogenicity is rare or non-existent when aptamers are applied to an in vivo system. Aptamers also enable easy control of bioavailability and delivery durability due to their small size (generally less than 20 kDa). This facilitates their penetration into cells and their delivery or immobilization in any medium, similar to liposomes (Kim & Gu 2014). Moreover, nucleic acid aptamers can be easily amplified by polymerase chain reaction (PCR), unlike other synthetic receptors such as antibodies, oligopeptides, or molecular

imprinted polymers (MIP), and can be expressed inside cells containing a plasmid that includes the aptamer sequence (You et al. 2003). Owing to all these characteristics, aptamers have been used in numerous investigations, as therapeutic (White et al. 2000) or diagnostic tools (Brody & Gold 2000) and for the development of new drugs. Moreover, aptamers have been recently used in analytical chemistry applications, as immobilized ligands or in homogeneous assays.

The primary limitation on the use of aptamers (mainly RNA aptamers) in bioanalytical methods has been their nuclease sensitivity, which is very critical for their use in vitro and in vivo applications (Famulok et al. 2000). However, it has been shown that the stability of such molecules can be improved by chemical modification of the ribose ring at the 2'-position (Pieken et al. 1991). A different approach to stabilize aptamers comes from selection of RNA aptamers that bind to stereoisomers of an intended target molecule, followed by chemical synthesis of the mirror image of the selected sequences. The mirror-image aptamer (L-ribose) binds to the natural target molecule due to molecular symmetry created as an example and hence substitution of the natural D-ribose with L-ribose makes the mirror image aptamer very stable (Klussmann et al. 1996).

A slowly increasing body of work indicates that aptamers are potentially important candidates for detection of parasites. The first ever application of aptamers in parasitology was selection of RNA aptamers targeting live trypanosomes (Homann & Göringer 1999). A subsequent study investigated the uptake and intracellular transport of RNA aptamers in African trypanosomes, demonstrating that RNA could be used as 'piggy-back' molecules to target aptamer-coupled compounds / toxins to the lysosomal compartment of the parasite (Homann & Goringer 2001). Areas of application of SELEX technique within the context of identifying high-affinity ligands against parasite target molecules has been reviewed (Goringer et al. 2003). Further works considered applications of RNA aptamers as potential pharmaceuticals against infections with African trypanosomes (Goringer et al. 2006). Moreover, in vitro selection of DNA aptamers, which bind with high affinity and specificity to *Leishmania infantum* H3 (Ramos et al. 2010) and oocysts of *Cryptosporidium parvum* (Iqbal et al. 2015) revealed some promising future with applications of aptamers in parasitology.

### *1.9.2.3. Application of lectins for parasite isolation and detection*

Lectins (from the Latin *legere*: to choose) (Boyd & Shapleigh 1954) are sugar-binding proteins or glycoproteins of non-immune origin which agglutinate cells and/or precipitates glycoconjugates. They bear at least two-sugar binding sites, agglutinate animal and plant cells, and/or precipitate polysaccharides, glycoproteins and glycolipids. The specificity of a lectin is usually defined in terms of the monosaccharide(s) or simple oligosaccharides that inhibit lectin-induced agglutination (precipitation or aggregation) reactions (Goldstein et al. 1980).

Lectins have a very long history of investigations with its origin in the discovery of a plant toxin, "ricin," in 1888 by Peter Hermann Stillmark in Russia when he tested the reactivity of partially purified protein extracts towards red blood cells and observed some clumping of the cells. Since then, a number of plant lectins have been identified with their detailed biochemical properties and biological implications in relevance to interaction with animal cell surface components (glycoconjugates). They have been used as useful tools in cell biology, basic biochemistry, and glycan/glycoprotein separation, as well as clinical applications such as cell stimulants (e.g., mitogen) (Van Damme 2014).

The applications of lectins to clinical microbiology has been reviewed (Slifkin & Doyle 1990) where it was noted that lectins have helped in bacteriology, mycology, parasitology and virology studies. Moreover, a review (Aksoy & Üner 2003) indicates that the role of lectins in parasitology has been steadily increasing. Due to their abilities to bind specific glycoconjugates, lectins have been effectively used with various organisms (*Trypanosoma*, *Cyclospora*, *Leishmania*, *Toxoplasma*, *Entamoeba*, *Cryptosporidium*, *Giardia*, *Trichomonas* etc.) to correlate virulence with their surface properties.

Table 3. Examples of lectin use in parasitology.

| Lectin (abbreviations below)* | Parasite | Application | Reference |
|---|---|---|---|
| Con A, DBA, PNA, SBA, UEA, WGA | *Spirocerca lupi* | *In vitro* binding study to the outer surface of the nematode at different stages | Aroch et al. (2017) |
| Con A, WGA | *Plasmodium berghei* in the mid gut of vector | Carbohydrate recognition mechanism | Basseri et al. (2016) |
| Con A, MPL, PNA, RCA | Gastrointestinal parasites | Identification based on binding characteristics to eggs in feces | Colditz et al. (2002) |
| Con A, DBA, DSA, GS-I, GS-II, Jacalin, LCA, LEA, PHA-E, PHA-L, PNA, RCA-I, SBA, SJA, STA, UEA I, VVA, WGA, | *Trichomonas fetus* | Assessment of saccharide binding pattern | Doumecq et al. (2014) |
| AAL, CCL2, CGL2, CGL3, Lb-Tec2;,MOA | *Haemonchus contortus* | Inhibition of larval development | Heim et al. (2015) |
| AAL, ConA, GSL-I, LCA, LTL, PNA; SNA; | *Teladorsagia circumcincta* and *H. contortus* | Distinguish third stage larvae and adult worms based on binding pattern | Hillrichs et al. (2012) |
| PNA | *H. contortus* | Identification of eggs | Jurasek et al. (2010) Palmer and McCombe (1996) |
| WGA | *Cyclospora cayetanensis* | Isolation of oocysts from fresh produce | Robertson et al. (2000) |
| WGA | *Giardia lamblia* | Study on cysts and trophozoite surfaces | Ortega-Barria et al. (1990) |
| Con A, WGA, SBA | *Toxoplasma gondii* | Agglutination of tissue cysts | Slifkin and Doyle (1990) |
| Con A, RCA | *Leishmania species* | Agglutination of blood stages | |

* *Aleuria aurantia* lectin (AAL); Concanavalin A (ConA); *Coprinopsis cinerea* lectin-II (β-trefoil) (CCL-2); *Coprinopsis cinerea* Galectin (CGL2); *Coprinopsis cinerea* Galectin-like (CGL3); *Datura stramonium* agglutinin (DSA); *Dolichos biflorus* agglutinin (DBA); *Griffonia simplicifolia* lectin-I (GS-I); *Griffonia simplicifolia* lectin-II (GS-II); *Laccaria bicolor* lectin (Lb-Tec2); *Lens culinaris* agglutinin (LCA); *Lotus tetragonolobus* lectin (LTL); *Lycopersicon esculentum* agglutinin (LEA); *Maclura pomifera* lectin (MPL); *Marasmius oreades* agglutinin (MOA); *Phaseolus vulgaris* Erythroagglutinin (PhA-E); *Phaseolus vulgaris* leucoagglutinin (PHA-L); Peanut agglutinin (PNA); *Ricinus communis* agglutinin (RCA), , *Ricinus communis* agglutinin (RCA-1); *Solanum tuberosum* agglutinin (STA); *Sambucus nigra* agglutinin (SNA); *Sophora japonica* agglutinin (SJA); Soybean agglutinin (SBA)*; Ulex europaeus* agglutinin-I (UEA-I); *Vicia villosa* agglutinin (VVA); Wheat germ agglutinin (WGA).

They bind with receptors associated with their highest affinity. As a result, making use of lectins in parasitology (Table 3) could provide certain advantages in routine diagnosis. Due their fixed structure, commercial feasibility, activity in low concentrations, as well as their ability to distinguish the difference between various isolates, lectins are expected to have great application in parasitology in the coming years (Aksoy & Üner 2003).

An earlier study on *Giardia* cyst walls (Ward et al. 1985) indicated that wheat germ agglutinin (WGA) with affinity for GlcNAc bound to the polymeric form of chitin. The work revealed that out of 13 lectins with varying sugar specificities, only the N-acetyl-D-glucosamine (D-GlcNAc)-specific lectins: wheat germ agglutinin (WGA), succinylated wheat germ agglutinin (S-WGA), and *Lycopersicon esculentum* (tomato) agglutinin (LEA) bound specifically to *Giardia* cyst walls. Likewise, pretreating of cysts using purified chitinase completely abolished the binding of WGA, confirming the presence of polysaccharide chitin as a major structural component of the cyst wall and the sugar N-acetyl-D-glucosamine (D-GlcNAc) is part of chitin (Ward et al. 1985). However, subsequent studies (Gerwig et al. 2002) suggest that the binding moiety was more likely to be a novel beta (1-3)-N acetyl-D-galactosamine polymer (GalNAc). Although the speculations from these two works appear contradictory to each other regarding the precise carbohydrate composition, it seems still possible to consider that WGA binds to the walls suggesting the presence of beta-linked poly-N-acetyl hexosamine containing polysaccharides.

Although evidences of earlier research indicate the application and usefulness of lectins to the tissue stages (not oocysts) of *T. gondii*, their application has been suggested for the isolation or purification of the oocysts of related protozoan parasites in the absence of suitable monoclonal antibodies. Consequently, the application of WGA especially for isolation of *Cyclospora* oocysts (Robertson et al. 2000) offered a promising result probably due to the affinity of WGA for beta-linked poly-N-acetyl hexosamine containing polysaccharides.

On the other hand, oocyst walls of *Toxoplasma* have two distinct layers that resemble those of fungi (β-1, 3-glucan in the inner layer) or mycobacteria (acid-fast lipids in the outer layer) (Samuelson et al. 2013). Likewise, the presence of β-1, 3-glucan in the oocyst walls of *Toxoplasma gondii* and *Eimeria* as part of a trabecular scaffold in the inner layer of the oocyst

wall has been described (Bushkin et al. 2012). In addition, β-glucans are polysaccharides of D-glucose monomers linked by β-glycosidic bonds. They are a diverse group of molecules that can vary with respect to molecular mass, solubility, viscosity, and three-dimensional configuration. *N*-Acetyl glucosamine (*N*-acetyl-D-glucosamine or GlcNAc) is a monosaccharide derivative of glucose. It is an amide between glucosamine and acetic acid and it is significant in several biological systems (Wikipedia: Free encyclopedia; 2015). Hence, the presence of β-1, 3-glucan in the inner wall of *T. gondii* is suggestive for the presence of potential binding target and seems a candidate for some lectin binding assays. Given the possibility of lectin binding to the oocyst walls of related protozoan (*Cyclospora*) and the potential of β-1, 3-glucan (glucose derivative) present in the oocyst walls of *T. gondii*, it is hypothesized that some lectins could bind to the walls of oocysts. This provides an insight into the selection of panel of lectins for binding to the walls of *T. gondii* oocysts to choose the best lectins and use them for immunofluorescence staining and visualization as well as apply further steps of separation in the absence of monoclonal antibodies.

# 2. Aims and objectives of study

The aim of this work is:

'To investigate different novel approaches and relevant aspects for the development of robust, reproducible method for the detection of *Toxoplasma gondii* oocysts in water that can be applied beyond the research lab for real-life analyses'.

Within this topic, the following sub goals were addressed:

- To investigate the surface-binding properties of *T. gondii* oocysts with respect to available monoclonal antibodies and different lectins, and the effect of oocyst age on these properties.
- To investigate the potential of microfluidic chips (Trilobite®) for the concentration of *Toxoplasma* oocysts in water
- To investigate the potential of nucleic acid aptamers for the isolation and detection of *Toxoplasma* oocysts, and to contribute towards preliminary attempts to develop this technology for this purpose
- To investigate the potential for separation of *Toxoplasma* oocysts using a lectin bound to magnetic beads
- To prove the applicability of Lectin-Magnetic separation (LMS) for detecting *Toxoplasma gondii* in environmental water samples.
- To identify, investigate, and address possible challenges in bringing LMS technique from the research lab to the analytical bench
- To use the technique developed in a pilot study of real life samples for investigating the contamination of drinking water sources in Norway with *Toxoplasma*

# 3. Summaries of individual papers

**Paper I**

**Surface binding properties of aged and fresh (recently excreted) *Toxoplasma gondii* oocysts.**

In this paper, we investigated the binding properties of the surfaces of aged (10 years) and fresh (recently excreted) oocysts of *Toxoplasma gondii* using monoclonal antibody (mAb) and lectin-binding assays. Fresh oocysts bound a wall-specific mAb labelled with fluorescein isothiocyanate while aged oocysts did not. In contrast, the walls of aged oocysts bound a lectin (wheat germ agglutinin, WGA), but not the walls of fresh oocysts. Exposure of oocysts to detergent solutions or trypsin did not affect the binding properties of the walls of the oocysts. However, exposure of fresh oocysts to acidified pepsin enabled labelling of the walls with WGA, presumably due to the relevant moieties on the oocyst walls becoming exposed. WGA binding, but not mAb binding, was partially abrogated with periodate exposure. These findings revealed a significant difference in the binding properties of oocyst walls from "aged" and "fresh" oocysts. The results are of relevance when considering technologies for isolating or detecting *T. gondii* oocysts in environmental samples based on oocyst surface properties, as used for other protozoan parasites. These results suggest the possibility of developing a WGA-based separation procedure for isolating *Toxoplasma* oocysts from environmental matrices, in which pepsin pre-treatment should be included to ensure that both fresh and aged oocysts would be isolated.

**Paper II**

**Lectin-magnetic separation (LMS) for isolation of Toxoplasma gondii oocysts from concentrated water samples prior to detection by microscopy or qPCR.**

This paper describes the proof-of-principle on the use of lectin-magnetic separation (LMS) for isolating *T. gondii* oocysts from water sample concentrates, with subsequent detection by microscopy or molecular methods. Four different types of magnetic beads coated with wheat erm agglutinin (WGA) were tested for capture of oocysts from clean or dirty water samples.

Dynabeads (Myone T1 and M-280) consistently provided mean capture efficiencies from 1 mL clean water in excess of 97%. High recoveries were also found with Tamavidin beads (in excess of 90%) when LMS was used for capture from a small (1 mL) volume. Dissociation (required for detection by microscopy) using 0.1N hydrochloric acid (HCl), as standard in IMS, was not successful, but could be achieved using a combination of acidified pepsin (AP) and N-acetyl D-glucosamine. Although simple centrifugation was as effective as LMS when concentrating high numbers of oocysts from clean water, LMS provided superior results when oocysts numbers were low or the water sample was dirty. Application of LMS integrated with qPCR enabled detection of 10 oocysts per 10 mL dirty water sample concentrate. These findings indicate that LMS with WGA coupled to magnetic beads could be an efficient isolation step in the analysis of water sample concentrates for *T. gondii* oocysts, with detection either by microscopy or by qPCR.

**Paper III**

**Use of Lectin-Magnetic Separation (LMS) for detecting *Toxoplasma gondii* oocysts in environmental water samples.**

This paper describes investigations on the application of lectin-magnetic separation (LMS) for isolating *Toxoplasma* oocysts from environmental water samples for subsequent detection by microscopy or molecular methods, and optimization for routine use in a water-analysis laboratory. In this work, initial studies attempted to apply the LMS technique to samples that had already been analyzed for contamination with *Cryptosporidium* and *Giardia* using standard methods, and the supernatant following immunomagnetic separation (IMS) retained. Mucilaginous material in these samples, which was thought to be exudate from bacterial (*Pseudomonas* and *Bacillus* species) and fungal overgrowth, affected use of the beads. Thus, such samples (that had been stored at room temperature and unwashed) are unsuitable for use with LMS or other magnetic bead-based methods. If such samples are to be analyzed using LMS, then they should be washed and kept refrigerated and analyzed as soon as possible post-IMS.

Experiments on AP-treatment of *Toxoplasma* oocysts *in situ* in such samples demonstrated that overnight incubation at 37°C was adequate, but excess AP had to be removed before continuing to LMS; neutralization in sodium hydroxide and a single wash step was found to be suitable. For detection, microscopy was found to be successful only suitable for clean samples, as debris occluded viewing in dirtier samples. Although qPCR was successful, for some samples non-specific inhibition occurred, as demonstrated by inhibition of an internal amplification control in the qPCR reaction. For some, but not all, samples this could be addressed by dilution. Finally, the optimized methodology was used for a pilot project in which 23 post-IMS water sample concentrates were analyzed. Of these, only 20 provided interpretable results (without qPCR inhibition) of which one sample was positive, and confirmed by sequencing of PCR product, indicating that *Toxoplasma* oocysts occur in Norwegian drinking water samples. In conclusion, we suggest that post-IMS samples may be suitable for analysis for *Toxoplasma* oocysts using LMS, only if freshly processed and washed before refrigerated. In addition, application of AP treatment requires a neutralization step before proceeding to LMS. For detection, qPCR, rather than microscopy, is the most appropriate approach, although some inhibition may still occur, and therefore inclusion of an internal amplification control is important. Our study indicates that, despite some limitation, this approach would be appropriate for further large-scale analysis of samples of raw and treated drinking water.

# 4. Materials and Methods

## 4.1. Diagnostic tools and sample preparation

### 4.1.1. Oocysts and cysts

Initially, obtaining *T. gondii* oocysts for performing each individual assay was not easy and hence I began my work using some surrogate organisms (e.g. *Eimeria* oocysts and *Giardia* cysts) based on their morphological and structural resemblance to *Toxoplasma* oocysts.

*Isolation*: Oocysts of *Eimeria* (unidentified species) were extracted from a preserved specimen originally collected and preserved (by a fellow PhD candidate; John J. Debenham) from the intestine of a turkey that reportedly died of coccidiosis. The step was briefly as follows. Using forceps and scissor, approximately five grams of tissue specimen in a preservative was transferred to a 50 mL centrifuge tube. It was then half-filled in Phosphate buffered saline (1× PBS) and vortexed. The wash suspension was pipette transferred to a new 50 mL centrifuge tube. The old tube was again half-filled, mixed and the wash transferred to the new tube. After centrifuging the new tube at 3000 rpm for 10 minutes, the supernatant poured off and the pellet (~5 mL) retained. The pellet was re-suspended in distilled water to 50 mL, mixed, centrifuged and the supernatant poured off as above. Then 33% zinc sulfate solution (specific gravity 1.18) was added to 30 mL mark, briefly mixed and carefully topped up with distilled water until 50 mL mark by letting the water run down the wall of the tube, so that two phases created (i.e. one with salt at the bottom and one with water on the top). The mixture was centrifuged at 3000 rpm for 10 minutes and the water phase in the upper most layer and the interface (the phase where water and salt meet) was transferred to the new tube using a plastic pipette. Following that, the new tube was topped up using distilled water, mixed, centrifuged as above and the supernatant poured off retaining 5 mL pellet. The last washing step was repeated twice more to remove any remnant salt and have a clean sample for analysis. Finally, the bottom 5 mL pellet was collected and kept in fridge in different storage medium until tests are performed.

Moreover, *Eimeria tenella* oocysts and *Giardia duodenalis* cysts were also obtained from the already isolated and stored source at parasitology lab (NMBU) that has been previously extracted from fecal samples using this centrifuge gradient flotation. The only difference is that the starting sample added was 3 gm of fecal material re-suspended in distilled water (3 gm/60 mL or 1:20 ratio). The source of fecal samples for obtaining *Giardia* cysts were cat or dog derived samples received for diagnostic purpose and positive for cysts.

*Toxoplasma* oocysts: the oocysts of *T. gondii* were obtained from two sources. While Dr. Kristin W. Prestrud provided aged oocysts collected back in 2005 (stored in 2% sulfuric acid at 4°C), we received new oocysts from J.P. Dubey in January 2015. Both aged and new oocysts were genotype II and obtained from infection of *T. gondii* free cats at the USDA facility in Beltsville, Maryland, USA. The procedures for collection and purification of oocysts have been described previously (Dubey, 1995). Details of further handling and storage of *Toxoplasma* oocysts at the parasitology laboratory (NMBU) are described in paper I.

*Enumeration*: Oocyst and cyst enumerations were performed by using KOVA GLASSTIC Slide 10 with grids (Fisher Scientific). For each suspension, a 10 µl sub sample was transferred to each chamber of KOVA GLASSTIC Slide 10 with grids for counting the number of oocysts/cysts. In each chamber, oocysts/cysts that appear in the four corner and the middle quadrant are counted. Then multiplying the total number counted in all chambers (each five quadrants) with the dilution factor and dividing it by 5 determines the number of oocysts/cysts per microliter.

*Sporulation*: Sub samples of newly received *Toxoplasma* oocysts were aliquoted and kept at room temperature for sporulation.

*Oocyst pre-treatments*: The subsamples of purified oocyst suspensions were then stored in $H_2O$, 1x PBS, 2% $H_2SO_4$, 1% SDS, 1% potassium dichromate, 0.1% hypochlorite (bleach), 1% Triton X-100, 1% deoxycholate, acidified pepsin and trypsin to investigate any effect on the oocyst surface properties, particularly their effects on lectin binding activities (Table 4).

Table 4. Different chemicals used for oocyst pre-treatment purposes.

| **Oocysts tested** | **Water and chemicals used for pre-treatment** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $H_2O$ | PBS 1x | 2% $H_2SO_4$ | SDS 1% | $K_2Cr_2O_7$ 1% | NaCLO 0.1% | Triton X-100 1% | $C_{24}H_{40}O_4$ 1% | AP | Trypsin |
| ***Eimeria*** | x | x | x | x | x | x | x | x | x | x |
| ***Toxoplasma*** | x | x | x | x | x | x | x | x | x | x |

To perform this, 1 mL suspension containing *Eimeria* or *Toxoplasma* oocysts was pipette transferred to a microfuge tube and centrifuged at 2000 ×g for 3 minutes. After pouring off the supernatant, the remaining pellet with oocysts was re-suspended in 1 mL of one of the storage medium stated above. The tubes were then allowed to stand overnight in a fridge (except for acidified pepsin and trypsin which were incubated at 37°C).

### 4.1.2. Lectins

A panel of 13 Lectins, conjugated to FITC (Vector Laboratories: FLK 2100 and FLK 3100) were investigated for potential binding activities to *Toxoplasma* oocysts (Table 5).

Each 1 mg of fluorescein-labelled lectin in a vial was first dissolved in phosphate buffered Saline (1× PBS) at a concentration of 2 mg/mL and kept in a refrigerator at 4°C. Following the supplier's recommendation (5-20 µg/mL), working dilutions were prepared for immediate uses. Usually, a 1:100 concentration was prepared in PBS (or 1:50 less often) to obtain a concentration of 5 µg/mL or 10 µg/mL for application.

Table 5. Panel of 13 lectins tested for binding to *T. gondii* oocysts.

| No. | **Lectin** | **Abbreviation** | **Primary sugar specificity** |
|---|---|---|---|
| 1 | Concanavalin A | CON A | α-Man, α-Glc |
| 2 | *Dolichos biflorus* agglutinin | DBA | α-GalNAc |
| 3 | Peanut agglutinin | PNA | Galβ3GalNAc |
| 4 | *Ricinus communis* agglutinin I | RCA I | Gal |
| 5 | Soybean agglutinin | SBA | α>βGalNAc |
| 6 | *Ulex europaeus* agglutinin I | UEA I | α-Fuc |
| 7 | Wheat Germ agglutinin | WGA | GlcNAc |
| 8 | *Griffonia simplicifolia* lectin I | GSL I | α-Gal, α-GalNAc |
| 9 | *Lens culinaris* lectin | LCA | α-Man, α-Glc |
| 10 | *Phaseolus vulgaris* Erythroagglutinin | PHA E | Galβ4GlcNAcβ2Manα6(GlcNAcβ4)(GlcNAcβ4Manα3)Manβ4 |
| 11 | *Phaseolus vulgaris* Leucoagglutinin | PHA L | Galβ4GlcNAcβ6(GlcNAcβ2Manα3)Manα3 |
| 12 | *Pisum sativum* agglutinin | PSA | α-Man, α-Glc |
| 13 | Wheat Germ agglutinin, succinylated | SWGA | GlcNAc |

Sugar abbreviations: Fuc: L-Fucose; Gal: D-Galactose; GalNAc: N-Acetylgalactosamine; Glc: D-Glucose; GlcNAc: N-Acetylglucosamine; Man: Mannose

### 4.1.3. Monoclonal antibodies

Two lots (Lot 02 & Lot 03) of anti-*T. gondii* monoclonal antibodies (mAb) conjugated to FITC (fluorescein-isothiocyanate) containing 15 µg/mL (α-*Toxoplasma* 3B1/-FL mAbs) raised against the surface epitopes of the oocyst wall kindly provided by Hal Stibbs (Waterborne Inc. New Orleans, USA) were used for testing in this study. The mAbs were originally developed by Dr. Furio Spano of Istituto Superiore di Sanità, Rome, Italy and obtained from Dr. Henry Stibbs of Waterborne Inc. (6045 Hurst Street, New Orleans, LA, USA) for performing this research work.

## 4.2. Assays based on oocyst surface binding properties

### 4.2.1. Lectin-binding assays

Lectin-binding studies were performed with both oocysts dried to slides and in suspension. Oocysts suspensions stored in different storage medium (pre-treatment), allowed standing overnight were spun at 2000 ×g for 5 minutes and the supernatant poured off. The pellet was again re-suspended in distilled water, vortexed, spun as above and the supernatant discarded. This oocyst-washing step was repeated at least four times using distilled water to remove the storage medium completely. The remaining pellet (300 µl) was then used for labelling process. For dry labelling, 50 µl of the oocyst suspension was transferred onto a 3 well 9mm NOVAKEMI AB (blue/black) microscope slide and dried in incubator at 37°C for 30 minutes and fixed by adding one drop of methanol solution. Following fixation, 50 µl of test lectin (working dilution) was added to the slide well. The slides were then incubated at 37°C for 30 minutes in a moist medium. The slides were then washed by applying 100 µl of 1× PBS to remove excess or unbound lectins. Finally, one drop (~10 µl) of DABCO glycerine mounting medium was added to the slide wells, then covered with a 50 × 24 mm cover glass and examined. For wet labelling, 50 µl of oocyst suspension was added to a microfuge tubes and 50 µl of test lectin (a working dilution) was added to it. All the microfuge tubes were then vortex mixed for 1 minute and incubated at 37°C for 30 minutes. About 10 µl of wet labelled suspension was transferred to a slide well, covered with a cover glass and screened as wet smear.

### 4.2.2. Monoclonal antibody binding assays

Both lots of mAb were tested by preparing working dilutions as 1:1, 1:5 or 1:10 using 0.1% bovine serum albumin (BSA) in PBS. Dry and suspension labelling experiments were performed using the same procedure as described for lectins above, but using the working mAb dilutions rather than lectins. Since preliminary trials displayed poor staining with Lot 02, all experiments involving mAb described here were performed with Lot 03. Based on results of mAb binding, preliminary investigations were made on developing an IMS system. Magnetic beads coated

with pan mouse IgG (ThermoFischer Scientific, Oslo, Norway) were prepared by washing in 0.1% BSA in PBS. They were mixed with oocyst suspensions, in which the oocysts had already been bound to mAb. The mixture was then incubated for 30 minutes at room temperature in a rotating mixer. Bead-bound oocysts were separated using magnetic particle separator (Dynal MPS-S) and then both the supernatant and the beads were examined by microscopy in two separate slide wells. Two independent trials of this procedure were conducted.

## 4.3. Methods applied to concentrate oocysts

### 4.3.1. Membrane filtration

For filtration, Millipore flatbed membrane filters (cellulose acetate, 142 mm diameter) with a pore size of 2 µm loaded onto stainless steel filter holder (Millipore®, 142 mm diameter) was used. A pressure tubing already connects the filter holder to a vacuum pressure pump (Varmeca, Watson Marlow, Heco, Oslo, Norway) at 1.5 Liters/minute. A 10-liter water sample in a container (a plastic 10 L carboy) spiked with a desired number of oocysts was pumped through the filter by means of vacuum. The container was rinsed in 2 liters of distilled water and the rinse was pumped similarly. Once the membrane filter was unloaded from the holder, it was washed in a beaker (high-density polyethylene) by addition of 25 mL of membrane filter elution buffer (USEPA, 2012) containing salts, detergents, and Antifoam A. After, transferring the eluate to a 50mL centrifuge tube, the membrane filter was then immersed in 0.1% Tween 80 and sonicated for 3 minutes (35 kHz, Transonic 310, Elma, Singen, Germany) and the rinse added to the 50 mL centrifuge tube. The eluate was then centrifuged at 1,100 ×g for 10 minutes and the supernatant poured off. The pellet was then stored in a fridge until further analysis.

### 4.3.2. Application of microfluidic chips (Trilobite® chips)

In collaboration with Brigitte Kasin Hønsvall, a PhD candidate from University college of Southeast Norway (HSN), we examined the potential of Trilobite® chip to concentrate *Giardia duodenalis* cysts and *Toxoplasma gondii* oocysts in water samples. The chip used for testing was

manufactured in silicon and glass at Micronit microfluidics (Enschede, The Netherlands). The whole chip measured 30 mm x 60 mm. The main channel within the chip measured 10 mm x 30 mm, and the field of separation units were located in the middle of the main channel, in a 10 mm x 10 mm area, as seen in Figure 7.

Figure 7. The Trilobite chip model in silicon and glass.

This field contained 113 trilobite separation units distributed in nine rows. Each unit was 1200 µm long and 500 µm wide, and the gaps between the blades were 5 µm wide. Micronit microfluidics (Enschede, The Netherlands) also provided a framework (Fluidic Connect Pro) to place the chip and connect to the associated tubes without leakage (Figure 8). We investigated the chip-based work in two separate occasions using two different setups for pumping. In the first with a syringe pump (tested to concentrate *Giardia* cysts), the volume of chip-treated fluid was limited while a peristaltic pump (used for experiments with *Toxoplasma gondii* oocysts) allowed more continuous chip-treatment and increased volume.

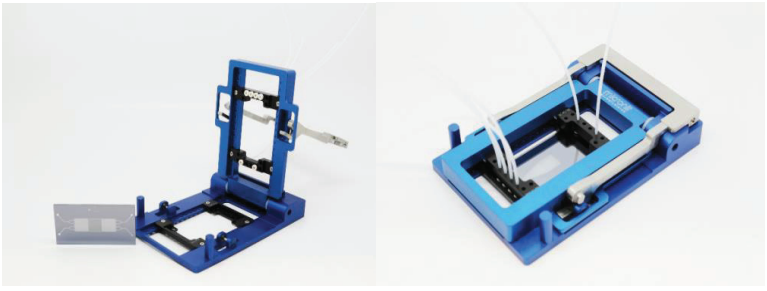


Figure 8 Trilobite® microfluidic chip and fluidic connect Pro framework from Micronit microfluidics (left). When the chip is placed in the framework, the inlet and outlet tubes are connected to the chip leak-free (right) (Pictures provided by Micronit microfluidics).

For the syringe pump setup, a plastic syringe (10 mL or 20 mL, Omnifix Luer Lock, B Braun, Melsungen, Germany) was filled with suspension, connected to the tubes from the chip framework and fitted onto a syringe pump (Aladdin AL-1000 syringe pump, World Precision Instruments, Inc., FL, USA), as seen in Figure 9. Each of the two outlet fractions (*concentrate* and *permeate*) were led out of the chip and framework through two tubes. The outlet fractions were collected in 15-mL tubes. Enumeration of *Giardia* cysts was carried out by drying and fixing 50µl sample onto a microscopic slide with wells, and IFAT staining using monoclonal antibodies (mAb) for *Giardia* (AquaGlo G/C direct, Waterborne Inc. New Orleans, LA; USA). Cysts were counted using a fluorescence microscope (Leica DM LB, Ortomedic, Oslo, Norway). Suspensions were prepared by suspending 200 cysts in 10 mL distilled water with 0.05 % Triton X-100 and 0.01 % Antifoam A (Fluka 10794), and added to a 10-mL syringe. The samples were run through the chip between one and four times.

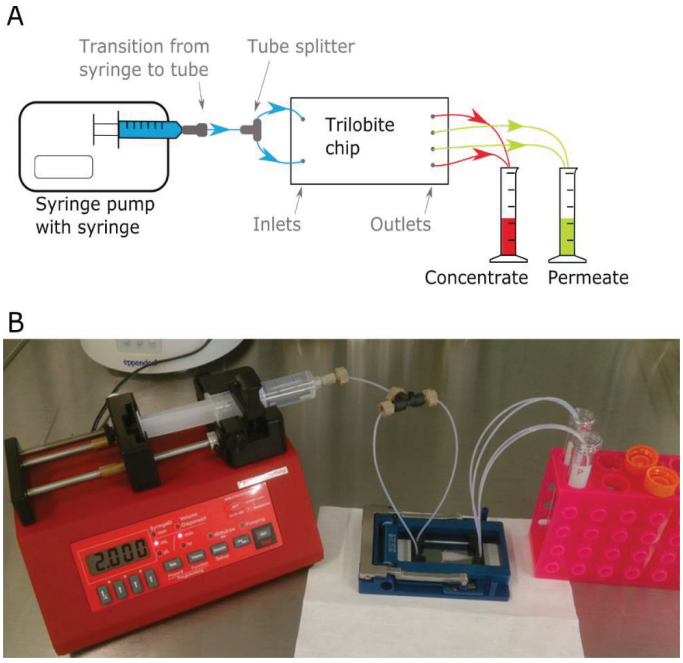



Figure 9 Schematic (A) and photographic (B) presentation of the experimental setup with a syringe pump.

In the experimental setup with a peristaltic pump, a 403/VM2 Watson Marlow pump (VWR) was used to pump the water samples though the Trilobite® chip (Figure 10). The chip was inserted in the frame system depicted in Figure 8, and the micro tubes were connected to larger tubes that were led through the peristaltic pump. The volume that was pumped through the chip was limited by the capacity of the peristaltic pump, with a flow rate of approximately 3 mL/min. Laboratory grade water in 50 mL centrifuge tubes were spiked with an estimated number of oocysts (0, 500, or 1000) and driven by a peristaltic pump through the chip.

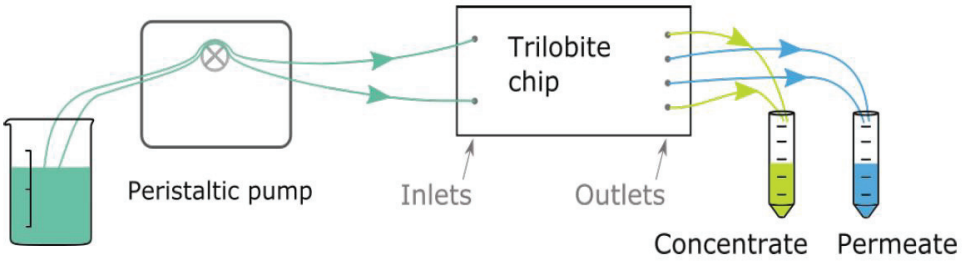



Figure 10. Experimental setup for chip treatment with a peristaltic pump

The outlet fractions were collected in 50 mL centrifuge tubes, and the collected *concentrate* was run once more through the chip. The chip was rinsed twice by running 4 mL 0.01 % Tween 80 water through the system, in order to capture any oocysts remaining in the tubes. All enumerations of oocysts were carried out using KOVA Glasstic Slide 10. Control samples were prepared in the same manner as the samples being chip-treated: 50 mL suspensions were prepared containing 0, 500, or 1000 oocyst in 50 mL centrifuge tubes. The oocysts of the control samples were concentrated by centrifugation at 3000 rpm (1550 ×g) for 10 min (Rotanta 460, Hettich, Tuttlingen, Germany), and discarded of supernatant down to a volume of 1 mL. The chip-treatment was compared with a traditional method for concentration using a filter membrane. It should be noted that the volume pumped through the chip (50 mL) was much lower than the volumes used in membrane filtration, due to limitation set by the chip and the peristaltic pump.

## 4.4. Methods for oocyst separation

### 4.4.1. Lectin-Magnetic Separation (LMS)

#### *4.4.1.1. Magnetic beads preparation and immobilization*

Four different magnetic beads were investigated (Table 6) in order to select the one with the most promising performance. Initially, three different sizes of beads coated with streptavidin or avidin (Dynabeads™ M-280 Streptavidin and Myone™ Streptavidin T1; ThermoFisher Scientific, Oslo, Norway and SPHERO™ Avidin magnetic particles; Spherotech Inc, Lake Forest, IL, USA) were used to investigate whether dissociation could be improved by the increased shear sources with larger beads. In addition, Tamavidin-coated beads (Tamavidin® 2-REV magnetic beads (Wako Pure Chemical Industries, Ltd., Japan) were investigated due to the lower biotin dissociation constant being hypothesized to improve dissociation (streptavidin and avidin have $K_D \approx 10^{-15}$ M; tamavidin $K_D \approx 10^{-7}$ M).

Table 6. Characteristics of the beads tested in this study for LMS following coating with WGA

| **Beads** | **Bead diameter (µm)** | **Concentration used in LMS** |
|---|---|---|
| Dynabeads™ M-280 Streptavidin | 2.8 | 100 µg/mL |
| Dynabeads™ Myone Streptavidin T1 | 1.0 | 100 µg/mL |
| SPHERO™ Avidin beads | 4.0-4.5 | 100 µg/mL |
| Tamavidin® 2-REV beads | 2.8 | 250 µg/mL |

For coupling with magnetic beads, wheat germ agglutinin lectin from *Triticum vulgaris* (wheat) conjugated to biotin (Sigma-Aldrich Norway AS), suspended in phosphate buffered saline (PBS, pH 7.4) at a concentration of 1 mg/mL was used. The beads were then bound to WGA by incubating for 1 hour on a rotating mixer at 15 rpm. While the concentration of biotin-WGA added to 200 µl 10 mg/mL stock Myone Streptavidin T1 beads was 40 µl, only 20 µl was added to the same volume (200 µl 10 mg/mL stock) of the other three beads used in the study. After

coating, the beads were washed four times in 1 mL of washing buffer (PBS, pH 7.4) using a magnet (Dynal MPC-S, ThermoFisher Scientific, Oslo, Norway) to separate beads from the supernatant and then re-suspended (in PBS) back to a concentration of 10 mg/mL.

#### *4.4.1.2. Separation of bead-bound oocysts (LMS)*

This experiment was evaluated in small (1 mL) and large (10 mL) volumes. All tests were performed in independent triplicates. Tests using 1 mL volume (clean water) were performed to assess all beads (Table 6) using different incubation buffers (0.1% BSA in PBS and SL Buffer, Dynal GC Combo kit). In order to investigate capture and recovery, 20 µl aliquots of the suspension containing an estimated 1000 oocysts was spiked into 970 µl incubation buffer (0.1% BSA in PBS or SL buffer Dynal GC Combo kit) in a 1.5 mL micro-centrifuge tube and 10 µl of prepared beads (Dynabeads or SPHERO Avidin) were added. The mixture was incubated for 1 hour at room temperature on a rotating mixer at 15 rpm. For the Tamavidin 2-REV magnetic beads, the amount used was 25 µl mixed with 955 µl incubation buffer (0.1% BSA in PBS or SL buffer, Dynal GC Combo) with the same quantity of oocysts and processed similarly. For capturing bead-bound oocysts, the tube was placed in a magnet (Dynal MPC-S) for 2-3 min and the supernatant transferred to a second tube (for enumeration of unbound oocysts).

For large volume (10 mL) processing, only two of the beads (M-280 Streptavidin and Tamavidin 2-REV) were evaluated using a protocol based partly on the method reviewed by Robertson et al. (2000). Oocysts (approximately 1000) were inoculated into 8.9 mL laboratory grade water. Briefly, the 8.9 mL sample was added to a L10 tube (Dynal, ThermoFisher Scientific, Oslo, Norway) with 1 mL of SL-buffer A (×10 concentrate). The WGA-coupled magnetic beads were thoroughly mixed, and 100 µl added to the L10 tube containing the sample and buffer. The tube was capped and its contents mixed by slow rotation (approx. 25 rpm) at room temperature for 1 hour using a Dynal sample mixer (Dynal, ThermoFisher Scientific, Oslo, Norway). The beads were then separated from other debris in the sample by placing the L10 tube in a specially designed magnetic particle concentrator (MPC; Dynal MPC-1, Dynal, ThermoFisher Scientific, Oslo, Norway), which was rocked gently by hand for 2-3 min (as used in standard IMS for

*Cryptosporidium* and *Giardia*). With the L10 tube remaining in the MPC, the fluid was transferred to a 15 mL test tube for further analysis for unbound oocysts (using centrifugation). The L10 tube was removed from the MPC, and the beads were gently resuspended in 1 mL of SL-buffer A (×1 concentrate). The buffer with beads were transferred to a 1.5 mL micro-centrifuge tube and the beads collected using another MPC (Dynal MPC-S) following a 2-3 min rocking period. The supernatant was again transferred to a different micro-centrifuge tube for recovering unbound oocysts.

#### *4.4.1.3. Dissociation of bead-bound oocysts*

Dissociation of oocysts from beads is important for detection by microscopy, as the beads can occlude the oocysts. Dissociation trials were made using hydrochloric acid (0.1 N, 0.2 N, 0.5 N, and 1 N), sulfuric acid solution (2 %), sodium hydroxide (1 M), N-acetyl D-glucosamine (0.5 M) containing acidified pepsin (0.5 %, 1 %), and biotin (2 mmol/l) solutions. Dissociation trials were conducted in triplicate. To each tube containing the magnetic concentrated oocyst-bead complexes was added 100 µl of test dissociation reagents, vortex-mixed thoroughly, and incubated for 20 min at room temperature. Tubes were vortex-mixed 3-4 times at regular intervals during the incubation period, the beads separated using the MPC-S magnet, and the oocysts in the dissociation reagent transferred to slide wells. This was repeated three times for most reagents to investigate the effect of triple dissociation. Finally, the beads themselves were resuspended in 50-100 µl PBS (depending on the quantity) and transferred to a new slide wells and the supernatant of the incubation buffer was collected, centrifuged and the pellet transferred to a different slide well to evaluate the oocysts that are unbound to the beads. All slides were then incubated for 30 min at 37 °C to dry. A drop of methanol solution was added to each slide well to fix and then overlaid by 10 µl of DABCO mounting medium, and covered with 50 x 24 mm cover glass.

#### *4.4.1.4. Capture in dirty water sample concentrates*

For dirty water capture, only M-280 Streptavidin beads were evaluated and tests were carried out in 10 mL and 50 mL samples. Concentrates of dirty water were obtained from 10 L of raw

water that was collected from a local lake (Sognsvann) and concentrated by membrane filtration using a 2.0 µm membrane filter. For 10 mL dirty water samples the procedure was the same as for 10 mL clean water samples. For 50 mL volumes, the samples were first centrifuged at 1000 ×g for 10 min and then aspirated to 10 mL (packed pellet of approx. 0.25 mL), before being processed as for 10 mL clean water samples. Furthermore, post-IMS supernatant samples collected after analysis for *Cryptosporidium* and *Giardia* were tested for *T. gondii* using lectin-magnetic separation and PCR to optimize for environmental water sample analysis.

### 4.4.2. DNA aptamers

Aptamer selection study was carried out at parasitology lab, Bureau of Microbial Hazards, Health Canada. The protocol followed was described earlier (Iqbal et al. 2015) by using synthetic single-stranded (ss) DNA library (Integrated DNA Technologies, CA, USA) consisting of a randomized region of 40 nucleotides (N40) flanked by two constant primer-hybridization sites, 5'-CTC CTC TGA CTG TAA CCA CG N40 GC ATA GGT AGT CCA GAA GCC-3'. Accordingly, the ssDNA library and aptamer pools were denatured by heating for 5 min at 95°C in Dulbecco's phosphate buffered saline with $CaCl_2$ and $MgCl_2$ (DPBS) (Sigma-Aldrich, ON, Canada), and then renatured on ice for 10 min.

Although the protocol usually requires about 10 rounds of alternating positive and negative selections at different research setups, it was only first round selection that was successfully implemented in this work. To this effect, $4\times10^5$ *T. gondii* oocysts (received from Dr. J.P. Dubey, USDA) were washed twice in DPBS at 3,500 x g for 5 min and resuspended in DPBS containing 5mg/mL yeast tRNA (EMD Millipore) and 1 mg/mL bovine serum albumin (New England BioLabs, MA, USA). The oocysts were then incubated in 100 µl of DPBS containing 1 µM of ssDNA library for 30 min at 25°C and then centrifuged at 14000 × g for 10 min at 15°C, to remove unbound aptamers, followed by rinsing twice in DPBS. Low binding microfuge (1.5 mL) tubes (VWR, Corning Life Sciences, NY, USA) were used in this starting round of selection.

*T. gondii* oocysts were resuspended in 20 µl of 1 × Tris-EDTA, buffer pH 8.0 (TE buffer solution, Sigma-Aldrich, ON, Canada) and heated for 10 min at 95°C to release the aptamers bound to

the oocysts. After the denaturing step, unbound oocysts were removed by centrifugation at 14,000 × g for 15 min, and the supernatant (containing the aptamers) was collected and stored at −20°C. Subsequently, oocyst-bound aptamers were amplified using both symmetric and asymmetric PCR cycles. For the symmetric PCR, 5 µL of the aptamer pool in TE buffer was mixed with 45 µL of symmetric PCR reaction mixture containing GoTaq Flexi buffer (Promega, WI, USA), 2.5 mM $MgCl_2$ (Promega, WI, USA), 200 µM of each of the four deoxynucleotide triphosphates (dNTP) (Promega, WI, USA), 0.5 µM of forward primer (5′-CTC CTC TGA CTG TAA CCA CG-3′), 0.5 µM reverse primer (5′-GGC TTC TGG ACT ACC TAT GC-3′) and 2.5 U of GoTaq Hot Start Polymerase (Promega, WI, USA). The resulting amplification product was used as a template in the asymmetric PCR where 5 µL of the symmetric PCR product was mixed with 45 µL of the asymmetric PCR reaction containing the same reagents as above; except 1 µM forward FAM primer (5′-56-FAM-CTC CTC TGA CTG TAA CCA CG-3′) and 0.05 µM reverse primers were used. All PCR amplifications were performed using the following temperature cycle program: preheating for 2 min at 95°C, followed by 25 cycles for symmetric PCR, and 20 cycles for asymmetric PCR, of denaturing for 30 sec, at 95°C, annealing for 15 sec at 66°C, and extension for 15 sec at 72°C. The final cycle was followed by an extension step of 1 min at 72°C, and a hold at 4°C.

Fluorescently labeled ssDNA was separated from the PCR mixture, primers, and dNTPs, with 30 kDa cut-off filters (Nanosep, PALL) by centrifugation at 3,800 x g for 15 min at 15°C and washing three times with 200 µL of DPBS buffer. The quality and band intensity of an individual aptamer pool were examined by electrophoresis on 3% agarose gels containing GelRed (5 mL/100 mL) (Biotium, Inc., CA, USA). Gels were run for 35 min at 120V with 1x TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). Subsequently, the purified aptamer pool was diluted in 100 µL of DPBS, and its concentrations was measured by a NanoDrop®ND-1000 spectrophotometer (NanoDrop Technologies, Inc., USA), and then stored at −20°C before continuation with the next round. Finally, 300 nM of the aptamer pool was recommended to be utilized for the next round of selection with the same procedure; however, no second round work was conducted as a matter of time limitation.

## 4.5. Detection

### 4.5.1. Microscopy

Slides were examined using fluorescence and ultraviolet (UV) illumination as well as Nomarski Differential Interference Contrast (DIC) microscopy using Leica DMLB Fluorescence Microscope equipped with the following fluorescence filters: an ultraviolet filter block (excitation 335 nm; emission 450 nm) and blue filter block for FITC (excitation BP 450 nm; emission 490 nm). Oocysts were first located by using UV illumination for characteristic blue autofluorescence (Lindquist et al. 2003) and then examined for FITC-intensity exhibited. All results were compared with staining of *Giardia* cysts labelled with FITC-WGA lectin (Ward et al. 1985), which was used as a model-staining organism, as the FITC fluorescence is bright and even over the entire cyst wall. The whole area of the slide well was screened in a systematic, side-to-side manner at ×200 and ×400 magnification, and oocysts counted using tally counter.

### 4.5.2. Polymerase Chain Reaction (PCR)

#### *4.5.2.1. DNA extraction*

Samples containing oocysts were re-suspended in 200 µl laboratory grade water before the commencement of DNA extraction steps. We followed a previously described protocol (Su & Dubey 2010) for DNA extraction with the QIAamp DNA Mini Kit (Qiagen, Norway), with few modifications. Briefly, the sample as oocyst suspension was re-suspended in 180 µL ATL lysis buffer, followed by six freeze-thaw cycles in liquid nitrogen and water bath set at 90°C. Heat-inactivated Phocine herpes virus 1 was included in each sample as internal control before mixing just before adding Proteinase K and incubation at 56 °C overnight. The samples were then vortexed vigorously and incubated with 200 µl Buffer AL at 70 °C for 1 hour. After adding ethanol (96-100 %), further steps of washing were performed using 500 µl of wash buffers (Buffer AW1 and AW2) according to the manufacturer's protocol. Using 50 µl ultrapure water, the DNA was eluted from the binding columns and stored at -20°C until performing PCR.

#### *4.5.2.2. Nested PCR*

As in aptamer selection, conventional nested PCR was used to compare amplification targeting both B1 gene and 529 bp repeat element using DNA extracted from both old and fresh oocysts of *T. gondii*. Previously published primers (Table 7) were evaluated in the amplification comparison for both targeted genes. Of these, Tox-9F and Tox-11R that are complementary to the 529-bp repeat element (GenBank AF487550) producing a target amplicon of 162bp were selected for further steps of qPCR detection in this study.

Table 7. Previously published primers used for comparison of amplification (Su & Dubey 2010).

| Markers (GenBank no.) | Primers | Primer sequences | PCR (bp) |
|---|---|---|---|
| B1 (AF179871) | External | T8: ATGTGCCACCTCGCCTCTTGG | 797 |
| | | T5: GCAATGCTTCTGCACAAAGTG | |
| | Internal | T2: TGCATAGGTTGCAGTCACTG | 126 |
| | | T7: TAAAGCGTTCGTGGTCAACT | |
| 529-bp (AF487550) | External | Tox-8: GACGTCTGTGTCACGTAG ACCTAAG | 450 |
| | | Tox-5: CTGCAGACACAGTGCATCTGGATT | |
| | Internal | Tox-9: AGGAGAGATATCAGGACTGTAG | 162 |
| | | Tox-11: GCGTCGTCTCGTCTAGATCG | |

#### *4.5.2.3. qPCR*

The qPCR assay for detection and quantification of *T. gondii* oocysts targeted the 529 bp repeat element (Homan et al. 2000). All PCR reactions were carried out in triplicate. The amplification mixtures consisted of 10 µl KiCqStart SYBR Green qPCR ReadyMix, Low ROX (2×) (KCQS01 SIGMA); 0.40 µM Tox-9F and 0.40 µM Tox-11R primers, molecular grade $H_2O$ and 5 µl DNA template to provide a final reaction volume of 20 µl. The PCR amplification was performed in a Mx3005P (Agilent Technologies, Inc.) thermal cycler with a SYBR green protocol with dissociation curve. Initial denaturation was at 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 15 sec, annealing at 58 °C for 1 min, and fluorescent measurement after a final extension step at 72°C for 15 sec. Dissociation curves were made by increasing the temperature from 55°C to 95°C with continuous fluorescent measurement. Negative controls (water) were added in each run.

The effect of PCR inhibitors was monitored by an internal amplification control included while DNA isolation. Amplification of the internal control DNA was performed by addition of primers provided for this purpose (PhHV-1F; PhHV-1R) in separate reaction wells simultaneous to the qPCR assay for *Toxoplasma*. A standard curve was prepared using serial dilutions of *T. gondii* DNA extracted from 10,000 oocysts diluted 1:5; 1:50; 1:500 and 1: 5000 (corresponding to 2000, 200, 20, and 2 oocysts). Samples recorded as positive when the Ct values were 40 cycles or less and exhibited a smooth exponential curve in at least two of the triplicate samples compared with the standards curves. Samples without amplification of template DNA but positive for internal amplification control DNA were considered negative.

# 5. Results and general discussion

In the sections below, results and discussion of the experiments that were conducted and have not been included in articles for publication are given. In particular, use of the Trilobite® chip for concentration of *Toxoplasma* oocysts from larger volume water samples (Section 5.1) and the initial investigations on the development of aptamers for capturing and/or detecting the oocysts (Section 5.2) were shown.

In addition, results and discussion are provided on the work that has resulted in scientific articles, with addition of some preliminary findings that were not included in the final articles that have been published or submitted for publication, and a brief overview of the results and discussion that are detailed in the three attached articles.

## 5.1. Use of microfluidic chips for concentrating *Toxoplasma* oocysts in water samples

### 5.1.1. Preliminary tests with Trilobite® chips using *Giardia* cysts as a model organism

Initial work used *Giardia* cysts rather than *Toxoplasma* oocysts, as a supply of this protozoan parasite was more readily available. Preliminary results were not promising, with only a few cysts recovered in the outlet fractions, indicating poor performance. As cysts were not detected at either outlet, it appeared that cyst losses had occurred within the chip system. Microscopic examination of the used chips revealed that salt crystals had been deposited inside the chip, apparently clogging the system; this may have caused a build-up of pressure, resulting in the chip cracking (Figure 11). The source of the salt precipitation could not be identified, as the cysts had been washed several times in laboratory grade water before being used in the spiking experiments, and could have been due to a manufacturing error.

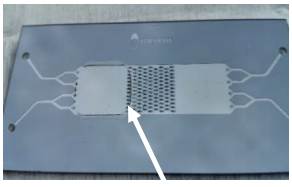


Figure 11. Broken chip (white arrow) as a result of increased pressure

Although our experiments indicated limited use of this system for concentration of *Giardia* cysts from water, previous studies have reported high recoveries of both *Cryptosporidium parvum* oocysts and *Giardia* cysts, and have suggested that this technology could be used as a pre-filter to improve recovery of *Cryptosporidium* oocysts from turbid water samples (Pires & Dong 2013; Pires & Dong 2014). However, we were unable to replicate these experiments and obtain equivalent results, and it was clear that the issue of salt precipitation should be addressed. It is possibly of relevance that the experiments of (e.g.Pires & Dong 2013; 2014) were conducted using chips from a different manufacturer, but of the same design, in China, and therefore are not entirely equivalent.

### 5.1.2. Preliminary tests with Trilobite® chips for concentrating *Toxoplasma* oocysts

Despite the lack of success with *Giardia* cysts, some preliminary trials were also conducted with *Toxoplasma* oocysts. The original *Toxoplasma* oocyst suspension (50 mL) was reduced to 7.5 mL following two rounds of chip processing. Although more successful than the experiments with *Giardia* cysts, *Toxoplasma* oocyst recovery was also low, with higher or more reproducible results being achieved by either membrane filtration or direct centrifugation (the control) (Figure 12 and Figure 13). With experiments using 500 oocysts, the average number of oocysts and (%) recoveries were 120 (24 %) using the chip, 181 (36 %) using membrane filtration, and 360 (72 %) for the control. When samples were spiked with 1000 oocysts, the equivalent recoveries were 427 (43 %), 445 (45 %) and 560 (56 %) oocysts, respectively. Thorough examination of the eluate fraction and the chip after processing by microscopy were negative for oocysts.

Although no significant difference in oocyst recovery efficiencies could be observed between the concentration methods (chip treatment and membrane filtration) and the control when 1000 oocysts were used in the spike, when 500 oocysts were used, the chip treatment performed relatively poorly. As 2 logs fewer oocysts would be expected in environmental samples, and the results tended to be inconsistent, further research on this technology as a concentration step was halted.

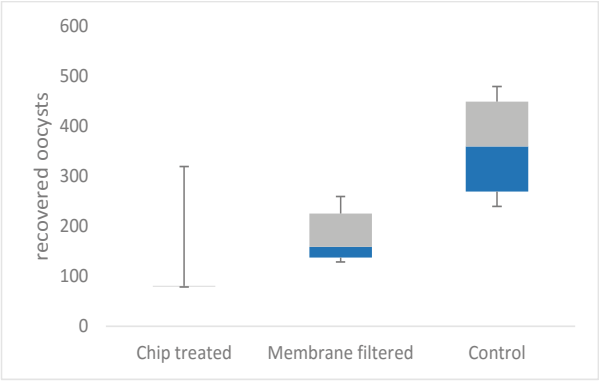



Figure 12. Comparison of recovery of *Toxoplasma* oocysts (spike size: 500 oocysts) by Trilobite® chips and membrane filtration with recovery from controls (suspensions concentrated to 1 mL by centrifugation).

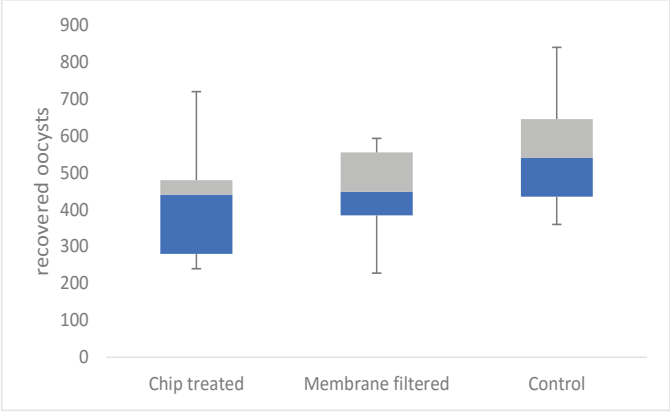



Figure 13. Comparison of recovery of *Toxoplasma* oocysts (spike size: 1000 oocysts) by Trilobite® chips and membrane filtration with recovery from controls (suspensions concentrated to 1 mL by centrifugation).

The efforts to explore the application of Trilobite® chips to concentrating *Toxoplasma* oocysts from water indicated that this technology is currently not suitable for this purpose, as demonstrated by the very low recovery efficiencies for the model organism (*Giardia* cysts) used initially, and the low recovery seen with *Toxoplasma* oocysts. Whereas for the *Giardia* cyst, cracking of the chips may have been partially to blame for the lack of success, this did not seem

to be the case for *Toxoplasma*. For both parasites, it seems likely that the poor recovery is due to loss of the parasites in the tubing system in and out of the chip. Although efforts were made to reduce possible points of oocyst trappings, it seems possible that they might adhere to the tubes themselves and also accumulate at the junctions connecting the micro-tubing to the tubing system of the pump itself. Micro-tubing could also be blocked by particles, and these were regularly rinsed throughout the treatments. Another possibility for losses is from leakage at these connection points. Although attempts were made to reduce the possibility of leakage by using hydrophobic silicone paste to seal the junctions, the parasites could also be lost due to adherence to the silicone paste.

Bubble formation in the larger tubing occurred frequently during the experiment, and might have contributed to losses, by providing extended surfaces for adherence. It should also be noted that the post-concentration centrifugation step, in which the volume was further reduced prior to enumeration, could also result in losses, as the recovery efficiency in the control was also low.

Another limitation of the technique was the limited capacity of the peristaltic pump (approximately 3-6 mL/min) and the handling volume of a single chip. Thus, the volume of the samples concentrated using the Trilobite® chip was only 0.05 L, whereas with membrane filtration the volume can be at least twenty times higher at 10 L.

Although the Trilobite® chip seems to have application at removing liquid from a sample of greater volume, and has been used successfully (at lab scale) to dewater algae (Hønsvall et al. 2016), in its current format, it seems less successful for concentrating organisms in which retention and recovery of all organisms is the aim, rather than removing excess fluid.

According to Ganz et al. (2015), 10 mL suspensions of *Giardia duodenalis* cysts were reduced to less than 1 mL in about 30 min by running the sample 6 times through the unit using an inertial microfluidic separation system. Using the system, they concentrated and detected *G. duodenalis* cysts with a recovery rate of 68.4 % for 1000 oocysts in 10 mL. Compared with, for example, immunomagnetic separation technology, this is not a very high recovery efficiency. In our experiment, we were able to use the Trilobite® chip to reduce the volumes from 50 mL to 7.5 mL by running the sample twice through the chip in 10 min. This reduction in volume is

comparable or better to that achieved by Ganz et al. (2015). However, our recovery rate was lower, presumably due to difference in the equipment set up and protocol.

Should further work on water analysis using microfluidic chips be attempted, upscaling the process by making larger chips, or putting several chips together into a system would be recommended such that larger volumes of water could be processed.

## 5.2. Selection of DNA aptamers

Selection of DNA aptamers is a lengthy and resource-expensive procedure, and therefore practical limitations meant that this work was not continued until completion, and only preliminary stages were conducted to investigate whether this could be an avenue worthy of further exploration.

The first round of selection was performed six times and initial results appeared promising, with clear bands in gel electrophoresis of the product (Figure 14), indicating that ssDNA had already been bound to *T. gondii* oocysts. The subsequent steps would include separation, purification and amplification by repeated rounds of positive and negative selection procedures.

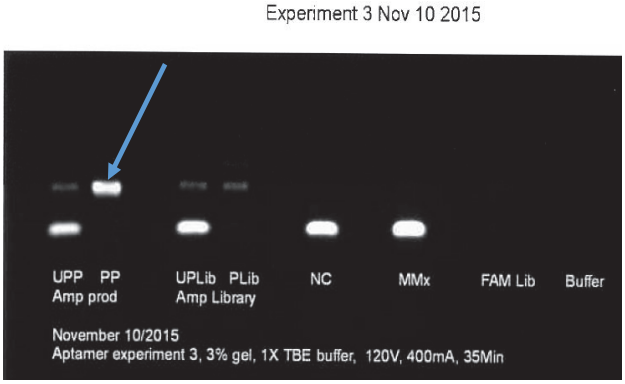



Figure 14. Aptamer selection experiment in first round with the purified product run on 3% agarose gel showing a clear band (PP: purified aptamer product).

NanoDrop® spectrophotometry of the purified product (total volume 25 µl) gave a result of 22.53 µg/µl presence of SS-DNA. The next stage would be to diluted this to 100 µl in DPBS then continue into a second round of selection, using the same protocol used by Iqbal et al. (2015) for selection of DNA aptamers to target *Cryptosporidium parvum* oocysts.
Constraints of time and resources meant that this line of research could not be continued, but it has clear potential.

## 5.3. Monoclonal antibody binding assay

Of the two Lots tested, Lot 02 revealed poor staining in preliminary trials. Hence, all experiments involving mAb were performed with Lot 03 alone. Control oocysts of *T gondii* (old and new) that had not been labelled with mAb did not fluoresce under the FITC filter block.

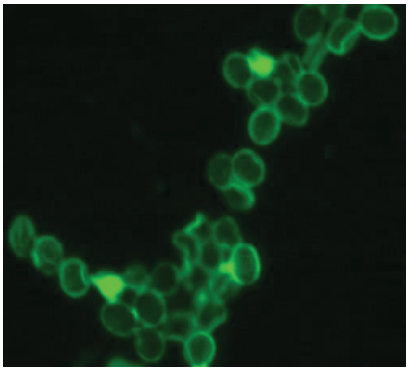


Figure 15. Freshly excreted *T. gondii* oocysts stained with mAb after drying

Dry labelling of aged oocysts with mAb was weak or poor, and weak when staining in suspension. However, dry labelling with fresh oocysts had relative intense staining (Figure 15). Although for dried oocysts the staining intensity decreased with decreasing concentration of mAb, labelling of fresh oocysts in suspension resulted in intense staining even when the concentrations of the mAb were reduced (1:1 or 1:5 using 0.1% BSA in PBS) (Figure 16).

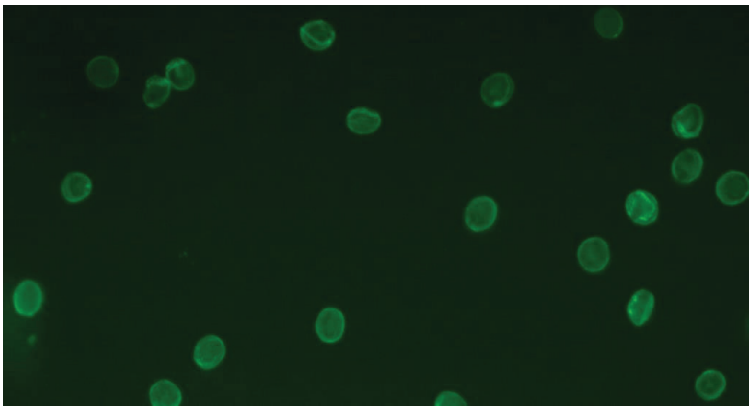


Figure 16. Fresh oocysts stained with mAb in suspension.

As the mAb binding activity with older oocyst was not sufficient, no further steps were taken. However, fresh oocysts, which showed intense, staining with the mAb were used in attempting to create an IMS system.

The results from the preliminary work on developing an IMS system were not encouraging, with less than 1% oocysts captured and the vast majority of oocysts remaining in the supernatant uncaptured. This result, together with ineffective binding of the mAbs to aged oocysts, indicated that the mAbs available for testing were probably not suitable for either detection of all oocysts or for creating an IMS system for fresh oocysts.

Although monoclonal antibodies (mAbs) are frequently used for identification of intestinal protozoa, there are relatively few studies regarding the use of such tools for *Toxoplasma* oocysts. Previously published studies have investigated the binding capacity of mAb 3G4 (Dumetre & Darde 2005), who found no alteration in binding capacity between new oocysts and those that had been stored for up to a year. The authors suggest that the oxidative conditions to which *Toxoplasma* oocysts are exposed during purification and storage had no effect on binding (Dumetre & Darde 2005) . The fact that our “aged” oocysts were even older (10 years refrigerated storage in 2 % sulfuric acid) may indicate that changes do occur over very prolonged periods. However, the mAb that we used may also bind to a less stable epitope. However, protease (trypsin and acid pepsin) exposure was not found to affect binding of mAb, indicating

that the binding occurred in domains that were not affected by these proteases. In contrast, mAb bound weakly to old oocysts but strongly to new oocysts, and this binding was not abrogated by pepsin treatment. Nevertheless, preliminary experiments indicated that it was not appropriate for use with magnetic bead capture and the potential development of IMS.

## 5.4. Lectin-binding assay

### 5.4.1. Lectin-binding test on *Giardia* cysts

Of the two lectins (Con A and WGA) applied for staining *Giardia* cysts, WGA provided strong labelling with a uniform, bright and clear FITC staining revealing a perfect reactivity at different dilution levels used (data not shown). However, with Con A FITC staining was only faint and sometimes dominated by particulate matter.

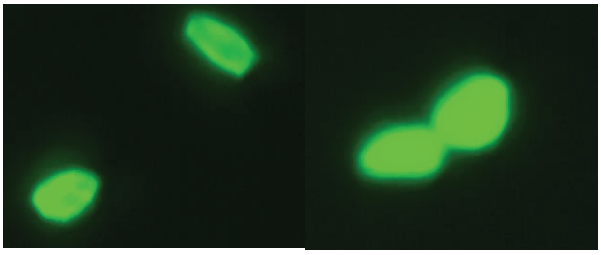


Figure 17. FITC-labelled WGA bound to *Giardia* cysts.

This finding supports the earlier report of Ward et al. (1985), in which it was suggested that the WGA binding to *Giardia* cyst walls indicate that they contain chitin as a structural component. Based on the results with *Giardia* cysts, this binding fluorescence was used in this work as a best model and a positive control in performing the subsequent trials with different lectins on oocysts of other protozoan parasites, particularly *Toxoplasma* oocysts.

### 5.4.2. Lectin-binding test on *Eimeria* oocysts

As with *Giardia* cysts, lectin-binding tests were performed on *Eimeria* oocysts (unidentified species and *Eimeria tenella*) using two lectins (Con A and WGA). Lack of binding of the

unidentified *Eimeria* species, resulted in investigation of various storage and treatment regimens to improve binding, with labeling both in suspension and following drying to slides. In general, these did not provide promising results although storage in 1% SDS and 1% bleach, which still gave slightly better, although variable and non-reproducible, results, with moderate FITC-staining for both Con A and WGA with dry labeling, but no improvement when labelling in suspension. It could be speculated that dried oocysts might have been staining due to the oocysts rupturing because of drying, enabling entrance of the lectins and access to the target carbohydrates.

The *Eimeria tenella* oocysts, which had been stored in 1% potassium dichromate solution, had good staining with Con A, although the staining intensity was higher for dry labeling than labeling in suspension. Addition of 100 mM glucose as a potential blocking agent abrogated the binding activity.

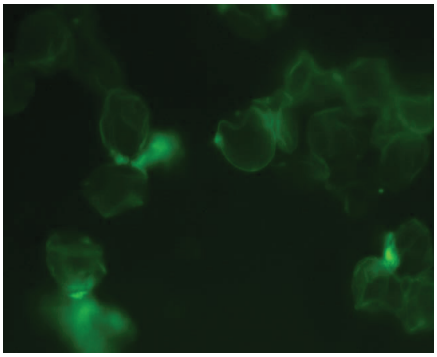


Figure 18. FITC-labelled Con A binding to *Eimeria tenella* oocysts

In an earlier study (Bushkin et al. 2012), the structural resemblance of the oocyst walls of *T. gondii* and *Eimeria* species has been described by the presence of beta-1, 3-glucan as part of a trabecular scaffold in the inner layer of the oocyst walls. Hence, it was initially speculated that this structural resemblance might be of relevance to our studies, as we though it may be possible to use the oocysts of *Eimeria* species as surrogates for *T. gondii* oocysts, given their phylogenetic relatedness and their presumed biochemical and structural similarities. Indeed, in various studies, *Eimeria* oocysts have been used as surrogates for *Toxoplasma* (Bushkin et al.

2012). However, the inconsistency in our results suggested that they would not necessarily mimic *Toxoplasma* oocysts sufficiently for our purposes. Thus, *Toxoplasma* oocysts were used for the remainder of the study, and I did not continue any further work with *Eimeria* oocysts.

### 5.4.3. Lectin-binding test on *Toxoplasma gondii* oocysts

#### *5.4.3.1. Application on aged oocysts*

Following tests using a panel of 13 lectins, dry labelling for aged oocysts of *Toxoplasma* revealed that only WGA and SWGA exhibited an intense staining (Figure 19) while the rest showed patchy, inconsistent, or poor staining patterns. Likewise, results remained consistent when both WGA and SWGA were used for labelling in suspension. A notable finding here was the identification of two distinct subsets of aged oocysts, distinguished by reactivity with WGA detected by FITC. Although the majority of aged oocysts displayed an intense staining, a considerable number of unbound oocysts were noticed as well (data not shown). This difference among the population of aged oocysts lacking uniformity was also noticed when examining UV autofluorescence emissions. The difference was that while oocysts binding the FITC-WGA had relatively thicker walls, the non-binding population had very light and transparent walls. As the aged oocysts had been stored for about 10 years, it could be speculated that the surfaces of the oocysts had changed over time or exposure to particular environments, resulting in the loss of some surface components.

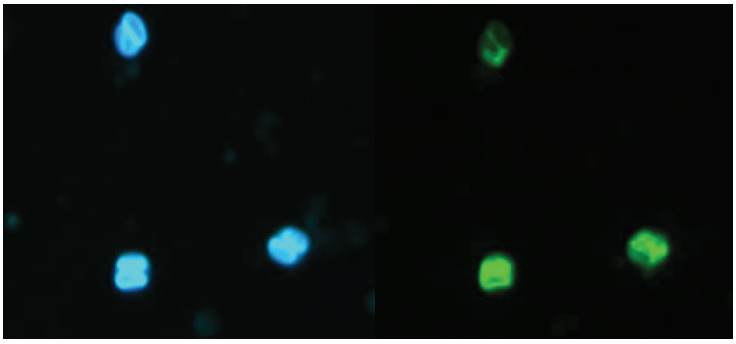


Figure 19. Aged *Toxoplasma* oocysts showing autofluorescence (left) and FITC labelled WGA stained (right).

*5.4.3.2. Application on freshly excreted oocysts*

In contrast to the observed results of two FITC-labelled lectins binding to aged oocysts, all thirteen lectins failed to show any reaction when applied directly to a dried slide containing freshly excreted oocysts and trials with wet labelling produced the same results. However, the fresh oocysts originally suspended in water, 2% sulfuric acid or 1% potassium dichromate solutions and then treated with varied concentrations of acid pepsin (AP) solutions for 18 to 24 hours exhibited an intense staining pattern (Figure 20) with two concentrations of WGA, although moderate with oocysts exposed for 9 hours and weak to none with lower exposure times. In general, the intensity of staining pattern showed an improvement throughout with directly proportional relationship to time length that the oocysts spent in AP solutions (24 hours maximum). The solutions of AP used in this study include; 0.25%, 0.5%, and 1%. However, the intensity of WGA staining did not seem to be affected by the concentrations of AP used as no significant variations were observed. Control experiments, using oocysts exposed to AP solution but examined without application of WGA, did not provide any signal. Thus, these results indicate that exposure of freshly excreted oocysts to AP enables binding of the WGA lectin, as opposed to aged oocysts that bind to WGA without any requirement for pre-treatment with AP. Moreover, the WGA binding was partially abrogated following the addition of sodium metaperiodate (50 mM and 100 mM solutions) to fresh oocysts (AP treated) and aged oocysts, presumably due to cleavage of the relevant sugar groups.

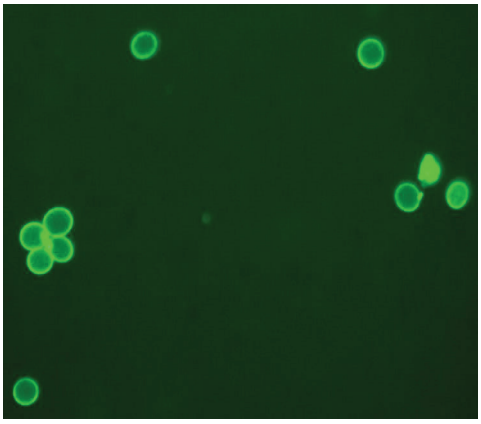


Figure 20. Fresh *Toxoplasma* oocysts treated with AP for 18 hours and labelled with WGA.

These results suggest that a material covering the outer wall surface of the fresh oocysts could have been removed by the action of AP, indicating that the sugar that binds WGA in this instance (possibly GlcNAC), occurs on the outer oocyst wall of *T. gondii* and is probably exposed by the application of AP solution to fresh oocysts. It is of interest to speculate what could be covering the relevant sugar moiety of fresh oocysts prior to AP treatment. One possibility is that fresh oocysts are still enclosed in tissue remnants of the host intestinal epithelial cells or, perhaps more probably, that the outer veil that covers the oocyst wall (Samuelson et al. 2013), preventing exposure of the moieties for lectin binding.

#### *5.4.3.3. Lectin-magnetic separation and microscopy*

With acidified pepsin (AP) pretreated and purified *Toxoplasma* oocysts, an effort for lectin-magnetic separation (LMS) using WGA coated Streptavidin (Myone T1 and M-280) beads showed significantly ($P < 0.05$) higher mean capture when used in buffered conditions. WGA-Tamavidin 2-REV beads also performed very well, but the efficiency was lower than the streptavidin beads ($P<0.05$). WGA-Avidin beads displayed some inconsistent and lower capture efficiencies.

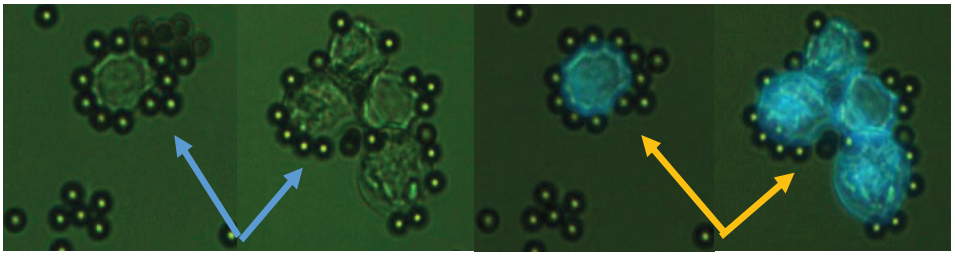


Figure 21. Appearance of bead-bound oocysts of *Toxoplasma* ready for separation using magnet (blue arrow: light microscopy; yellow arrows: UV autofluorescence).

Although capture of oocysts with some of the tested beads was extremely high (typically in excess of 97 %), the oocysts need to be dissociated from the beads for optimal detection by microscopy. Using the conventional HCl-based dissociation was not satisfactory, and remained problematic except some moderate dissociation for M-280 beads with triplicate attempts.

Alternatively, 1 % AP containing 0.5 M GlcNAc provided high (88.8 %) dissociation for M-280/WGA bound oocysts and also 2.0 mmol/L biotin for oocysts bound to Tamavidin 2-REV beads. Oocysts bound to other beads could not be dissociated. In further larger volume applications, investigation for the potential use of Tamavidin beads was also stopped since the bead-oocyst complex was unstable, and this resulted in loss of oocysts during manipulations. Therefore, capture of oocysts from larger volumes (10 mL) of both clean and dirty water samples was investigated only for M-280 streptavidin beads. The work provided encouraging results, with recovery in excess of 90 % (clean) and 85% (dirty water) using experimental samples.

As a result, a proof-of principle for the development of a novel LMS method has been established. It then enabled us to isolate and concentrate *Toxoplasma* oocysts from water sample concentrates for their subsequent detection either by microscopy or by molecular methods, with WGA-coated Dynabeads$^{TM}$ M-280 Streptavidin bead (the best for capture and dissociation), displaying a stable bead-oocyst complex when processing in a larger volume.

Under natural circumstances, oocysts usually occur in low concentrations in water. Hence, it is important that the limit of detection is low. Furthermore, environmental water samples may contain a variety of particulate and other contaminants, and it is important that analytical methods can operate not only in ideal clean spiked “lab” samples, but also in natural samples. To address these issues, optimization steps on the LMS technique for application in the routine water-analysis laboratory were carried out by incorporating AP-treatment of *Toxoplasma* oocysts *in situ* in contaminated water samples. The experiment demonstrated that overnight incubation of oocysts with AP at 37°C in a rotating mixer was adequate. However, this should be followed by neutralization in sodium hydroxide and a single wash step to remove the excess AP. Removal of excess AP prior to LMS was important, as initial experiments indicated that excess AP affected the action of WGA during LMS.

For clean water samples (reverse osmosis (RO) water or tap water) in larger volumes (10 mL), spiking of untreated oocysts followed by AP treatment, alkali neutralization and single washing in 0.05% PBST, and then LMS resulted in high recovery efficiencies (in excess of 97 %). This finding did not noticeably differ from recovery efficiencies when the same samples were spiked

using AP-pretreated oocysts. This indicated that microscopy, as a detection method was successful for clean samples with initial oocyst separation by LMS. However, with dirtier samples microscopy was less successful due to occlusion of oocysts by other material.

In dirty samples, recovery efficiency as determined by microscopy was found to be inversely proportional to pellet size with a significant ($p<0.05$) reduction in recovery efficiency as pellet size increased (Figure 22). Although oocysts may have been captured and separated, they remained hidden in excess particulate matter and could therefore not be enumerated.

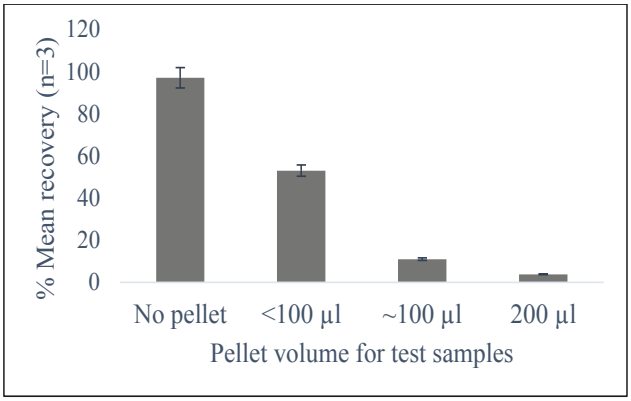



Figure 22. LMS recovery of oocysts from freshly processed post-IMS samples with different pellet sizes, and AP treatment of oocysts *in situ*.

Although, satisfactory recovery results were obtained by LMS for freshly-processed IMS samples using our AP-treatment protocol, it should be noted that the non-specific nature of LMS does mean that other biological materials may co-isolate with *Toxoplasma* oocysts. This was particularly noticeable with larger pellet sizes, which means that microscopy, as the detection method is only appropriate for clean samples. Our results emphasize the importance of coupling LMS with molecular detection techniques rather than relying on microscopy for detection (Hohweyer et al. 2016).

Initial studies attempted to apply the LMS technique to samples that had already been analyzed for contamination with *Cryptosporidium* and *Giardia* using standard methods, and the supernatant following immunomagnetic separation (IMS) retained. The reason why using post-IMS water samples (supernatants) from environmental sources was chosen was to optimize the

utilization of resources and to facilitate the simultaneous analysis and detection of different waterborne pathogens in the same samples. However, the appearance of mucilaginous material in these samples, which was found to probably be an exudate from bacterial (*Pseudomonas* and *Bacillus*) species and fungal overgrowth, affected use of the LMS beads. This was especially the case when post-IMS samples were kept at room temperature and unwashed. Thus, such samples (that had been stored at room temperature and unwashed) may be unsuitable for use with LMS or other magnetic bead-based capture methods. If such samples are to be analyzed using LMS, then they should be washed and kept refrigerated and analyzed as soon as possible post-IMS. In conclusion, we suggest that post-IMS samples may be suitable for analysis for *Toxoplasma* oocysts using LMS, only if freshly processed and washed before refrigerated.

#### *5.4.3.4. Lectin-magnetic separation and molecular detection*

As noted above, using microscopy as a detection method following separation of *Toxoplasma* oocysts from water samples, is adversely affected by the non-specific binding of particulate matter occurring in dirty water samples from environmental sources, although is a suitable method for clean water samples. Thus, it was anticipated that molecular methods would be a more suitable method for detection following LMS. Moreover, a dissociation step is not required following separation if molecular detection methods are used, as is required for microscopy. A further benefit from using molecular methods is that they preclude any concern about misidentification of *Toxoplasma* oocysts (due to their morphological similarity to other coccidian oocysts such as from *Hammondia* species or *Neospora caninum*). The oocysts of these coccidian may also be isolated by LMS due to the lack of specificity of WGA; its binding to *Cyclospora* oocysts has already been noted (e. g. Robertson et al. 2000). Thus, by using appropriate primers, only *Toxoplasma* oocysts will be detected with molecular methods. Alternatively, less specific primers may be used, but identification confirmed by subsequent sequencing of amplified DNA. In the current study, we targeted the 529-bp repeat element using previously published qPCR primers as this has been demonstrated to be more sensitive technique than ITS nested PCR (Wells et al. 2015). The ability to detect 10 oocysts /10 mL in a turbid water sample concentrate (experimentally in the lab) using this qPCR following LMS

provided an important indication of the potential power of this method for the analyses of water samples for contamination with *Toxoplasma* oocysts.

Although PCR can be very effective with pure solutions of nucleic acids, its sensitivity may be reduced dramatically when applied directly to biological samples due to the presence of PCR inhibitors (Lantz et al. 2000; Rådström et al. 2004), and thus any pre-isolation method that removes potential inhibitors is likely to improve sensitivity. Although results from our initial studies indicated that LMS could facilitate separation of the bead-bound oocysts from potential PCR inhibitors that might be present in turbid water samples from environmental sources, we nevertheless found that the same challenge persisted in some samples even after LMS coupled with qPCR. This could be due to the non-specific binding characteristics of WGA, which may result in isolation of material that also inhibits PCR. Dilution of the template enabled us to overcome this problem for some samples, but was not always sufficient. For future experiments, we recommend that qPCR optimization, particularly to reduce the effect of inhibitors, is investigated more closely. Given that the copy number of the target is likely to be low, dilution is probably not the most appropriate approach for addressing inhibition. We therefore suggest that other approaches, such as use of PCR enhancers (additives) such as T4 gene 32 protein, or using digital-droplet PCR as a detection methodology, as this has been considered to be superior for inhibition-prone samples (e. g. Dingle et al. 2013), would be worthy of investigation.

Finally, having developed and provided proof-of-principle of the LMS-approach coupled with qPCR, and then optimized the approach for analysis of real samples, identifying and addressing the problems as they arose. With this, I was finally able to apply the method in a pilot project involving 23 water sample concentrates (post-IMS supernatants) that had already been analyzed for *Cryptosporidium* and *Giardia*. The samples were obtained from 9 different drinking water sources in Norway. Among the 20 samples with conclusive results, one positive sample was detected, and was also confirmed by sequencing of the PCR product. This confirms that our method could be implemented in water-analysis labs to perform analysis of environmental water samples. Although only a limited study, our finding suggests that *Toxoplasma* contamination of drinking water samples does occur in Norway, probably at levels that are

similar to those recently reported from Scotland (Wells et al. 2015). Although we have no information on the infectivity of the *Toxoplasma* detected, our knowledge on the robustness and longevity of *Toxoplasma* oocysts in the environment indicates that there is a need to analyze further water samples and to determine the extent of contamination and relevant risk factors.

# 6. Conclusions and future directions

Consumption of water contaminated by sporulated oocysts of *Toxoplasma gondii* has become a major concern worldwide since it has a significant public heath impact. While detection methods have been developed and validated for other waterborne protozoan parasites, notably *Cryptosporidium* and *Giardia*, and have been adopted worldwide, this has not been the case for *T. gondii*. Such methods not only enable surveillance studies and risk assessment, but also provide robust tools for investigating outbreaks; thus, information regarding the contamination of water with *Cryptosporidium* oocysts and *Giardia* cysts far outweighs that available regarding *Toxoplasma*.

The studies described in this thesis have provided a significant step towards development of reliable methods for analyzing water samples for contamination with *T. gondii* oocysts. First, the surface binding properties of *Toxoplasma* oocysts were investigated using both available monoclonal antibodies and a panel of lectins, and included various treatments to affect the surfaces of the oocyst walls. Based on the results obtained we were able to develop a method for oocyst separation from contaminated water using magnetic beads coated with a lectin (wheat germ agglutinin, WGA), with detection by microscopy or molecular methods. The proof-of-principle of this method (lectin-magnetic separation, LMS) was established, and was tested for capturing oocysts from both clean and dirty (environmental) water samples. Although microscopy was satisfactory as a detection tool when isolating *Toxoplasma* oocysts from clean water samples, it was less successful for dirty water samples. Thus, for successful application to environmental samples, LMS should be coupled to molecular detection. Hence, this study proved that LMS coupled with qPCR detection would be appropriate approach to apply for large-scale analysis of samples of raw and treated drinking water. Such a large-scale water analysis is currently called for, as a pilot study conducted during the course of this work indicated that some drinking water sources in Norway are contaminated by *Toxoplasma* oocysts. Additional work during this project made preliminary investigations on the use of a microfluidic chip (Trilobite®) for initial concentration procedures and the use of aptamers for

binding to oocyst walls. Both these strands of study were not completed in depth due to preliminary results with the microfluidic chip being poor, and due to time and material constraints regarding the aptamers.

Despite a successful method being developed, further work is suggested by the results of the studies. In particular, I think that the work described here suggests the following areas as being of importance for further investigation:

- Microfluidic chips
    - This study used only one sort of available chip; other chip types are available and would be worthy of investigation. Although the use of these chips has not yet been brought into the field even for monitoring for *Cryptosporidium* or *Giardia,* due to scale-up problems, there is considerable potential. Importantly, such chips are suitable for including into complete LOC systems.
- Aptamers for *Toxoplasma* oocysts
    - Although initial studies were promising, my work on this area was compromised by time and material constraints. As it has been a successful avenue for other protozoan parasites, it would be worthwhile considering further, particularly as mAb against the oocyst wall are not widely available.
- Addressing inhibitor challenges in molecular detection
    - In some samples we found that, despite the LMS step, inhibitors were a challenge for molecular detection. Studies that investigate procedures that are less vulnerable to inhibitors would be valuable (e.g., digital-droplet PCR or use of PCR enhancers).
- Investigation of other molecular-based detection techniques
    - There is a plethora of molecular detection techniques, and my study was limited to qPCR. Other detection technologies may be more easily combined with LMS and some may also be applicable to use in the field, or in combination with microfluidics in LOC technology. Such approaches could include isothermal techniques (e.g., LAMP, NASBA, TWIST), and this would be worthy of further investigation.

- Improving AP treatment for LMS
    - Further investigation to improve or speed up the AP treatment of oocysts for LMS could be of value.
- Combining analysis of water for *Toxoplasma* with analysis for other pathogens, including protozoa
    - While my work ultimately used post-IMS supernatants from water samples that had already been analyzed for *Cryptosporidium* and *Giardia*, the ideal analytical tool would investigate for several pathogens simultaneously. Studies to enable simultaneous analysis for all three pathogens together, and, ideally, others (e.g. *Cyclospora*, waterborne viruses, etc.) in a multiplex system could be very valuable.
- Further investigation of *Toxoplasma* oocyst walls
    - My studies on oocyst walls threw up some interesting findings that were not investigated further during my work. In particular, the unexpectedly high binding affinity between the WGA and the moieties that it bound on the oocyst wall would be worthy of further investigation. A greater knowledge of the biophysical properties of the wall may assist in our understanding of why these parasite are so persistent in the environment and provide potential avenues to explore for enhanced identification, or even removal or destruction.
- Further studies to obtain information on occurrence in drinking water sources and factors associated with contamination
    - The results of my preliminary pilot study on the occurrence of *Toxoplasma* oocysts in water sources in Norway indicate that there is a need for a large-scale survey. Such a survey could focus not only on the extent of contamination, but could also indicate those factors that are associated with increased likelihood of contamination, including temporal factors (weather) and site-specific factors (exposure to sewage, domestic or wild felids etc.).

As indicated from the list above of possible future work, my studies described in this thesis towards increasing our knowledge on waterborne transmission of *Toxoplasma*, is just a small contribution towards unraveling the complexities of this parasite and its transmission routes.

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# 8. Scientific papers

# Paper I

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# Surface binding properties of aged and fresh (recently excreted) *Toxoplasma gondii* oocysts

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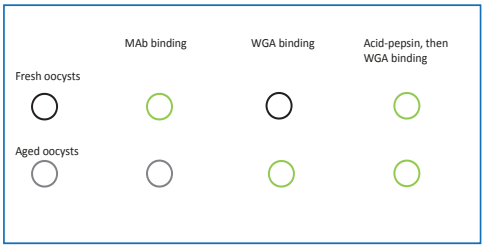
## HIGHLIGHTS

- Age of Toxoplasma oocysts affects surface-binding properties.
- Fresh oocysts bound monoclonal antibody, but not old oocysts.
- Old oocysts bound wheat germ agglutinin (WGA), but not fresh oocysts.
- Treating fresh oocysts with detergent or trypsin did not affect WGA binding.
- Fresh oocysts bound WGA after treatment with acidified pepsin.

## GRAPHICAL ABSTRACT

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## ABSTRACT

The surfaces of aged (10 years) and fresh (recently excreted) oocysts of *Toxoplasma gondii* were investigated using monoclonal antibody (mAb) and lectin-binding assays. Fresh oocysts bound a wall-specific mAb labelled with fluorescein isothiocyanate while aged oocysts did not. In contrast, the walls of aged oocysts bound a lectin (wheat germ agglutinin, WGA), but not the walls of fresh oocysts. Exposure of oocysts to detergent solutions or trypsin did not affect the binding properties of the walls of the oocysts. However, exposure of fresh oocysts to acidified pepsin enabled labelling of the walls with WGA, presumably due to the relevant moieties on the oocyst walls becoming exposed. WGA binding, but not mAb binding, was partially abrogated with periodate exposure. These findings reveal a significant difference in the binding properties of oocyst walls from "aged" and "fresh" oocysts. The results are of relevance when considering technologies for isolating or detecting *T. gondii* oocysts in environmental samples based on oocyst surface properties, as used for other protozoan parasites. Our results suggest the possibility of developing a WGA-based separation procedure for isolating *Toxoplasma* oocysts from environmental matrices, in which pepsin pre-treatment would be included to ensure that both fresh and aged oocysts were isolated.



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## 1. Introduction

*Toxoplasma gondii* is a protozoan parasite that infects humans and other warm-blooded animals, including birds and marine mammals worldwide (Dubey, 2010). The life cycle of the parasite consists of asexual reproduction in the intermediate hosts and sexual reproduction in the intestinal mucosa of the definitive host. Felids (wild and domestic) are the only known definitive hosts and therefore serve as the main reservoir of infection shedding millions of oocysts in their faeces (Frenkel et al., 1970; Dubey et al., 1970). There are three infectious stages of the parasite for all hosts: tachyzoites (the rapidly dividing form) in tissues, bradyzoites (the slowly dividing form) inside cysts in tissues, and sporozoites in the oocyst (Jones and Dubey, 2010; Petersen and Dubey, 2001).

Postnatal transmission occurs by eating raw or undercooked meat containing viable tissue cysts, or ingestion of water, soil, or food contaminated with sporulated oocysts derived from the environment (Jones and Dubey, 2010; Dumetre and Darde, 2003; Dubey, 2004). A foetus may be infected by tachyzoites crossing the placental barrier, possibly leading to congenital defects (Sullivan and Jeffers, 2012). *T. gondii* oocysts are highly resistant to the environment and can remain viable in water sources for years. Some published reports suggest that *T. gondii* oocysts can survive both chemical and physical inactivation steps often employed in drinking water treatment processes (Dumetre and Darde, 2003; Kuticic and Wikerhauser, 1996; Wainwright et al., 2007a, 2007b) and sporulated *T. gondii* oocysts survived in seawater for up to 2 years (Lindsay and Dubey, 2009).

Toxoplasmosis resulting from ingestion of oocysts can be clinically severe, and epidemics of waterborne toxoplasmosis have been reported from several countries (Dardé et al., 1998; Benenson et al., 1982; Bahia-Oliveira et al., 2003; Keenihan et al., 2002; de Moura et al., 2006; Gattás et al., 2000; Heukelbach et al., 2007; Dubey et al., 2012; Bowie et al., 1997; Aramini et al., 1999; Burnett et al., 1998. Balasundaram et al., 2010; Hall et al., 1999; Palanisamy et al., 2006). Despite the substantial risks and health implications of waterborne toxoplasmosis in humans and animals, detection of *T. gondii* oocysts in environmental samples (water, soil, fruits or vegetables) is a considerable challenge due to the lack of sensitive and specific methods (Jones and Dubey, 2010; Dumetre and Darde, 2003; Shapiro et al., 2009, 2010). Standard methods for detection of other protozoan parasites, such as *Cryptosporidium* oocysts and *Giardia* cysts, in environmental matrices have been developed, standardized, and adopted globally (Anonymous, 2006, 2012, 2015). These methods rely on specific isolation techniques, such as immunomagnetic separation (IMS), and similarly specific detection techniques such as immunofluorescent antibody tests (IFAT). Other approaches, including fluorescence-activated cell sorting and polymerase chain reaction, have shown some promise in detecting protozoa from complex environmental matrices (Dumetre and Darde, 2003), but do not compete with IMS and IFAT regarding sensitivity. However, neither IMS nor IFAT are currently commercially available for *T. gondii* oocysts (Jones and Dubey, 2010.).

Improved knowledge on the antigenic and biochemical composition of the oocyst wall will provide valuable information for developing similar techniques for *T. gondii* oocysts. It should be noted that although the oocysts of *Toxoplasma* are very robust, like *Cryptosporidium* oocysts and *Giardia* cysts, an important difference is that *Toxoplasma* oocysts need time to sporulate in the environment. This implies that metabolic processes are ongoing within the *Toxoplasma* oocyst, and therefore may not be as inert as the transmission stages of *Cryptosporidium* and *Giardia*.

The aim of this study was to investigate the surface binding properties of *T. gondii* oocysts using antibody-binding and lectin-binding studies, and to investigate whether the surfaces altered with ages of oocysts and following different treatments. The information obtained could be of value for developing methods based on the oocyst surfaces for concentrating and detecting them in environmental matrices.

## 2. Materials and methods

### 2.1. T. gondii oocysts

Two batches of oocysts were included in this study. Both batches of oocysts were genotype II and obtained from infection of *T. gondii* free cats at the USDA facility in Beltsville, Maryland. The procedures for collection and purification of oocysts have been described previously (Dubey, 1995, 2010).

Batch 1 oocysts, derived from an Arctic fox strain, were collected in 2005 (Prestrud et al., 2008). They were sporulated in 2% sulphuric acid at room temperature for 1 week and then stored at 4 °C. Based on characteristic UV autofluorescence, this population of oocysts was heterogeneous, with autofluorescence that ranged from an extremely faint, ghost-like appearance to a dense blue UV emission. They contain two ellipsoidal sporocysts and appear subspherical or ellipsoidal in shape. Throughout the text, these oocysts are referred to as “aged oocysts”.

Batch 2 oocysts, derived from the ME 49 strain, were collected on 13 January 2015, suspended in water, and kept cold to prevent sporulation. The oocysts were unsporulated when received in Norway within 2 days of shipment from USA. The characteristic UV autofluorescence was less intense than that of the aged oocysts, but there was greater homogeneity within the population, with the majority (>99%) of oocysts showing similar characteristic autofluorescence. Throughout the text, these oocysts are referred to as “fresh oocysts”.

### 2.2. Storage of oocysts

Upon arrival at the parasitology laboratory in Oslo, the stock of fresh oocysts was subdivided into 500 μl suspensions each containing approximately $5 \times 10^7$ oocysts. These were re-suspended in either 4.5 ml laboratory-grade water, 4.5 ml 2% sulphuric acid, or 4.5 ml 1% potassium dichromate solutions. All suspensions were kept refrigerated in the dark at 4 °C. The aged oocysts were held refrigerated in 2% sulphuric acid.

Oocysts were washed (tubes centrifuged at 4000 rpm (~1560 *g*) for 3 min and the supernatant removed) at least four times using laboratory-grade water before being used in the various experiments. During and following experiments and washing procedures all potentially contaminated liquids were collected into 50 ml tubes that were subsequently sealed and disposed of by incineration. Oocyst enumeration was performed both using KOVA GLASSTIC Slide 10 with grids (Fisher Scientific).

### 2.3. Pre-treatments of oocysts prior to binding assays

#### 2.3.1. Detergent treatments

Oocyst suspensions were exposed to the following detergent solutions: sodium dodecyl sulphate (SDS; 1%, 5%), and 1% alkaline SDS (dissolved in 0.2 N NaOH rather than water), Triton X-100 (1%), sodium deoxycholate (0.5%), Tween 80 (1%), and Tween 20 (1%).

Briefly, 100 μl of oocyst suspension pre-stored in water, sulphuric acid, or potassium dichromate solutions and washed in laboratory grade water was added to a 900 μl detergent solution in a microfuge tube. The sample was then incubated for 18–24 h at 37 °C then washed three times as described above using laboratory grade water. Treated, washed oocysts were stored in distilled water at 4 °C and then used for mAb and lectin-binding assays (see

Sections 2.4 and 2.5).

*2.3.2. Protease treatments*

Three concentrations of acid pepsin solution were prepared: 0.25% (0.875 μl 1 M HCl, 0.625 g NaCl, 0.325 g pepsin mixed with distilled water to 125 ml), 0.5%, and 1.0% as described (Dubey, 1998; Jacobs et al., 1960). The pepsin was from porcine stomach (1:10,000 biological activity, BIOFAC A/S, Englandsvej 350-356/DK-2770 Kastrup, Denmark).

The acid pepsin solutions were prepared 1 h before use and pre-warmed to 37 °C. Then 900 μl of acid pepsin solution mixed with 100 μl of the purified *T. gondii* oocyst suspensions in Eppendorf tubes and incubated at 37 °C for from 1 to 24 h (sampling at 0, 1, 3, 5, 7, 9, 18 and 24 h).

The trypsin used was Gibco® Trypsin powder, an irradiated mixture of proteases derived from porcine pancreas (2.50% Trypsin (10×), catalogue No. 15090046, Life Technologies, Thermo Fisher Scientific Inc., UK). Three concentrations were prepared in phosphate buffered saline (PBS): 0.25%, 0.5%, and 1.0%. The solutions were pre-warmed to 37 °C and then 900 μl mixed with 100 μl of purified *T. gondii* oocyst suspensions in Eppendorf tubes and incubated at 37 °C as above.

Following incubation with either acidified pepsin or trypsin the oocysts were washed four times in distilled water and the final volume adjusted to 100 μl. Finally, washed oocysts were evaluated for surface binding properties using anti-*T. gondii* monoclonal antibody (mAb) and/or lectin-binding assays as described in Sections 2.4 and 2.5 below.

*2.3.3. Periodate treatment*

In conjunction with the binding assays, sodium metaperiodate solution was used to evaluate whether oxidation with periodate alters the mAb binding or lectin-binding surface properties of oocysts, including oocysts that had been previously treated with acid pepsin. Prior to staining with either the mAb or the lectin wheat germ agglutinin (WGA), purified oocysts were dried to slide wells, methanol fixed, and treated with 50 mM and 100 mM solutions of sodium metaperiodate for 1, 3, or 6 h and then washed twice in PBS. WGA and mAb binding assays were then performed as in Sections 2.4 and 2.5 below and visualized accordingly.

*2.4. Monoclonal antibody assay*

Two lots (02 and 03) of anti-*T. gondii* mAb conjugated to FITC (fluorescein isothiocyanate) containing 15 μg/ml (*α-Toxoplasma* 3B1/-FL mAbs) raised against the surface epitopes of the oocyst wall were kindly provided by Waterborne Inc. (New Orleans, USA) for testing in this study. Working dilutions were prepared as 1:1, 1:5 or 1:10 in 0.1% bovine serum albumin (BSA) in PBS.

Preliminary trials indicated poor staining with Lot 02, so all experiments involving mAb described here were performed with Lot 03.

Labelling experiments were performed on both oocysts dried and fixed to slides (dry-labelling) and on oocysts in suspension (suspension-labelling). For dry-labelling, slides were prepared by adding a 50 μl aliquot of the purified oocyst suspension onto a 3-well 9 mm NOVAKEMI AB (blue/black hydrophobic surface coated) microscope glass slide and dried at 37 °C for 30 min. Following fixation in methanol, 50 μl of working dilution of mAb was added to each well and incubated at 37 °C for 30 min in a humid chamber. The slides were then washed by applying 100 μl of PBS to remove excess or unbound mAb. For examination, one drop of mounting medium (PBS-glycerol with DABCO) was added to slide wells, covered with a 50 × 24 mm cover slip and examined by fluorescent microscopy (see Section 2.6).

For suspension-labelling, 25 μl aliquots of the purified oocyst suspension were transferred to Eppendorf tubes and an equal volume of working dilution of mAb was added. The Eppendorf tubes were then vortex mixed for 1 min and incubated at 37 °C for one hour. 5 μl aliquots of this suspension were then transferred onto a slide well, covered with a 50 × 24 mm cover slip, sealed by nail varnish and examined as wet mounts. The remaining contents of the tubes were added to slide wells and kept in the dark to dry, after which a drop of mounting medium was applied, covered with a cover slip and examined by fluorescent microscopy.

*2.5. Lectin-binding assay*

Lectins conjugated with FITC (Vector Laboratories: FLK 2100 and FLK 3100) were used in this study (Table 1). Each 1 mg of fluorescein-labelled lectin in a vial was first diluted in PBS to a concentration of 2 mg/ml and stored refrigerated at 4 °C.

Working dilutions of these stock solutions of lectins were prepared for immediate applications. Usually, a 1:100 (10 μg/ml) or 1:50 (20 μg/ml) concentration was prepared in PBS for labelling in both dry and wet preparations.

Dry-labelling and suspension-labelling experiments were performed, using the same procedure as described in Section 2.4 for mAb, but using the working dilutions of lectins rather than mAb.

*2.6. Examination of oocysts by microscopy*

Slides were examined using fluorescence and ultraviolet (UV) illumination as well as Nomarski Differential Interference Contrast (DIC) microscopy using Leica DMLB Fluorescence Microscope equipped with the following fluorescence filters: an ultraviolet filter block (excitation 335 nm; emission 450 nm) and blue filter block for FITC (excitation BP 450 nm; emission 490 nm). Oocysts were first located by using UV illumination for characteristic blue autofluorescence (Lindquist et al., 2003) and then examined for FITC-intensity exhibited.

All results were compared with staining of *Giardia* cysts labelled with FITC-WGA lectin (Ward et al., 1985), which was used as a model-staining organism, as the FITC fluorescence is bright and even over the entire cyst wall. Oocyst staining in both dry-labelling and suspension-labelling was evaluated in at least three experiments, and staining was classified qualitatively in comparison with *Giardia* cyst WGA-staining as intense (+++), moderate (++), weak (+), doubtful (+/−), or none (−), as previously described in several lectin-binding publications (Leal et al., 2011, 2012) (Fig. 1).

## 3. Results

*3.1. Monoclonal antibody staining*

Control oocysts (aged and fresh) that had not been labelled with mAb did not fluoresce under the FITC filter block.

Labelling of aged oocysts with mAb, with both dry-labelling and suspension-labelling, was weak, almost non-existent.

With dry-labelling, fresh oocysts were intensely stained with the mAb. However, stain intensity diminished with decreasing mAb concentration. Fresh oocysts labelled in suspension exhibited a consistent, intense staining despite reducing the concentrations of mAb (1:1, 1:5 or 1:10 using 0.1% bovine serum albumin in PBS).

*3.2. Lectin-binding assay on untreated oocysts*

The lectins WGA and SWGA provided an intense staining of the aged untreated oocysts in dry labelling, while binding to GSL I and SBA presented weak staining patterns. In suspension-staining,

**Table 1**
Panel of lectins tested for binding to *T. gondii* oocyst walls.

| Lectin | Abbreviation | Primary sugar specificity |
|---|---|---|
| Concanavalin A | CON A | α-Man, α-Glc |
| *Dolichos biflorus* agglutinin | DBA | α-GalNAc |
| Peanut agglutinin | PNA | Galβ3GalNAc |
| *Ricinus communis* agglutinin I | RCA I | Gal |
| Soybean agglutinin | SBA | α>βGalNAc |
| *Ulex europaeus* agglutinin I | UEA I | α-Fuc |
| Wheat germ agglutinin | WGA | GlcNAc |
| *Griffonia simplicifolia* lectin I | GSL I | α-Gal, α-GalNAc |
| *Lens culinaris* lectin | LCA | α-Man, α-Glc |
| *Phaseolus vulgaris* Erythroagglutinin | PHA E | Galβ4GlcNAcβ2Manα6(GlcNAcβ4)(GlcNAcβ4Manα3)Manβ4 |
| *Phaseolus vulgaris* Leucoagglutinin | PHA L | Galβ4GlcNAcβ6(GlcNAcβ2Manα3)Manα3 |
| *Pisum sativum* agglutinin | PSA | α-Man, α-Glc |
| Wheat germ agglutinin, succinylated | SWGA | GlcNAc |

Sugar abbreviations: Fuc: L-Fucose; Gal: D-Galactose; GalNAc: N-Acetylgalactosamine; Glc: D-Glucose. GlcNAc: N-Acetylglucosamine; Man: Mannose.

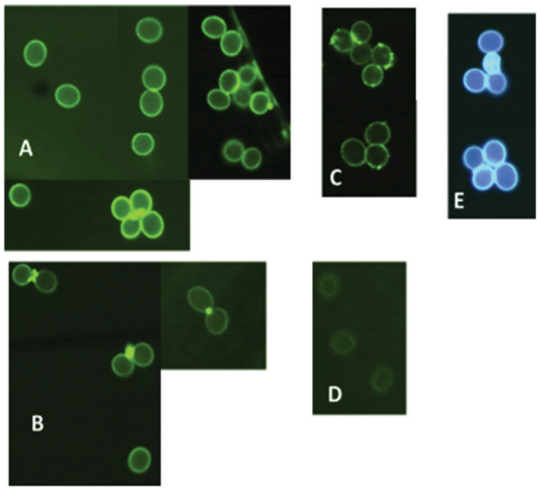


**Fig. 1.** Scoring of staining patterns of *Toxoplasma* oocysts. A: Intense (+++), B – Moderate (++), C – Weak (+), D – Doubtful (+/–), E – Autofluorescence.

most (>90%) oocysts displayed intense staining with both WGA and SWGA, but some oocysts did not stain with WGA. In these oocysts, staining of sporocyst walls could sometimes be observed. Based on the results obtained here, further lectin-binding studies used solely WGA.

None of the 13 lectins bound to the fresh untreated oocysts, with either dry-labelling or suspension-labelling.

### 3.3. Effect of oocyst pretreatment with detergents on labelling with mAb or lectins

Detergent pre-treatment of both aged and fresh oocyst suspensions had no effect on labelling with either mAb or lectins.

### 3.4. Effect of oocyst pretreatment with proteases on labelling with mAb or lectins

There was no change in either lectin or mAb staining of aged oocysts following pretreatment with proteases (Table 2).

Exposure of fresh oocysts to trypsin solutions did not result in any change in the staining with both mAb and WGA. However, fresh oocysts originally suspended in water then treated with varied concentrations of acid pepsin solutions for 18–24 h had an intense staining pattern with two concentrations of WGA. All observed oocysts stained. This staining was time-dependent, being moderate with oocysts exposed for 9 h and weak to none when exposure time was less (Table 2). Overall, the intensity of staining increased with the period of oocyst exposure to the acid pepsin solution, but did not depend on the concentrations of acid pepsin, as no significant variation was observed.

Fresh oocysts exposed to acid pepsin solutions and that had been stored in sulphuric acid and potassium dichromate solutions stained more intensely with WGA (1:50 concentration) starting from 7 h post exposure to acid pepsin than those stored in water (Table 2).

Staining with mAb was unaffected by the exposure of fresh oocysts to acid pepsin.

### 3.5. Effect of oocyst treatment with periodate on labelling with lectins or mAb

Although periodate treatment of oocysts had no effect on mAb activity in either aged or fresh oocysts, it reduced WGA labelling of aged oocysts (Table 3).

Fresh oocysts that had been exposed to acid pepsin and then labelled with WGA had their labelling slightly abrogated following exposure to sodium metaperiodate (Table 3).

## 4. Discussion

The main findings from these studies are a significant difference in the binding properties of oocyst walls from “aged” and “fresh” *T. gondii* oocysts. Whereas fresh oocysts bound a wall-specific mAb, aged oocysts did not. In contrast, the walls of aged oocysts bound a lectin (WGA), but fresh oocysts did not. However, exposure of fresh oocysts to acidified pepsin enabled labelling with WGA (which could be partially abrogated with periodate exposure), but did not affect mAb binding. These results are of relevance when considering technologies for isolating or detecting *T. gondii* oocysts in environmental samples, such as water, that can be based on oocyst surface properties (as used for *Cryptosporidium* and *Giardia*). It should be noted that the studies described here are laboratory-based, and the binding properties of oocysts isolated from environmental samples (e.g. from water samples) has yet to be performed.

**Table 2**
Effect of acid pepsin exposure on staining intensity in labelling of *Toxoplasma gondii* oocysts stored in water, sulphuric acid and potassium dichromate solutions with wheat germ agglutinin (WGA; 1:50) and monoclonal antibody (mAb).

| Storage medium | Treatment time/hour | 0.25% acid pepsin | | 0.5% acid pepsin | | Untreated | |
|---|---|---|---|---|---|---|---|
| | | WGA | mAb | WGA | mAb | WGA | mAb |
| Fresh oocysts in water | 1 | +/− | +++ | +/− | +++ | − | +++ |
| | 3 | + | +++ | + | +++ | − | +++ |
| | 5 | + | +++ | + | +++ | − | +++ |
| | 7 | + | +++ | ++ | +++ | − | +++ |
| | 9 | ++ | +++ | ++ | +++ | − | +++ |
| | 18 | +++ | +++ | +++ | +++ | − | +++ |
| | 24 | +++ | +++ | +++ | +++ | − | +++ |
| Fresh oocysts in sulphuric acid | 1 | + | +++ | + | +++ | − | +++ |
| | 3 | ++ | +++ | ++ | +++ | − | +++ |
| | 5 | ++ | +++ | ++ | +++ | − | +++ |
| | 7 | +++ | +++ | +++ | +++ | − | +++ |
| | 9 | +++ | +++ | +++ | +++ | − | +++ |
| | 18 | +++ | +++ | +++ | +++ | − | +++ |
| | 24 | +++ | +++ | +++ | +++ | − | +++ |
| Fresh oocysts in potassium dichromate | 1 | ++ | +++ | ++ | +++ | − | +++ |
| | 3 | ++ | +++ | ++ | +++ | − | +++ |
| | 5 | ++ | +++ | ++ | +++ | − | +++ |
| | 7 | +++ | +++ | +++ | +++ | − | +++ |
| | 9 | +++ | +++ | +++ | +++ | − | +++ |
| | 18 | +++ | +++ | +++ | +++ | − | +++ |
| | 24 | +++ | +++ | +++ | +++ | − | +++ |
| Aged oocysts in sulphuric acid | 1 | +++ | + | +++ | + | +++ | + |
| | 3 | +++ | + | +++ | + | +++ | + |
| | 5 | +++ | + | +++ | + | +++ | + |
| | 7 | +++ | + | +++ | + | +++ | + |
| | 9 | +++ | + | +++ | + | +++ | + |
| | 18 | +++ | + | +++ | + | +++ | + |
| | 24 | +++ | + | +++ | + | +++ | + |

Staining intensity scores.
+++: Intense.
++: Moderate.
+: Weak.
+/−: Doubtful.
−: No staining observed.

**Table 3**
Effect of sodium metaperiodate exposure on staining intensity in labelling of *Toxoplasma gondii* oocysts with monoclonal antibody (mAb) and wheat germ agglutinin (WGA).

| Oocysts | Acid pepsin | Monoclonal antibody (mAb) | | | Wheat germ agglutinin (WGA) | | |
|---|---|---|---|---|---|---|---|
| | | **P−/Mab+** | **P+/Mab+** | **P+/Mab− (controls)** | **P−/W+** | **P+/W+** | **P+/W− (controls)** |
| Fresh | Untreated | +++ | +++ | − | − | − | − |
| | Treated | +++ | +++ | − | +++ | ++ | − |
| Aged | Untreated | + | + | − | +++ | + | − |
| | Treated | + | + | − | +++ | + | − |

**P+** = periodate treated; **P−** = periodate untreated.
**W+**= WGA applied, **W−** =WGA not applied.
**mAb+** = monoclonal antibody applied; **mAb−** = monoclonal antibody not applied.
Staining intensity scores.
+++: Intense.
++: Moderate.
+: Weak.
−: No staining observed.

One previously published study investigated the binding capacity of mAb 3G4 (Dumetre and Darde, 2005), and found no alteration in binding capacity between fresh oocysts and those that had been stored for up to a year. The authors postulate that the oxidative conditions to which *T. gondii* oocysts are exposed during purification and storage did not affect the binding (Dumetre and Darde, 2005). The fact that our "aged oocysts" were even older (ten years) refrigerated (storage in 2% sulphuric acid) may indicate that changes do occur over very prolonged periods. However, the mAb that we used may also bind to a less stable epitope. However, protease (trypsin and acid pepsin) exposure did not affect binding of mAb, indicating that the binding occurred in domains that were not affected by these proteases.

Applications of lectins to clinical microbiology have been reviewed elsewhere (Slifkin and Doyle, 1990). Lectins (from the Latin *legere*: to choose) (Boyd and Shapleigh, 1954) are sugar-

binding proteins or glycoproteins of non-immune origin that agglutinate cells and/or precipitate glycoconjugates. Their specificities are usually defined in terms of the monosaccharide(s) or simple oligosaccharides that inhibit lectin-induced agglutination (precipitation or aggregation) reactions. They bear at least two-sugar binding sites, agglutinate animal and plant cells and/or precipitate polysaccharides, glycoproteins and glycolipids (Goldstein et al., 1980). The binding of the lectins WGA and SWGA to aged oocysts might suggest that N-acetyl-D-glucosamine (GlcNAC), specific for both lectins is expressed as a structural component of the walls. The lack of staining in walls of some aged oocysts might indicate gradual corrosion of its thickness and loss of the surface components, perhaps due partly to prolonged preservation. β-glucans are polysaccharides of D-glucose monomers linked by β-glycosidic bonds and are a diverse group of molecules with variable characteristics regarding molecular mass, solubility, viscosity, and three-dimensional configuration. *N*-acetyl-D-glucosamine (GlcNAc) is a monosaccharide derivative of glucose, an amide between glucosamine and acetic acid and the binding studies described here indicate that the indirect expression of GlcNAc, which is probably present as component of the β-1,3-glucan in the inner wall of *T. gondii* oocysts, as well as the sporocyst wall of sporulated oocysts.

Oocyst walls of *T. gondii* have been predicted to have two distinct layers that resemble those of fungi (β-1, 3-glucan in the inner layer) or mycobacteria (acid-fast lipids in the outer layer) (Dubremetz and Ferguson, 2009). Furthermore, the presence of β-1, 3-glucan in the oocyst walls of *T. gondii* (and *Eimeria*) as part of a trabecular scaffold in the inner layer of the oocyst wall has been described (Samuelson et al., 2013).

Previous work on *Giardia* cyst walls also assumed that WGA-binding indicated the presence of GlcNAc in the polymeric form of chitin (Ward et al., 1985). However, subsequent studies (Gerwig et al., 2002) suggest that the binding moiety was more likely to be a novel beta (1-3)-N-acetyl-D-galactosamine polymer (GalNAc), so here we do not try to define the precise carbohydrate composition, but rather indicate that WGA binds to the walls suggesting the presence of beta-linked poly-N-acetyl hexosamine containing polysaccharides.

In contrast, all fresh oocysts failed to show any binding for all tested lectins including WGA and SWGA. However, exposure to different concentrations of acid pepsin solutions enabled such labelling to occur, suggesting that a material covering the outer wall surface of the fresh oocysts could have been cleaved off by the action of acid pepsin. This indicates that the sugar binding WGA, i.e. possibly GlcNAC, occurs on the outer oocyst wall of *T. gondii* probably exposed by the application of acid pepsin solution. One possibility is that fresh oocysts are still enclosed in tissue remnants of the host intestinal epithelial cells or just the outer veil that covers the oocyst wall (Samuelson et al., 2013), preventing exposure of the moieties for lectin binding.

Moreover, lectins have previously been suggested for use for detection or purification of protozoan parasites in the absence of suitable mAb, and WGA binding has been used to isolate *Cyclospora* oocysts (Robertson et al., 2000), probably due to the affinity of WGA for beta-linked poly-N-acetyl hexosamine containing polysaccharides, which are likely to occur in the walls of *Cyclospora* oocysts (Bushkin et al., 2012).

From an epidemiologic viewpoint, the distinction of viable versus nonviable oocysts in environmental samples is important. Although we did not test the viability of either the aged or fresh oocysts, we assume that the aged oocysts were nonviable and the fresh oocysts were viable; the longest survival of oocysts at 4 °C was 1620 days (Dubey, 2010). mAbs are frequently used for identification of intestinal protozoa, but there are relatively few studies regarding the use of such tools for *T. gondii* oocysts.

In conclusion, the current study involving the binding properties of *T. gondii* oocyst walls to both mAbs and lectins provides information on the surface-binding properties of *T. gondii* oocysts suggesting potential approaches for the development of oocyst detection methods. Aged, stored oocysts and fresh (recently excreted) oocysts showed different binding characteristics, and pepsin treatment of fresh oocysts enabled lectin binding to resemble that of aged oocysts. Further investigations involving characterizations of the carbohydrate compositions and surface antigens of the oocyst walls of *T. gondii* at various ages could provide further elucidation of the binding mechanisms. The possibility of using a WGA-based separation procedure for isolating *T. gondii* oocysts from environmental matrices is suggested by these studies, provided that pepsin pretreatment is included to ensure that the moieties are available on all oocyst walls for the lectin to bind.

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# Paper II

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# Lectin-magnetic separation (LMS) for isolation of *Toxoplasma gondii* oocysts from concentrated water samples prior to detection by microscopy or qPCR

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ABSTRACT

Although standard methods for analyzing water samples for the protozoan parasites *Cryptosporidium* spp. and *Giardia duodenalis* are available and widely used, equivalent methods for analyzing water samples for *Toxoplasma gondii* oocysts are lacking. This is partly due to the lack of a readily available, reliable immunomagnetic separation technique (IMS). Here we investigated the use of lectin-magnetic separation (LMS) for isolating *T. gondii* oocysts from water sample concentrates, with subsequent detection by microscopy or molecular methods. Four different types of magnetic beads coated with wheat germ agglutinin (WGA) were tested for capture of oocysts from clean or dirty water samples. Dynabeads (Myone T1 and M-280) consistently provided mean capture efficiencies from 1 ml clean water in excess of 97%. High recoveries were also found with Tamavidin beads (in excess of 90%) when LMS was used for capture from a small (1 ml) volume. Dissociation (required for detection by microscopy) using 0.1N hydrochloric acid (HCl), as standard in IMS, was not successful, but could be achieved using a combination of acidified pepsin (AP) and *N*-acetyl D-glucosamine. Although simple centrifugation was as effective as LMS when concentrating high numbers of oocysts from clean water, LMS provided superior results when oocysts numbers were low or the water sample was dirty. Application of LMS integrated with qPCR enabled detection of 10 oocysts per 10 ml dirty water sample concentrate. These findings indicate that LMS with WGA coupled to magnetic beads could be an efficient isolation step in the analysis of water sample concentrates for *T. gondii* oocysts, with detection either by microscopy or by qPCR.



## 1. Introduction

The protozoan parasite *Toxoplasma gondii* has a worldwide distribution and is one of the most frequent parasitic infections (Dubey, 2004; Robert-Gangneux et al., 2015). Felids serve as definitive hosts and the environmentally resistant oocysts are excreted in their feces (Hutchison et al., 1969; Frenkel et al., 1970). Oocysts become infectious after sporulation in the environment and may survive for months in soil and water, thereby enhancing the probability of transmission to intermediate hosts. Almost all warm-blooded animals, including humans, can serve as intermediate hosts harboring *T. gondii* in the form of tissue cysts (Dubey, 2010).

Toxoplasmosis is a significant public health concern as infection can lead to serious consequences (Aramini et al., 1999), especially when the infection is acquired prenatally due to infection of naïve pregnant women shortly before or after conception. Such infection can lead to congenital toxoplasmosis, with consequences ranging from gross fetal abnormalities and spontaneous abortion to neonates being asymptomatic at birth but manifesting problems such

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as mental retardation and ocular disease much later in life (Gajadhar et al., 2006). *Toxoplasma* infections can also be acquired postnatally through various routes, including direct contact with cat feces containing sporulated oocysts, ingestion of sporulated oocysts from contaminated food or water or ingestion of tissue cysts in raw or undercooked meat (Dubey, 2010; Fayer et al., 2004). Infection in immunocompromised individuals can be life threatening, with encephalitis and brain abscess among the most serious manifestations (Hill et al., 2005). Although most infections in healthy individuals are asymptomatic, certain *T. gondii* strains (genotype 1) may cause symptoms in otherwise immunocompetent humans (Grigg et al., 2001).

Waterborne toxoplasmosis has emerged as a significant public health concern following the occurrences of large human outbreaks linked to oocyst contamination of water supplies as a source of infection in several countries (Jones and Dubey, 2010; Shapiro et al., 2010). Circumstantial evidence suggests that oocyst-induced infections in humans are clinically more severe than tissue cyst-acquired infections (Jones and Dubey, 2010). Oocysts can remain viable for longer periods in water and resist both freezing and moderately high water temperatures (Dubey, 1998; Frenkel and Dubey, 1973). In addition, oocysts are able to survive the effects of chemical and physical treatments currently applied in water treatment plants, including chlorination and ozone treatment (Dumetre et al., 2008).

Detecting contamination of water supplies with *T. gondii* oocysts is difficult because no standardized methods are available. For other protozoan parasites associated with waterborne transmission, *Cryptosporidium* spp. and *Giardia duodenalis*, standardized methods have been developed and are applied widely (e.g., ISO, 2006; US EPA, 2012). These methods were developed from relatively crude techniques involving high volume filtration, elution, concentration, and detection by light microscopy to the development and use of immunofluorescent antibody tests for detection in the late 1980s (e.g., Hayes et al., 1989) to the more recent use of molecular assays for genotyping. One important improvement in the analytical procedure was the development of immunomagnetic separation (IMS) for isolation of *Cryptosporidium* oocysts from water in the late 1990s (Campbell et al., 1997). Use of IMS, enabled oocysts to be separated from background debris, improving detection efficiency. However, there are currently no commercially available IMS techniques or immunofluorescent staining reagents for *T. gondii* oocysts (Dumetre and Darde, 2005).

Recent investigations on the surface-binding properties of *T. gondii* oocysts have indicated that they can bind to the lectin wheat germ agglutinin (WGA), and it has been suggested that this binding could be utilized to develop a lectin-magnetic separation (LMS) technique for improving the analysis of water samples, and other environmental samples, for *T. gondii* oocysts (Harito et al., 2016).

The current study investigates the performance of different magnetic beads coupled to WGA in the capture and separation of *Toxoplasma* oocysts from water samples (LMS) and detection using both microscopy and real-time PCR.

## 2. Materials and methods

### 2.1. Oocyst spiking and recovery

#### 2.1.1. T. gondii oocysts

Oocysts used in this study were from the ME 49 strain, as described previously, and were stored as described earlier (Harito et al., 2016).

Sporulation was induced by incubating aliquots of oocyst suspension at room temperature (15–25 °C). After confirming oocyst sporulation, all suspensions were kept refrigerated in the dark at 4 °C until the current study. Before use, aliquots of oocyst suspension (100 μl) were resuspended in 900 μl of 0.5% acidified pepsin, incubated at 37 °C for 24 h and washed four times in 0.1% BSA in PBS. Treated oocysts were then resuspended in 1 ml 0.1% BSA in PBS.

Oocyst enumeration was performed using KOVA GLASSTIC Slide 10 with grids (Fisher Scientific).

#### 2.1.2. Magnetic beads and their preparation

Four different magnetic beads were investigated (Table 1). Three different sizes of beads coated with streptavidin or avidin (Dynabeads™ M-280 Streptavidin and Myone™ Streptavidin T1; ThermoFisher Scientific, Oslo, Norway and SPHERO™ Avidin magnetic particles; Spherotech Inc, Lake Forest, IL, USA) were used to investigate whether dissociation could be improved by the increased shear sources with larger beads. In addition Tamavidin-coated beads (Tamavidin® 2-REV magnetic beads (Wako Pure Chemical Industries, Ltd., Japan)) were also investigated due to the lower biotin dissociation constant being hypothesized to improve dissociation (streptavidin and avidin have $K_D \approx 10^{-15}$ M; tamavidin $K_D \approx 10^{-7}$ M).

#### 2.1.3. Lectins

Wheat germ agglutinin lectin from *Triticum vulgaris* (wheat) conjugated to biotin (Sigma-Aldrich Norway AS), suspended in phosphate buffered saline (PBS, pH 7.4) at a concentration of 1 mg/ml was used for coupling with magnetic beads pre-coated by one of the biotin-binding proteins (Streptavidin or Avidin or Tamavidin 2-REV).

All beads (Table 1) were prepared according to the manufacturers' protocols, and then bound to biotin-WGA, by incubating for 1 h on a rotating mixer at 15 r.p.m. as follows: for the Myone Streptavidin T1 beads, 40 μl of Biotin-WGA was added to 200 μl 10 mg/ml stock beads; for the other three beads (M-280 Streptavidin beads, the avidin beads and the Tamavidin beads) 20 μl of Biotin-WGA was added to 200 μl 10 mg/ml stock beads. After coating, the beads were washed four times in 1 ml of washing buffer (PBS, pH 7.4) using a magnet (Dynal MPC-S, ThermoFisher Scientific, Oslo, Norway) to separate beads from the supernatant and then re-suspended back to a concentration of 10 mg/ml.

#### 2.1.4. Isolation of bead-bound oocysts (LMS)

*2.1.4.1. Capture in clean water.* This experiment was evaluated in small (1 ml) and large (10 ml) volumes. All tests were performed in independent triplicates. Tests using 1 ml volume (clean water) were performed to assess all beads (Table 1) using different incubation buffers (0.1% BSA in PBS and SL Buffer, Dynal GC Combo kit). In order to investigate capture and recovery, 20 μl aliquots of the suspension containing an estimated 1000 oocysts was spiked into 970 μl incubation buffer (0.1% BSA in PBS or SL buffer Dynal GC Combo kit) in a 1.5 ml microfuge tube and 10 μl of prepared beads (Dynabeads or SPHERO Avidin) were added. The mixture was incubated for 1 h at room temperature on a rotating mixer at 15 r.p.m. For the Tamavidin 2-REV magnetic beads, the amount

**Table 1**
Characteristics of the beads tested in this study for LMS following coating with WGA.

| Beads | Bead diameter (μm) | Concentration used in LMS |
|---|---|---|
| Dynabeads™ M-280 Streptavidin | 2.8 | 100 μg/ml |
| Dynabeads™ Myone Streptavidin T1 | 1.0 | 100 μg/ml |
| SPHERO™ Avidin beads | 4.0–4.5 | 100 μg/ml |
| Tamavidin® 2-REV beads | 2.8 | 250 μg/ml |

used was 25 μl mixed with 955 μl incubation buffer (0.1% BSA in PBS or SL buffer, Dynal GC Combo) with the same quantity of oocysts and processed similarly.

For capturing bead-bound oocysts, the tube was placed in a magnet (Dynal MPC-S) for 2–3 min and the supernatant transferred to a second tube (for enumeration of unbound oocysts).

For large volume (10 ml) processing, only two of the beads (M-280 Streptavidin and Tamavidin 2-REV) were evaluated using a protocol based partly on the method reviewed by Robertson et al. (2000). Oocysts (approximately 1000) were inoculated into 8.9 ml laboratory grade water. Briefly, the 8.9 ml sample was added to a L10 tube (Dynal, ThermoFisher Scientific, Oslo, Norway) with 1 ml of SL-buffer A ( × 10 concentrate). The WGA-coupled magnetic beads were thoroughly mixed, and 100 μl added to the L10 tube containing the sample and buffer. The tube was capped and its contents mixed by slow rotation (approx. 25 rpm) at room temperature for 1 h using a Dynal sample mixer (Dynal, ThermoFisher Scientific, Oslo, Norway). The beads were then separated from other debris in the sample by placing the L10 tube in a specially designed magnetic particle concentrator (MPC; Dynal MPC-1, Dynal, ThermoFisher Scientific, Oslo, Norway), which was rocked gently by hand for 2–3 min (as used in standard IMS for *Cryptosporidium* and *Giardia*). With the L10 tube remaining in the MPC, the fluid was transferred to a 15 ml test tube for further analysis for unbound oocysts (using centrifugation). The L10 tube was removed from the MPC, and the beads were gently resuspended in 1 ml of SL-buffer A ( × 1 concentrate). The buffer with beads were transferred to a 1.5 ml microfuge tube and the beads collected using another MPC (Dynal MPC-S) following a 2–3 min rocking period. The supernatant was again transferred to a different microfuge tube for recovering unbound oocysts.

*2.1.4.2. Dissociation of bead-bound oocysts captured from clean water.* Dissociation of oocysts from beads is important for detection by microscopy, as the beads can occlude the oocysts. In preliminary trials, dissociation was attempted on single replicates using different concentrations of hydrochloric acid (0.1 N, 0.2 N, 0.5 N, and 1 N), sulfuric acid (2%), sodium hydroxide (1 M), *N*-acetyl D-glucosamine (GlcNAc) (0.5 M) with acidified pepsin (0.5%, 1%), and biotin (2 mmol/l) solutions. Based on preliminary data (not shown) more thorough dissociation trials were attempted using hydrochloric acid (0.5 N), GlcNAc (0.5 M) mixed with acidified pepsin solution, or biotin (only for Tamavidin beads).

Dissociation trials were conducted in triplicate. To each tube containing the magnetic concentrated oocyst-bead complexes was added 100 μl of test dissociation reagents, vortex-mixed thoroughly, and incubated for 20 min at room temperature. Tubes were vortex-mixed 3–4 times at regular intervals during the incubation period, the beads separated using the MPC-S magnet, and the oocysts in the dissociation reagent transferred to slide wells. This was repeated three times for most reagents to investigate the effect of triple dissociation. Finally, the beads themselves were resuspended in 50–100 μl PBS (depending on the quantity) and transferred to a new slide wells and the supernatant of the incubation buffer was collected, centrifuged and the pellet transferred to a different slide well to evaluate the oocysts that are unbound to the beads. All slides were then incubated for 30 min at 37 °C to dry. A drop of methanol solution was added to each slide well to fix and then overlaid by 10 μl of DABCO mounting medium, and covered with 50 × 24 mm cover glass.

*2.1.4.3. Capture in dirty water concentrates.* For dirty water capture, only M-280 Streptavidin beads were evaluated and tests were carried out in 10 ml and 50 ml sample volumes. Concentrates of dirty water were obtained from 10 L of raw water that was collected from a local lake (Sognsvann, Oslo) and concentrated by membrane filtration using a 2.0 μm membrane filter. For 10 ml dirty water samples the procedure was the same as for 10 ml clean water samples (section 2.1.4.1). For 50 ml volumes, the samples were first centrifuged at 1000 × g for 10 min and then aspirated to 10 ml (packed pellet of approx. 0.25 ml), before being processed as for 10 ml clean water samples.

*2.1.4.4. Oocysts concentrated by centrifugation.* For comparative experiments of LMS with concentration for detection of oocysts from water without using LMS (NLMS; i.e. non-LMS), centrifugation was used and the final pellet obtained used for detection. For dirty water, this resulted in a pellet of between 200 and 250 μl. For microscopy, the whole pellet was carefully transferred to a slide well, dried, and examined as a whole to determine the comparative recovery efficiency.

### 2.2. Oocyst detection and quantification

Oocysts were detected and quantified using either microscopy and counting or qPCR.

#### 2.2.1. Detection and enumeration of oocysts by microscopy

Oocysts were visualized using ultraviolet (UV) illumination based on characteristic blue autofluorescence that they emit (Lindquist et al., 2003) as well as Nomarski Differential Interference Contrast (DIC) microscopy using Leica DMLB Fluorescence Microscope equipped with an ultraviolet filter block (excitation 335 nm; emission 450 nm). The whole area of the slide well was screened in a systematic, side-to-side manner at × 200 and × 400 magnification, and oocysts counted using a tally counter.

#### 2.2.2. Detection and quantification of oocysts by qPCR

*2.2.2.1. Sample processing and DNA extraction.* Samples for qPCR assay were prepared simultaneously in two groups; 1) oocysts captured using LMS and 2) oocysts concentrated by centrifugation (NLMS).

*2.2.2.1.1. Spiking experiment in clean water.* This test was performed in triplicate for LMS (WGA-M-280 beads) and NLMS with 1 ml clean water samples spiked with concentrations of approximately 1000, 100, 10, 1 and 0 oocysts. LMS samples were processed as described in section 2.1.4.1 with DNA extraction directly from the bead-bound oocyst complexes (no dissociation step). NLMS samples were centrifuged first and DNA extracted from the pellet.

For the LMS samples, the bead-oocyst complexes were resuspended in 200 μl laboratory grade water before the commencement of DNA extraction steps. For both LMS and NLMS samples, a previously described protocol (Su and Dubey, 2010) was used for DNA extraction with the QIAamp DNA Mini Kit (Qiagen, Norway), with few modifications. Briefly, the bead/oocyst suspension (LMS) or the pellet obtained by centrifugation (NLMS) were resuspended in 180 ml ATL lysis buffer, followed by 6 freeze-thaw cycles in liquid nitrogen and water bath set at 90 °C. Heat-inactivated Phocine herpes virus 1 was included in each sample as internal control before mixing just before adding Proteinase K and incubation at 56 °C overnight. The samples were then vortexed vigorously and incubated with 200 μl Buffer AL at 70 °C for 1 h. Before adding ethanol (96–100%), the beads (LMS) or insoluble particles (NLMS) were removed, either by placing the sample tubes on a magnet (MPC-M) or centrifugation at 11,000 × g for 10 min, respectively. The supernatant was then transferred to a new tube and the old tube with beads or insoluble particles discarded. Further steps of washing were performed using 500 μl of wash buffers (Buffer AW1 and AW2) according to the manufacturer's protocol. Using 50 μl ultrapure water, the DNA was eluted from the binding columns. The

DNA was stored at −20 °C until qPCR was performed.

*2.2.2.1.2. Spiking experiments in dirty water samples.* As with clean water samples, dirty water samples (50 ml), prepared as described in section 2.1.4.3, were performed in triplicate with LMS or NLMS (centrifugation). Each 50 ml sample, was first spiked with 1000, 100, 10, 1, or 0 oocysts. The tubes were then centrifuged at 1000 × g for 10 min and the supernatant discarded. For LMS, the pellet was transferred to L10 tube for LMS in 10 ml volume, whereas for NMLS the pellet was transferred to 1.5 ml micro-centrifuge tube and re-centrifuged at 2000 × g for 10 min to obtain a 200 μl pellet for DNA extraction. The LMS sample in the L10 tube was processed as described for LMS in Section 2.1.4.3. For both concentrates (pellet or oocyst/bead complex for NMLS and LMS, respectively) DNA extraction was as described in Section 2.2.2.1.1.

*2.2.2.2. Real-time PCR (qPCR assay).* The qPCR assay for detection and quantification of *T. gondii* oocysts targeted the 529 bp repeat element (Homan et al., 2000). The choice of target and primers was based on in-house experiments using conventional nested PCR to compare amplification (data not provided) of both B1 gene and 529 bp repeat element using DNA extracted from both old and fresh oocysts of *T. gondii*. Previously published primers were used (Tox-9F; AGGAGAGATATCAGGACTGTAG and Tox-11R; GCGTCGTCTCGTC TAGATCG) (Opsteegh et al., 2010; Su and Dubey, 2010) that are complementary to the 529-bp repeat element (GenBank AF487550) producing a target amplicon of 162bp (Reischl et al., 2003; Su and Dubey, 2010). All PCR reactions were carried out in triplicate. The amplification mixtures consisted of 10 μl KiCqStart SYBR Green qPCR ReadyMix, Low ROX (2×) (KCQS01 SIGMA); 0.40 μM Tox-9F and 0.40 μM Tox-11R primers, molecular grade $H_2O$ and 5 μl DNA template to provide a final reaction volume of 20 μl. The PCR amplification was performed in a Mx3005P (Agilent Technologies, Inc.) thermal cycler with a SYBR green protocol with dissociation curve. Initial denaturation was at 95 °C for 3 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 1 min, and fluorescent measurement after a final extension step at 72 °C for 15 s. Dissociation curves were made by increasing the temperature from 55 °C to 95 °C with continuous fluorescent measurement. Negative controls (water) were added in each run.

To monitor the effect of PCR inhibitors in the samples, an internal amplification control was included during DNA isolation (Section 2.2.2.1.1) as described previously (Opsteegh et al., 2010; Wells et al., 2015). Amplification of the internal control DNA was performed by addition of primers provided for this purpose (PhHV-1F; PhHV-1R) in separate reaction wells simultaneous to the qPCR assay for *Toxoplasma*.

A standard curve was prepared using serial dilutions of *T. gondii* DNA extracted from 10,000 oocysts diluted 1:5; 1:50; 1:500 and 1:5000 (corresponding to 2000, 200, 20, and 2 oocysts). Samples recorded as positive when the Ct values were 40 cycles or less and exhibited a smooth exponential curve in at least two of the triplicate samples compared with the standards curves. Samples without amplification of template DNA but positive for internal amplification control DNA were considered negative.

### 2.3. Data management

All data generated were imported to Microsoft Excel spreadsheet and analyzed using STATA version 14.0 for Windows (Stata Corp. College Station, TX, USA). One-way analysis of variance (ANOVA) was used to investigate the effects of bead type and buffers used on recovery efficiency using proportions as individual data input. The effects of addition or exclusion of buffers were compared by student t-tests. Statistical significance was set at $P < 0.05$.

Descriptive statistics such as mean and confidence intervals were calculated to show the effects of considered variables with respect to oocyst recoveries in a summarized form using tables and graphs. Mean capture efficiency was calculated using the average counts of three trials for each test, considering all oocysts counted by bead capture and unbound oocysts recovered simultaneously.

$$\text{Mean capture efficiency } (\%) = [A/\ (A + B)] * 100$$

where A is the mean (n = 3) count of oocysts (bead-bound) captured and separated; B is the mean (n = 3) count of unbound oocysts recovered by analysis of supernatants, and A + B is the number of oocysts in the reaction.

The limit of detection (LOD) was determined for qPCR results by using oocysts diluted in a series. qPCR results following either LMS and NLMS are presented by ranges of threshold cycle (Ct) values.

## 3. Results

### 3.1. Oocyst capture from 1 ml volumes of clean water

The capture results by buffer, bead type, and oocyst sporulation status are summarized in Table 2. Both WGA-Streptavidin (Myone T1 and M-280) beads showed a significantly ($P < 0.05$) higher mean capture when used in buffered conditions than in laboratory grade water (Fig. 1). There was no significant difference ($P > 0.05$) in the performance between two streptavidin beads. Buffer formulation (0.1% BSA/PBS, 1× SL Buffer A, 1× SL Buffer A + B) did not affect capture efficiency ($P > 0.05$).

WGA-Tamavidin 2-REV beads also performed very well and consistently captured and separated a high proportion of oocysts, but the efficiency was lower than with the streptavidin beads ($P < 0.05$).

WGA-Avidin beads displayed some inconsistent and lower capture efficiencies, apart from in Dynal SL buffer A where capture was above 70%, but still lower than achieved with the other beads.

Oocyst sporulation status apparently had no effect ($P > 0.05$) on the binding and capture activity of M-280 Streptavidin and Tamavidin 2-REV beads (Table 2).

Analysis of the supernatant for unbound oocysts, indicate that the total of oocysts found (captured oocysts and oocysts in the supernatant) was very close to the estimated number of oocysts spiked. Thus, there seems to have been minimal oocyst loss due to adsorption to the walls of the tubes and pipette tips during the capture procedures.

### 3.2. Dissociation of bead-bound oocysts

Although capture of oocysts with some of the tested beads was extremely high (typically > 97%), for optimal detection by microscopy the oocysts need to be dissociated from the beads. Using the conventional 0.1 N HCl-based dissociation, as used in IMS for *Cryptosporidium* and *Giardia*, oocyst dissociation from the beads was low, particularly for Myone Streptavidin T1 and SPHERO Avidin beads (data not shown). For higher concentrations (0.5 N HCl), dissociation remained low for Myone Streptavidin T1 and SPHERO Avidin beads, even when the dissociation was repeated in triplicate, but was moderate for M-280 beads (Table 3). Among the other dissociation reagents investigated (sulfuric acid solution (2%), sodium hydroxide (1 M), *N*-acetyl D-glucosamine (0.5 M), acidified pepsin (0.5%, 1%), and biotin (2 mmol/l)), sulfuric acid and sodium hydroxide did not result in good dissociation. However, 0.5M GlcNAc and 1% acidified pepsin solution (1:1) provided relatively high dissociation for M-280/WGA bound oocysts and 2.0 mmol/L

**Table 2**
*Toxoplasma gondii* oocyst capture efficiency from 1 ml clean water using four different WGA-labelled magnetic beads.

| Reaction buffer | Oocyst status | Mean % capture efficiency of beads (95% CI) | | | |
|---|---|---|---|---|---|
| | | Dynabeads Myone T1 | Dynabeads M-280 | SPHERO Avidin | Tamavidin 2-REV |
| BSA/PBS | Unsporulated | 98.65 (97.95–99.35) | 98.06 (97.16–98.96) | 49.34 (45.84–52.84) | ND |
| BSA/PBS | Sporulated | ND | 97.16 (95.88–98.44) | ND | 89.85 (87.44–92.26) |
| SL Buffer A | Unsporulated | 99.63 (99.29–99.97) | 99.76 (99.48–100) | 77.9 (75.4–80.4) | ND |
| SL Buffer A + B | Unsporulated | 99.55 (99.16–99.94) | 98.58 (97.92–99.24) | 28.58 (25.75–31.41) | 90.38 (88.49–92.27) |

ND: not done; CI: confidence interval.

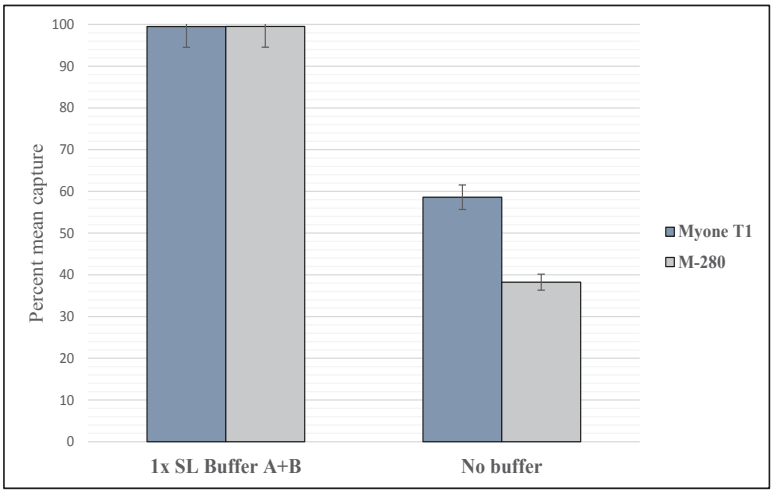


**Fig. 1.** Effect of buffer on capture of unsporulated *T. gondii* oocysts with two different WGA-streptavidin beads.

biotin resulted in high dissociation of oocysts from Tamavidin 2-REV beads (Table 3).

### 3.3. Oocyst capture from larger volumes of clean and dirty water

Capture of oocysts from larger volumes of clean water was investigated for the highest-performing WGA-coated beads from 1 ml volumes of clean water (M-280 streptavidin beads and the Tamavidin beads). For the Tamavidin beads, which showed both high capture in 1 ml and good dissociation using biotin, the bead-oocyst complex was found to be unstable when transferring from the L10 tube to the microfuge tube, with loss of oocysts. Thus, focus was then placed on M-280 beads and these were the only beads tested in the large volumes of dirty water, with LMS using the WGA-coated M-280 beads compared with centrifugation as a concentration method (NLMS). In clean water samples, LMS and NMLS had comparable efficiencies, but for dirty water samples, recovery was significantly higher with LMS than by centrifugation (Fig. 2).

### 3.4. Bead-based oocyst recovery assessment using qPCR

Recovery of oocysts from a small volume of clean water (laboratory grade) was similar using both bead-based (LMS) and non-bead based (NLMS) concentration procedures, and DNA was detected at a level of 10 oocysts per 1 ml in both experiments (Table 4). However, only one of three samples was detectable at this low level when concentration was by NLMS (centrifugation) compared with LMS (3 out of 3 samples). In the experiments with

**Table 3**
Dissociation of oocysts from different WGA-magnetic beads using different potential dissociation agents (proportion of oocysts released from the beads).

| Reagent | Mean dissociation (95% CI) | | | |
|---|---|---|---|---|
| | Dynabeads Myone T1 | Dynabeads M-280 | SPHERO Avidin | Tamavidin 2-REV |
| HCl (0.5N) | 14.43 (12.22–16.64) | 52.51 (49.33–55.69) | 9.22 (6.34–12.1) | ND |
| GlcNAc (0.5 M) + AP (1%) | ND | 88.87 (86.31–91.43) | ND | ND |
| Biotin (2 mmol/L) | ND | ND | ND | 95.46 (94.05–96.87) |

ND: not done; CI: confidence interval.

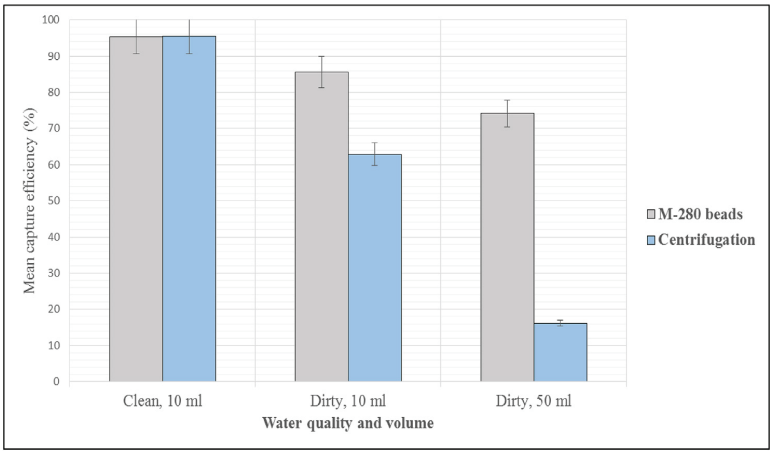


**Fig. 2.** Effect of particulate matter in water samples on *T. gondii* oocyst recovery using LMS (WGA-M-280 streptavidin beads) compared with centrifugation.

**Table 4**
Detection of DNA from oocysts by qPCR with oocyst concentration prior to DNA isolation by LMS using WGA-magnetic beads (M-280) or NLMS (centrifugation).

| Method | Water | Number of oocyst spiked and detection limits | | | |
|---|---|---|---|---|---|
| | | 1000 | 100 | 10 | 1 |
| | | Positive/N (CT range) | Positive/N (CT range) | Positive/N (CT range) | Positive/N (CT range) |
| LMS | Clean, 1 ml | 3/3 (22.59–23.21) | 3/3 (25.15–26.18) | 3/3 (28.3–29.19) | 3/3 (30.14–34.01) |
| | Dirty 10 ml | 3/3 (22.55–27.46) | 3/3 (29.90–33.13) | 2/3 (30.80–32.45) | ND |
| NLMS | Clean, 1 ml | 3/3 (23.11–23.22) | 3/3 (25.66–25.87) | 3/3 (27.78–29.79) | 1/3 (29.23) |
| | Dirty 10 ml | 3/3 (25.53–28.04) | 3/3 (30.73–32.10) | 0/3 – | ND |

dirty water, use of LMS enabled detection of 10 oocysts in 10 ml sample concentrate volumes, whereas with NLMS it was not possible to detect such low concentrations, and 10 oocysts per 10 ml dirty water sample concentrate were not detected when NMLS (centrifugation) was used to concentrate the oocysts (Table 4; Fig. 3).

Based on amplification of the internal control, none of the reactions seemed to be affected by inhibitors (data not shown).

## 4. Discussion

The main result from this study is the development of an LMS method for isolating and concentrating *Toxoplasma* oocysts from water sample concentrates for their subsequent detection by either microscopy or molecular methods. The best capture and dissociation was obtained using WGA-coated Dynabeads™ M-280 Streptavidin beads. Furthermore, the bead-oocyst complex obtained with capture of oocysts with these beads was stable during transfer from L10 tubes to microfuge tubes.

Although the use of LMS in clean water to recover the oocysts did not show any significant advantage over simple centrifugation when oocyst concentrations were high, at low concentrations of oocysts (theoretical dilution of 1 oocyst per ml) LMS was more likely to provide a positive result than centrifugation. As oocysts can be expected to occur in low concentrations in water, it is important that the limit of detection is low. Furthermore, environmental water samples may contain a variety of particulate and other contaminants, and it is important that analytical methods can operate not only in ideal clean lab samples, but also in natural samples. The LMS method was able to detect *Toxoplasma* oocysts even when present at relatively low numbers (10 oocysts/10 ml) in dirty water concentrates, which was not achieved when LMS was not used.

The application of magnetic beads for isolation of protozoan parasites from water is not new, as it has been used for isolation of *Cryptosporidium* oocysts and *Giardia* cysts during the analysis of water samples and fresh produce samples for several decades, and been incorporated into the standard methods (ISO, 2006; US EPA, 2012). However, for these parasites immunomagnetic separation (IMS) is used as the beads are coated with monoclonal antibodies (mAb) specific to these protozoa. However, mAbs against *T. gondii* oocysts are not commercially available and one mAb that we tested previously (Harito et al., 2016), and gave promising results against fresh oocysts did not bind older oocysts. As *Toxoplasma* oocysts can survive for prolonged periods in the environment, it is important that any capture or detection method is equally suitable for both old and fresh oocysts. In previous studies for development of IMS for *Toxoplasma* (Dumetre and Darde, 2005) using an in-house mAb, oocyst age does not seem to have had any effect. However, although some IMS results appeared promising in clean samples, when samples containing other debris were used, the recovery efficiency decreased substantially (Hohweyer et al., 2016). For a study using IMS for which the mAb is not freely available, or the IMS is not commercially produced, it is not possible for independent

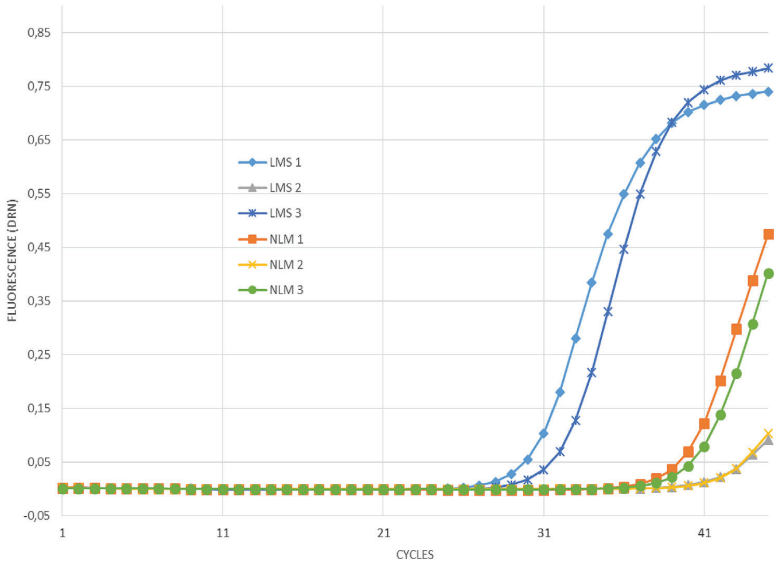


**Fig. 3.** Amplification curve illustrating detection of DNA from spike of 10 oocysts into turbid water sample concentrates Prior to detection the oocysts were concentrated either by LMS (LMS1 (blue), LMS2 (grey), LMS3 (blue)) or by NLMS (NLM1 (orange), NLM2 (yellow), NLM 3 (green)). Samples considered positive at Ct values of 40 cycles or less.

researchers to replicate the results. However, with LMS it is possible for researchers to buy the necessary reagents and create their own beads.

Development of an LMS method requires testing of different lectins, bead types, capture volumes, buffers, and dissociation reagents (if required). These factors will affect not only binding and capture efficiency, but also the efficiency of dissociation. For example, it may be easier to dissociate oocysts from larger beads due to greater shear forces being produced with shaking. Previous work had already demonstrated that WGA was the lectin of choice (Harito et al., 2016), and although recoveries with SPHERO Avidin beads was relatively low and variable, using Dynabeads Streptavidin magnetic beads (Myone T1 and M-280) coupled to WGA consistently provided mean capture efficiencies from 1 ml clean water in excess of 97%. This result appears comparable or better to previous report regarding to oocyst capture and separation. High recoveries were also found with the Tamavidin beads (in excess of 90%) when a single LMS was used for capture from a small (1 ml) volume, but was compromised when the oocysts were spiked into larger volumes than 1 ml, requiring initial capture in 10 ml, followed by transfer to a smaller tube. Our results indicate that when using the Tamavidin beads the oocyst-bead complex was insufficiently stable to remain intact during the transfer procedure, as most oocysts seemed to dissociate before the final separation.

Unpublished data from Campbell (1999, cited by Robertson et al. (2000)), used beads coated with a lectin (WGA) and reported a recovery efficiency in excess of 60% for isolation of *Cyclospora* oocysts from 10 ml water samples. The higher recovery achieved for *Toxoplasma* oocysts than for *Cyclospora* oocysts may reflect the unexpectedly high avidity of WGA for *Toxoplasma* oocyst walls, as discussed in greater detail with respect to dissociation. Robertson et al. (2000) also used lectin (WGA)-coated paramagnetic beads for the isolation of *Cyclospora* oocysts from fruits and vegetables with an overall recovery efficiency of 12%. Although this indicates the potential for LMS of these related coccidian parasites, the reduction in recovery efficiency from 60% to 12% indicates also that some types of interfering debris, particularly that associated with fresh produce, may have a significant effect on performance.

Our experiments demonstrated the importance of using a buffered suspension for capture. This phenomenon has been noted previously for IMS of *Toxoplasma* oocysts (Dumetre and Darde, 2005) and also for *Cryptosporidium* and *Giardia* (Utaaker et al., 2015). In our experiment we found that use of the buffers supplied with the Dynabeads Crypto and Giardia IMS kit (SL buffers) or a self-made buffer (0.1% BSA in PBS) were both equally effective.

For analysis of water samples for *Cryptosporidium* and *Giardia*, immunofluorescent antibody tests (IFAT) is usually used (ISO, 2006; US EPA, 2012). This probably reflects the availability of very good mAb that can be labelled with fluorogenic markers, as well as the historical reasons of IFAT being optimized for parasites while molecular methods were in their infancy. In the absence of suitable fluorochrome-labelled mAb for *Toxoplasma* oocysts, detection in water samples may rely on microscopy-based methods (e.g., Lora-Suarez et al., 2016), epifluorescent microscopy to detect autofluorescent *T. gondii* oocysts (Shapiro et al., 2010; Verant et al., 2014), or molecular methods (Mahmoudi et al., 2015; Shapiro et al., 2010; Wells et al., 2015). Although molecular methods are of increasing importance, and enable genetic characterization, Shapiro et al. (2010) reported higher recovery efficiencies when microscopy was used. However, Verant et al. (2014) noted the importance of confirming the detection of *Toxoplasma* oocyst-like structures by polymerase chain reaction and sequence analyses. One problem with using a bead-based method for separation followed by microscopy for detection is that the beads themselves may occlude the oocysts and thereby limit detection. For *Cryptosporidium* and *Giardia* that are separated by IMS, an acid dissociation step is included in the procedure, such that the beads are removed from the concentrate prior to detection by IFAT. Thus, we were also interested in including a dissociation procedure in our method, should the detection method of choice be microscopy-based.

In our work, we found that the conventional acid dissociation approach using 0.1N hydrochloric acid (HCl) was not successful. Antibody-antigen links are readily affected by pH with high acidity

or alkalinity causing conformational changes in antibodies that affects antigen complementarity (Chivers et al., 2011; Reverberi and Reverberi, 2007). The binding between the *Toxoplasma* oocyst and the beads used here is based on two links, the biotin-(strept)avidin link and the WGA-oocyst link. The biotin-streptavidin is one of the strongest non-covalent links in nature (Stayton et al., 1999), and is unlikely to be disrupted by shaking in acid, but carbohydrate-protein links are generally rather weak (Lienemann et al., 2009). Although, the WGA-oocyst links might not be expected to be as strong as antibody-antigen binding (antibodies with high affinity have dissociation constants of around $10^{-7}$ M, compared with $5 \times 10^{-5}$ M for WGA and its sugar targets; Lienemann et al., 2009), we were unable to use alkalinity or acidity accompanied by shear forces to break them. However, we were able to achieve satisfactory dissociation from WGA-streptavidin magnetic beads (M-280) using a combination of acidified pepsin and GlcNAc, a sugar predicted to have affinity for WGA, was anticipated to block free WGA sites on the beads, thereby preventing re-binding.

It is probable that the effect from acidified pepsin is due to its role in the digestion of protein components, resulting in the removal of some binding components in the oocyst-bead complex. Although dissociation of oocysts from the Tamavidin bead-bound oocysts using 2 mmol/L biotin (which would compete with the Tamavidin binding) was also successful (in excess of 95%) when tested in 1 ml clean water, the lack of stability of the bead-oocyst complexes when transferring from larger to smaller tubes excluded further use of these beads.

For molecular detection methods, a dissociation step is not required as the DNA can be isolated from the separated target (Olsvik et al., 1994). In addition, molecular methods are useful as they preclude any concern about misidentification of *Toxoplasma* oocysts due to their morphological similarity to other coccidian oocysts such as *Hammondia* species or *Neospora caninum*, that may also be isolated by LMS due to the lack of specificity of WGA; its binding to *Cyclospora* oocysts has already been noted (Robertson et al., 2000). In the current study we targeted the 529bp repeat element using previously published qPCR primers as this has been demonstrated to be more sensitive technique than ITS1 nested PCR (Wells et al., 2015). The ability to detect 10 oocysts in 10 ml dirty water sample concentrate using this qPCR following LMS provides an important indication of the potential power of this method for the analyses of water samples for contamination with *Toxoplasma* oocysts. Although PCR can be very effective with pure solutions of nucleic acids, its sensitivity may be reduced dramatically when applied directly to biological samples due to the presence of PCR inhibitors (Lantz et al., 2000), and thus any pre-isolation method that removes potential inhibitors is likely to improve sensitivity. Hence, the current method also seems promising as it facilitates detection by separating the bead-bound oocysts from potential inhibitors that might be present in samples containing inhibitors, such as environmental water samples.

Our results provide evidence that LMS involving WGA-coated magnetic beads can be an efficient approach for the isolation of *T. gondii* oocysts from water sample concentrates, with detection either by microscopy (following dissociation) or by qPCR (without dissociation necessary). The possibility of combining LMS for *Toxoplasma* with IMS for *Cryptosporidium* species and *Giardia* cysts to enable the simultaneous detection of all three parasites would be worthy of further exploration.

## 5. Conclusions

This work describes a method of isolating *T. gondii* oocysts from water sample concentrates for improved detection, either by microscopy or by qPCR. The greater detection sensitivity provided by this method should enable us to obtain more reliable data on the potential for different water bodies to act as transmission routes of this protozoan parasite, which is of both veterinary and public health importance. Accurate knowledge of the extent of contamination of particular water bodies is important in deciding on appropriate interventions.

Developments in water analysis methods for the protozoan parasites *Cryptosporidium* spp. and *G. duodenalis* have been key in the control of their waterborne transmission, and the LMS method described here may be equally useful for *Toxoplasma* as IMS has been for *Cryptosporidium* and *Giardia*.

## Funding information

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# Paper III

# Use of Lectin-Magnetic Separation (LMS) for detecting *Toxoplasma gondii* oocysts in environmental water samples.

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**Highlights**

- ✓ Acidified pepsin treatment of *T. gondii* oocysts *in situ* in water samples was achieved
- ✓ Excess acid pepsin must be neutralized before proceeding to LMS
- ✓ Following LMS, detection by qPCR could be used on both clean and dirty samples
- ✓ Pilot analyses indicate *T. gondii* occurs in some Norwegian drinking water sources
- ✓ Mucilaginous secretions from microorganisms may hamper LMS for *Toxoplasma* oocysts

**Abstract**

Proof-of-principle of lectin-magnetic separation (LMS) for isolating *Toxoplasma* oocysts (pre-treated with 0.5% acidified pepsin (AP)) from water for subsequent detection by microscopy or molecular methods, has been shown. However, application of this technique in the routine water-analysis laboratory requires that the method is tested, modified, and optimized. Initial studies attempted to apply the LMS technique to samples that had already been analyzed for contamination with *Cryptosporidium* and *Giardia* using standard methods, and the supernatant following immunomagnetic separation (IMS) retained. Mucilaginous material in these samples, which was found to be probably an exudate from bacterial (*Pseudomonas* and *Bacillus* species) and fungal overgrowth, affected use of the beads. Thus, such samples (that had been stored at room temperature and unwashed) are unsuitable for use with LMS or other magnetic bead-based methods. If such samples are to be analyzed using LMS, then they should be washed and kept refrigerated, and analyzed as soon as possible post-IMS. Experiments on AP-treatment of *Toxoplasma* oocysts *in situ* in such samples demonstrated that overnight incubation at 37°C was adequate, but excess AP had to be removed before continuing to LMS; neutralization in sodium hydroxide and a single wash step was found to be suitable. For detection, microscopy was found to be successful only suitable for clean samples, as debris occluded viewing in dirtier samples. Although qPCR was successful, for some samples non-specific inhibition occurred, as demonstrated by inhibition of an internal amplification control in the qPCR reaction. For some, but not all, samples this could be addressed by dilution. Finally, the optimized methodology was used for a pilot project in which 23 post-IMS water sample concentrates were analyzed. Of these, only 20 provided interpretable results (without qPCR inhibition) of which one sample was positive, and confirmed by sequencing of PCR product, indicating that *Toxoplasma* oocysts occur in Norwegian drinking water samples. In conclusion, we suggest that post-IMS samples may be suitable for analysis for *Toxoplasma* oocysts using LMS, only if freshly processed and washed before refrigerated. In addition, application of AP treatment requires a neutralization step before proceeding to LMS. For detection, qPCR, rather than microscopy, is the most appropriate approach, although some inhibition may still occur, and therefore inclusion of an internal amplification control is important. Our study indicates that, despite some limitation, this approach would be appropriate for further large-scale analysis of samples of raw and treated drinking water.
Key words: Contamination, isolation, neutralization, qPCR

## 1. Introduction

Ensuring an adequate supply of fresh water of appropriate quality has been a major challenge globally in maintaining or improving public health and prosperity, as good public health and productive animal husbandry require safe water. In developed regions of the world, attention on infectious disease agents in water has been prompted by large disease outbreaks due to waterborne pathogens (e.g. *Cryptosporidium, Giardia*, *E. coli* O157:H7, *Campylobacter*, *Salmonella* and *Norovirus)* (Coffey et al., 2007). Such outbreaks presumably also occur at least as frequently in less-developed regions of the world but are less often reported. Investigation of waterborne outbreaks and monitoring of water for contamination such that interventions may be implemented require the development of robust, standardized detection methods for the detection of these pathogens in water samples.

*Cryptosporidium* and *Giardia* are the most commonly reported intestinal protozoan pathogens, with estimated prevalences of 3-5% and 10%, respectively, in the world's human population, and they are also among the most commonly reported waterborne infectious agents (Efstratiou et al., 2017). However, other protozoan pathogens may also be transmitted by water but are less likely to be recognized. This lack of recognition is partly due to the symptoms of infection often being less acute and partly due to the lack of appropriate analytical tools. Of particular relevance is the zoonotic protozoan *Toxoplasma gondii,* which is often more commonly thought to be transmitted by ingestion of bradyzoites in undercooked meat, than by water. The importance of waterborne transmission became particularly recognized in the wake of a large human outbreak linked to contamination of a municipal water reservoir in Canada by wild felids, and also due to the identification of widespread infection of marine mammals in the USA (Aramini et al., 1999; Jones and Dubey, 2010).

*T. gondii* is an obligate intracellular apicomplexan parasite that infects all warm-blooded vertebrates, including humans and birds, worldwide (Dubey, 2010; Yan et al., 2016). It is currently estimated that one-third of the world's human population is infected by this parasite, indicating its incredible success in nature (Dubey, 2010; Innes, 2010). Transmission occurs via three primary pathways: ingestion of uncooked meat containing viable tissue cysts, ingestion of sporulated oocysts (often via contaminated food or water) from the feces of infected felids, and congenital transmission from an infected mother to her fetus during pregnancy (Dubey, 2010).

Contamination of the environment with oocysts is widespread, as the definitive hosts (domestic cats and other members of the family Felidae) shed millions of oocysts during a short period (about 1-2 weeks) of their lifetime (Hill and Dubey, 2002). *T. gondii* oocysts are highly robust and resistant in the environment and can survive for months to years in soil and water (Dubey, 2010). Rain and surface water runoff may transport infective oocysts into drinking water supplies, recreational sites, including fresh and marine waters, and irrigation waters, which, in turn, can contaminate the food supply (Petersen et al., 2010). Hence, ingestion of oocysts may be an important route of infection for humans, and several waterborne outbreaks of toxoplasmosis linked to oocyst contamination of drinking water have now been documented (Dubey, 2010).

Infection with *T. gondii* is typically mild or asymptomatic, resulting in a life-long latent infection in immunocompetent individuals. A more serious problem is that chronic infection can be reactivated and cause encephalitis or death in subsequently immunocompromised hosts, such as AIDS patients (Pereira-Chioccola et al., 2009). In addition, congenital transmission from mother to fetus, following primary exposure in pregnancy, may result in miscarriage, and congenitally infected children may suffer from ocular disease, including blindness, and mental retardation (Jones and Dubey, 2010). Furthermore, severe and even lethal toxoplasmosis has been observed in immunocompetent patients infected with atypical strains (type IV) (Carme et al., 2002).

Analysis of environmental matrices for *T. gondii* has been the focus of very few studies due to the lack of standardized methods to identify contamination with oocysts. Methods for identifying other potentially waterborne protozoan parasites (*Cryptosporidium* species and *Giardia duodenalis*) in water samples were developed, standardized, and validated several decades ago, and are available globally (e.g., US EPA Methods 1622 and 1623 first published in 2005 (US EPA, 2012), and ISO Method 15553 (ISO, 2006)). However, similar methods for detection of *T. gondii* oocysts in environmental matrices have not been established. This is partly because immunomagnetic separation (IMS) techniques and immunofluorescent antibody testing (IFAT) using monoclonal antibodies, both of which are key components of the methods for *Cryptosporidium* and *Giardia*, are lacking. Although attempts have been made to develop antibodies specific for *T. gondii* oocysts (Dumétre and Dardé, 2005; Possenti et al., 2010), development of IMS and IFAT that can be used for high efficiency separation and identification of *Toxoplasma* oocysts has yet to be achieved (Dumétre and Dardé, 2007; Harito et al., 2016).

Recent studies with lectin-binding assays, using purified oocysts pretreated with acidified pepsin (AP) and spiked into water samples, have indicated that there is a potential for efficient oocyst capture and separation using lectin magnetic separation (LMS) (Harito et al., 2016; 2017). However, although proof-of-principle has been achieved, further investigation is still required to bring this methodology to the routine water-analysis laboratory. One important aspect to be explored is that *Toxoplasma* oocysts occurring in water sources or other environmental samples are not chemically pretreated with AP, as described in Harito et al. (2016; 2017). Furthermore, 'real' water sample concentrates may contain a variety of different biological and chemical components that may preclude satisfactory use of the method or hamper recovery efficiency. It should be noted that IMS and IFAT for *Cryptosporidium* and *Giardia* are based on monoclonal antibodies, and, thus, being targeted towards particular wall epitopes, are relatively organism specific. In contrast, WGA binds a range of glycoconjugates, including 2-acetamido-2-deoxy-n-glucose (GlcNAc) and its P-1, 4-linked oligomers (Bhavanandan and Katlic, 1979), and these may be components of a range of different organisms that occur in water. Therefore, binding of non-target organisms may occur, and this could affect the efficiency of LMS and could also inhibit detection by qPCR in the final concentrate.

Thus, the purpose of the work described here was to refine the proposed method for use on "real life" water sample concentrates, to identify potential challenges and investigate methods to overcome them, and also to undertake a preliminary analysis of some "real life" water sample concentrates as a pilot project using the optimized method derived in the experimental work.

## 2. Materials and methods

### 2.1. *T. gondii* oocysts

Oocysts used in this study were, as described previously (Harito et al., 2016), genotype II (derived from the ME 49 strain) obtained from infection of *T. gondii*-free cats at the USDA facility in Beltsville, Maryland. The oocysts were collected and purified according to the procedures described elsewhere (Dubey, 1995, 2010) and shipped, unsporulated and thus non-infectious, to Norway in January 2015. At NMBU parasitology lab, the freshly received stock of oocysts were stored in water as described earlier (Harito et al., 2016) and used in the current work with or

without pretreatment (using 0.5% AP). Oocyst enumeration to adjust the number to a chosen concentration was performed by using KOVA GLASSTIC Slide 10 with grids (Fisher Scientific).

2.2. Lectin-coated beads for LMS

Wheat germ agglutinin (WGA)-coated beads were prepared as previously described (Harito et al, 2017). Briefly, WGA conjugated to biotin (Sigma-Aldrich Chemie GmbH, D-91625 Schnelldorf, Germany), was suspended in phosphate buffered saline (PBS; pH 6.8, as recommended by the supplier) at a concentration of 1 mg/ml (L5142 SIGMA) and coupled to Dynabeads™ M-280 Streptavidin magnetic beads (M-280 Streptavidin beads) purchased from Invitrogen (ThermoFisher Scientific; Oslo, Norway). The M-280 Streptavidin beads were washed then coupled with biotin-conjugated WGA as previously described (Harito et al, 2017). The bead-biotin-WGA complex was washed four times in buffer (PBS, pH 7.4) and re-suspended in 1 ml PBS (pH 7.4).

2.3. Water samples

2.3.1. Laboratory grade

Reverse osmosis (RO) water (Millipore Direct-Q™ 3, Merck Life Science AS, Oslo, Norway) was used throughout this study as test control against environmental source samples and for preparing different reagents.

2.3.2. Environmental samples (real samples)

2.3.2.1. Tests performed on stored post-IMS water samples

The Parasitology laboratory (NMBU) regularly analyses drinking water samples (either raw, untreated or treated potable supply) from different municipalities in Norway for contamination with *Cryptosporidium* and *Giardia*. The laboratory has been officially accredited (by Norwegian Accreditation, http://www.akkreditert.no/en/) for analysis of water samples for these parasites since 2010. The method used for analysis is closely based on that described in ISO 15553 (ISO, 2006), but with membrane filtration (142 mm Isopore membrane, 2.0 µm pore size; Millipore corp.) of the 10 L water samples as the preliminary step.

For the previous 2 years, the post-IMS supernatant from most water samples analyzed for *Cryptosporidium* and *Giardia* had been collected and stored. Usually these post-IMS supernatants

are discarded, but these were stored at room temperature. However, they were not washed prior to storage, and thus contained the buffers used in the IMS (Dynal GC-Combo kit, IDEXX Laboratories Inc.).

2.3.2.2. Tests performed on freshly processed post-IMS water samples

Due to problems with LMS in the stored post-IMS samples (see Sections 2.3.2.1 and 3.1.1), further post-IMS samples, obtained from water analyzed for *Cryptosporidium* and *Giardia* during the study period, were washed and re-suspended in RO water and stored at 4°C until analysis. A total of 23 post-IMS samples were obtained, all were from raw (untreated) water to be used for potable supply post-treatment, and came from a total of 9 different water sources. The turbidity of the post-IMS samples ranged from 8.6 to 16 nephelometric turbidity units (NTU).

2.4. Sample processing and LMS

2.4.1. Evaluation of prewashing of sample matrices prior to LMS

As preliminary results (see Section 3.1.1) indicated matrix interference effects on LMS, attempts were made to remove potential inhibitors by pre-washing with various chemicals. These included: detergents (0.1%Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.05% PBS Tween 20), acid (0.5 N hydrochloric acid), alkali (0.2 M sodium hydroxide), bleach solutions (0.1% and1% sodium hypochlorite), and a blocking agent (PBS containing 0.1% bovine serum albumin). In each case, subsamples of post-IMS pellets were spiked with AP-pretreated oocysts and then exposed to the reagents by incubation at room temperature or 37°C for 1 hour. The spiked samples were then washed once in RO water before proceeding to LMS as described in Section 2.4.4.1.

In addition, five freshly processed post-IMS samples were divided into two duplicate sub-samples, one of which was washed once in 0.05% PBST and stored refrigerated, and the other subsample was stored, unwashed, at room temperature. The subsamples were spiked with AP-pretreated oocysts, and after approximately 4 weeks, these spiked, stored duplicate subsamples were analyzed in parallel using LMS as described (Section 2.4.4.1), with detection by microscopy (Section 2.6.1)

2.4.2. Acidified pepsin treatment of oocysts in contaminated water samples

Although all previous successful spiking experiments for LMS have used AP-pretreated oocysts (Harito et al., 2017), in "real-life" water samples that may contain *Toxoplasma* oocysts, these

oocysts will not be pretreated with AP. It was therefore important that AP treatment of oocysts already contained in water samples or pellets was investigated and optimized. Preliminary assessments on treatment of oocysts already *in situ* in water samples were carried out using both RO water and dirty water sample concentrates, the latter of which were categorized as low turbidity (<10 NTU) or highly turbid (>10 NTU) according to turbidity measurements (Hach turbidimeter 2100A). Thus, non-pretreated oocysts were spiked into water sample concentrates (freshly prepared post-IMS sample concentrates) and 0.5% AP added. The samples were incubated overnight at 37°C, either stationary in a tube rack or rotated at 20 rpm (using an ELMI ROTAMIX RM1 (1 ml) or Dynal Sample mixer (10 ml) depending on the volume processed).

2.4.3. Removal or neutralization of excess acidified-pepsin from treated water samples

After AP treatment of oocysts that had been spiked into water samples (see Section 2.4.2.), LMS was performed. Poor recovery results (see Section 3.2.2.) suggested that excess AP that had not been removed prior to LMS affected recovery efficiency. Approaches to removing or neutralizing excess AP prior to LMS included triplicate washing with PBS containing 0.05% Tween 20 (PBST) or with PBS containing 0.1% bovine serum albumin, and neutralization with 1 M sodium hydroxide (NaOH) followed by a single wash in PBST. Initial experiments were conducted in 1 ml sample volumes (using microscopy for detection – see section 2.6.1), and the method assessed as best was then attempted in 10 ml using both microscopy and qPCR for detection (see section 2.6.1. and 2.6.2).

2.4.4. Lectin-Magnetic Separation (LMS)

2.4.4.1. Using AP-pretreated oocysts in experiments on matrix pretreatments

Following experimental sample pretreatments (section 2.4.1) in samples spiked with AP-pretreated oocysts, LMS was performed as described by Harito et al. (2017). Briefly, following spiking of the treated sample concentrates or controls with oocysts pretreated with AP, the spiked sample was washed then resuspended again in the SL buffers (Dynal GC-combo kit, IDEXX Laboratories), to which was added 20 µl of the M-280 Streptavidin beads (40 µg/ml) coupled to WGA and incubated for an hour at room temperature in a rotating mixer at 20 rpm (ELMI ROTAMIX RM1). The bead-bound oocysts were then separated using a magnet (Dynal MPC-S, Dynal, ThermoFisher Scientific, Oslo, Norway) and resuspended in 100 µl of RO water and transferred to welled-slides

(Novakemi AB, Träffgatan 2, 136 44 Handen, Sweden), dried, and examined by microscopy (section 2.6.1).

2.4.4.2. Using non-pretreated oocysts for determining recovery efficiency and pilot analyses of water samples

LMS for capture of oocysts that had not been pretreated with AP was evaluated in 1 ml and 10 ml volumes, and also used for a pilot project analysis of some "real life" samples. Briefly, for 1 ml processing, 200 µl water sample concentrate was added to 1.5 ml microfuge tubes in triplicate and spiked with a known number of untreated oocysts (1200 stored in water). Samples were resuspended in 900 µl 0.5% AP, mixed, and incubated for 24 hours at 37°C in a rotating mixer (20 rpm) (ELMI ROTAMIX RM1). Following incubation, the activity of the AP was neutralized by adding 22 µl 1.0 M NaOH (see sections 2.4.3 and 3.2.2) and mixing on the rotating mixer for 30 minutes, followed by a single wash in 0.05% PBST. The treated samples were then centrifuged at 2000 ×g (VWR® MicroStar 17R Micro-centrifuge, VWR International AS, Oslo, Norway) for 10 minutes and the supernatant discarded. Pellets were resuspended in 900 µl 0.05% PBST, mixed vigorously and the washing steps repeated once. After resuspending the final pellets in RO water, 100µl 10× SL buffer A (Dynal GC combo) and 20 µl M-280 WGA-Streptavidin beads were added, and samples incubated on a rotating mixer (20 rpm) for 1 hour at room temperature. Following incubation, the bead-bound oocysts were separated using a magnet (Dynal MPC-S) and resuspended in 100 µl RO water. This was either transferred to the three wells on a welled slide, dried, and examined by microscopy (section 2.6.1) or was used for DNA extraction and qPCR detection (section 2.6.2).

For 10 ml experiments, spiked samples and pilot study water samples were processed in 15 ml plastic centrifuge tubes. The spiked samples were prepared by adding 1200 untreated oocysts (stored in water) to 2 ml water sample concentrates in triplicate. Samples were resuspended in 9 ml 0.5% AP, mixed, and incubated for 24 hours at 37°C on a rotating mixer (Dynal Sample Mixer, Dynal, ThermoFisher Scientific, Oslo, Norway) at 20 rpm. In order to neutralize AP, 220 µl of 1.0 M NaOH was added to each tube and mixed for 30 minutes, before centrifugation at 3000 rpm (1550 ×g) for 10 minutes (Rotanta 460, Hettich, Tuttlingen, Germany). The supernatant aspirated to approximately 2 ml and the pellets washed once in 9 ml 0.05% PBST. The final pellet was resuspended in RO water, transferred to L-10 tube (Dynal, ThermoFisher Scientific, Oslo,

Norway). The volume was adjusted to 10 ml with RO water, and 1 ml 10× SL buffer A (Dynal GC Combo) added as previously described for *Cyclospora* oocysts experiments (Robertson et al., 2000). Briefly, 200µl M-280 Streptavidin bead coupled to WGA was added to the mixture in L-10 tube and incubated in rotating mixer at 20 rpm for 1 hour at room temperature. Bead-bound oocyst separation was performed as previously described (Harito et al., 2017), using two magnets (Dynal MPC-1, Dynal, ThermoFisher Scientific, Oslo, Norway and Dynal MPC-S ). The bead-bound oocysts in 1.5 ml microfuge tubes were then either resuspended in 150 µl RO water, transferred to slide wells, dried and examined using microscopy (section 2.6.1) or resuspended in 200 µl RO water before DNA extraction and qPCR detection (section 2.6.2).

2.5. Partial characterization of organisms from stored post-IMS samples potentially affecting LMS activity

As preliminary results (see Section 3.1.1) indicated matrix interference effects on LMS, particularly on stored post-IMS water samples from which the buffers had not been removed (see section 2.3.2.1), an effort was made to partially characterize those organisms that may have interfered with the LMS binding to oocysts. Three stored water samples for which low LMS recovery of oocysts had been recorded in spiking experiments (see Section 2.4.1 and 3.1.2) were concentrated by centrifugation and then plated directly onto lysogeny broth (LB) agar using disposable inoculation loops (VWR). Plates were incubated at room temperature and monitored for three days. Bacterial colonies were analyzed at the microbiology laboratory-NMBU using blood agar and MacConkey agar (blue) plates followed by Gram staining. Identification to the genus level was by oxidase tests and a further analytical profile index using API® 20 NE kit (http://www.biomerieux-usa.com/clinical/api).

2.6. Detection and quantification

2.6.1. Microscopy

Oocysts were visualized using ultraviolet (UV) illumination based on their characteristic blue autofluorescence (Lindquist et al., 2003) using a Leica DMLB Fluorescence Microscope equipped with an ultraviolet filter block (excitation 335 nm; emission 450 nm). Oocyst counting was performed with a tally counter, following a systematic side-to-side screening of the slide wells at ×200 and ×400 magnification.

2.6.2. Quantitative polymerase chain reaction (qPCR)

2.6.2.1. DNA extraction

DNA was isolated from post-LMS samples as previously described (Harito et al., 2017). Briefly, the bead-oocyst complexes were resuspended in 200 µl RO water, mixed with 180 µl ATL lysis buffer and subjected to 6 freeze-thaw cycles in liquid nitrogen and water bath set at 90°C. Before adding proteinase K, heat-inactivated Phocine herpes virus 1 was included as an internal control and samples incubated at 56°C overnight. After a brief centrifugation, the tubes were held in a magnet (Dynal MPC-S) to remove the beads. The supernatant was transferred to a 2 ml microfuge tube and DNA extracted in an automatic DNA purification instrument (QIAcube, QIAGEN, Biotechnology Company, 40724 Hilden, Germany) using a modified version (30-100 µl elution volume) of the QIAamp Mini Tissue protocol (QIAamp® DNA Mini kit (50)). The resulting DNA was eluted in 50 µl, and stored at -20°C until further processing by qPCR.

Before analysis of "real life" samples as a pilot study, nine freshly processed post-IMS sub-samples were assessed for turbidity and pellet content, after which they were spiked with estimated 100 untreated oocysts and processed as described in section 2.4.4.2 coupled with alkali neutralization (section 2.4.3). DNA extraction was then performed as described above and the performance of LMS evaluated using qPCR.

For the pilot study, 23 freshly processed post-IMS samples (section 2.3.2.2.) were processed according to the final method (AP-treatment, neutralization, LMS, qPCR) for determination of actual *Toxoplasma* contamination.

2.6.2.2. DNA amplification and quantification (qPCR assay)

Real-time PCR (qPCR) detection was performed on DNA extracted from the post-LMS concentrates by targetting the 529 bp repeat element according to Harito et al. (2017). The amplification mixtures consisted of 10 µl KiCqStart SYBR Green qPCR ReadyMix, Low ROX (2×) (KCQS01 SIGMA); 0.40 µM Tox-9F and 0.40 µM Tox-11R primers (Life Technology), molecular grade $H_2O$ and 5 µl DNA template to provide a final reagent volume of 20 µl. The PCR amplification was performed in an Mx3005P (Agilent Technologies, Inc.) thermal cycler using a SYBR green protocol with dissociation curve. Initial denaturation was at 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 15 sec, annealing at 58 °C for 1 min, and fluorescent

measurement after a final extension step at 72°C for 15 sec. Dissociation curves were made by increasing the temperature from 55°C to 95°C with continuous fluorescent measurement. All reactions were performed in triplicate and PCR-grade water was added as no template control in each run.

The effect of PCR inhibitors in the samples was indirectly monitored by inclusion of an internal amplification control during DNA isolation as described previously (Opsteegh et al., 2010; Wells et al., 2015). Amplification of the internal control DNA was performed by addition of primers provided (PhHV-1F; PhHV-1R) in separate reaction wells simultaneous to the qPCR assay for *Toxoplasma*. A standard curve was prepared using serial dilutions of *T. gondii* DNA extracted from 10,000 oocysts diluted 1:5; 1:50; 1:500 and 1: 5000 (representing 2000, 200, 20, and 2 oocysts). Samples were considered positive when the threshold cycle (Ct) values were 40 cycles or less and exhibited a smooth exponential curve in at least two of the triplicate samples compared with the standards curves. Samples without amplification of template DNA, but positive for the internal amplification control DNA, were considered negative (Harito et al., 2017). Samples that were negative for both the internal amplification control and the target were considered to contain inhibitors. For such samples, 50% dilutions were performed such that the equivalent of 2.5 µl of template was used instead of 5 µl, and the amplification repeated. If the internal control remained negative, then the sample was categorized as uninterpretable.

For the pilot study with analysis of environmental water samples, confirmation of any positive sample was performed by sequencing the PCR product in both directions by Sanger sequencing (Macrogen Europe, Amsterdam Netherland). Sequences were aligned and manually checked using Geneious version 9.1.3 (https://www.geneious.com, Kearse et al., 2012), before comparison with already published sequences in GenBank using BLAST search.

### 2.7 Data management

All data generated were imported to Microsoft Excel spreadsheet, analyzed using descriptive statistics (mean, percentages and confidence intervals), and summarized using tables and graphs. Mean capture efficiency was calculated from average counts of three trials for each test. The effect of pellet size on recovery efficiency of oocysts treated with AP after spiking was analyzed by one-way ANOVA using STATA 14.2 for Windows (Stata Corp. College Station, TX, USA). To

compare recovery efficiencies between duplicate sub-samples stored at room temperature or washed and stored refrigerated, a paired T-test was used. Statistical significance was set at $P< 0.05$. The limit of detection (LOD) was determined for qPCR results by using 100 oocysts spiked in subsamples selected at random and processed using LMS in 10 ml volume. Quantitative PCR (qPCR) results were expressed in terms of number of triplicate wells in which DNA amplification signals were detected and the range of corresponding threshold cycle (Ct) values.

**3. Results**

3.1. Tests with stored post-IMS water samples

3.1.1 Recovery of AP-pretreated oocysts from stored post-IMS samples

Although recovery of AP-pretreated oocysts using LMS has previously been shown to be very high in clean water samples, being often in excess of 97 % (Harito et al., 2017), when the AP-pretreated oocysts were spiked into stored post-IMS samples, the recovery was below 1 % (data not shown). A mucilaginous mass trapped the LMS beads and other debris. This spread over the whole area of the microscope well, occluding viewing.

3.1.2. Effect of various pretreatment of matrices on recovery of AP-pretreated oocysts from stored post-IMS water samples

Pretreatment of the post-IMS samples with various chemicals (section 2.4.1) prior to spiking with the AP-pretreated oocysts did not improve recovery efficiency or have a notable effect on matrix quality (data not shown).

3.1.3. Partial characterization of microorganisms from stored post-IMS samples potentially affecting LMS activity

All three post-IMS samples analyzed were found to contain large amounts of bacteria (*Pseudomonas* and *Bacillus* species) and one sample also contained unidentified fungi.

3.2. Recovery of *Toxoplasma* oocysts from clean water and freshly processed post-IMS water samples

3.2.1. Recovery of AP-pretreated oocysts

With freshly processed water sample concentrates, use of LMS for recovery of AP-pretreated oocysts resulted in a recovery efficiencies ranging from 54.2% to 99.4% depending on pellet volume (Figure 1). Samples with high pellet volumes (200 µl) had lower recovery efficiency than control samples without a pellet, and samples containing pellets (ranging in size from <100 µl - ~200 µl) showed a gradual drop in recovery efficiency.

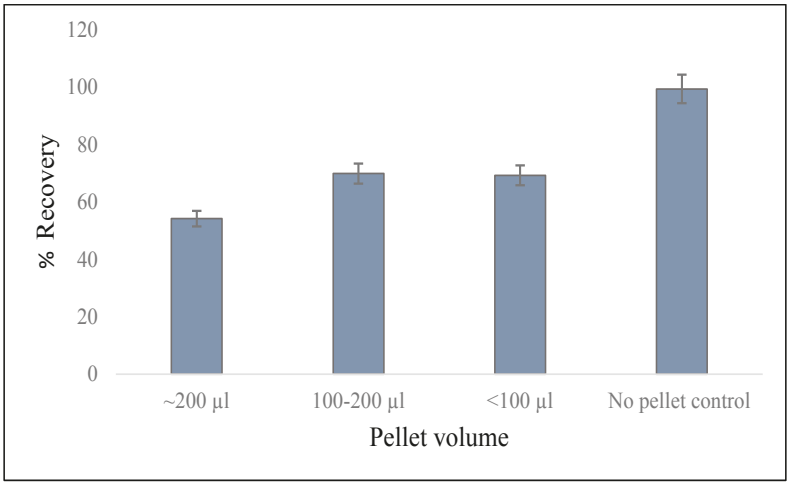


Figure 1. Recovery of spiked AP-pretreated oocysts from freshly processed post-IMS samples with different pellet sizes (mean recovery with SD bars).

Additionally, investigation of storage of post-IMS supernatant (stored at room temperature for one month or washed and stored refrigerated for one month) on recovery of spiked oocysts by LMS demonstrated a higher recovery of the latter (mean recovery of 28.0% (CI 1.7-54.0%) for samples stored at room temperature and 54.58% (CI 32.9-76.2) for washed samples stored refrigerated). However, the paired T-test did not show statistical significance (P=0.06).

3.2.2. Recovery of oocysts treated with AP post-spiking of 1 ml volumes; experiments to remove or neutralize excess AP

Initial AP-treatment of oocysts *in situ* was conducted with tubes standing in a rack. As recovery efficiency was low from this approach (data not shown), tubes were incubated on a rotator to facilitate greater contact between AP and the oocysts.

Recovery of oocysts from 1 ml spiked water samples (RO water or dirty according to turbidity measurements; see section 2.4.2) and then treated with AP *in situ* was not successful (data not shown), it was surmised that the excess AP must have affected the subsequent LMS. Attempts to remove or neutralize the excess AP demonstrated that acid neutralization followed by a single wash step resulted in satisfactory recovery by LMS (Table 1).

Although recovery efficiency in dirty samples was lower than in clean samples, with some oocysts occluded by particulate matter, which made microscopy difficult, it was nevertheless considered satisfactory to proceed to 10 ml volumes (Table 1).

Table 1. Oocyst recovery after AP treatment in experimentally contaminated 1 ml water samples; comparison of methods to remove/neutralize excess AP (n=3)

| **Treatment to remove or neutralize excess AP following treatment of water sample containing oocysts** | **Recovery efficiency of *Toxoplasma* oocysts spiked into RO and dirty (freshly processed post-IMS) water samples with AP treatment post-spiking** | |
|---|---|---|
| | **RO water samples Mean % recovery (CI)** | **Dirty water samples Mean % recovery (CI)** |
| Washed three times in BSA in PBS | 54.2 (54.17-54.23) | Not done |
| Washed three times in PBST | 63.75 (61.03-66.47) | 4.58 (3.3-5.86 ) |
| Neutralization + washed once in PBST | 74.75 (72.29-77.21) | 56.08 (53.28-58.88) |

3.2.3. Recovery of oocysts treated with AP post-spiking of 10 ml volumes using clean water samples.

For 10 ml clean water samples (RO water or tap water), spiking of untreated oocysts followed by AP treatment, alkali neutralization and single washing in 0.05% PBST, and then LMS resulted in recovery efficiencies that did not noticeably differ from recovery efficiencies when the same samples were spiked using AP-pretreated oocysts (Table 2).

Table 2. Oocyst recovery after AP treatment in experimentally contaminated 10 ml clean water samples (n=3)

| Sample description | Mean % recovery efficiency | Confidence intervals |
|---|---|---|
| RO water spiked with untreated oocysts and AP-treated post-spiking | 97.4 | 96.61-98.21 |
| Tap water spiked with untreated oocysts and AP-treated post-spiking | 97.27 | 96.47-98.07 |
| Control (AP-pretreated oocysts spiked into RO water) | 97.35 | 96.49-98.21 |

3.2.4. Recovery of oocysts treated with AP post-spiking of 10 ml volumes using dirty water samples and detection by microscopy or qPCR

In these samples recovery efficiency as determined by microscopy was found to be inversely proportional to pellet size with a significant ($P<0.05$) reduction in recovery efficiency as pellet size increased (Figure 2). Although oocysts may have been captured and separated, they remained hidden in excess particulate matter and could therefore not be counted.

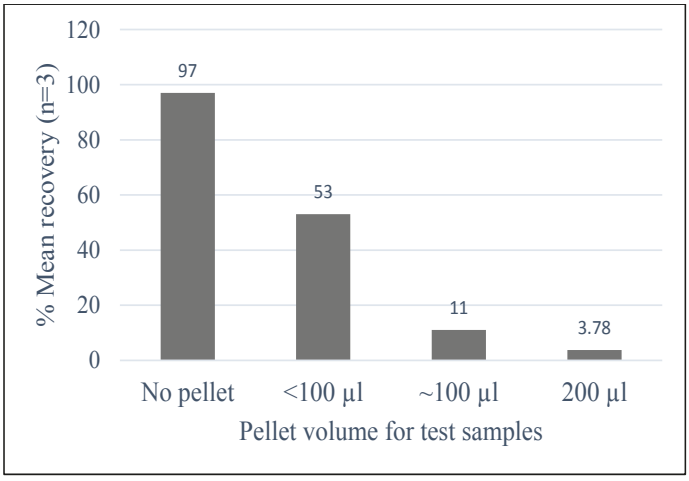


Figure 2. Recovery of oocysts from freshly processed post-IMS samples with different pellet sizes, with treatment with AP after spiking prior to LMS – enumeration of oocysts by microscopy

When qPCR was used for detection in these samples, positive results were obtained despite differences in pellet volume (Table 3). Signal detection was achieved for only two triplicate out of four (<100 µl pellet) and one triplicate out of three (~100 µl pellet) samples and the absence of amplification signals for the corresponding internal amplification controls indicated PCR inhibition. By dilution of the template volume by 50%, down to 2.5 µl equivalent instead of 5 µl, amplification signals were detected in all three wells and also for the internal amplification controls.

Table 3. Detection of oocysts by qPCR from freshly processed post-IMS samples with different pellet sizes, with treatment with AP after spiking prior to LMS

| **Pellet size** | **Turbidity (NTU)** | **Signal detection (no. positive/no. tests per sample)** | **Threshold cycle range** |
|---|---|---|---|
| <100 µl (n = 4) | 6.1-10.1 | 3/3 | 27.71- 35.74 |
| ~100 µl (n =3) | 12-16 | 3/3 | 31.01- 35.03 |
| Control (no pellet) | - | 3/3 | 27.97- 28.32 |

3.2.6. Pilot analysis of environmental water samples

Out of 23 environmental water samples (freshly processed post-IMS) collected and tested using AP-treatment, neutralization, and LMS, followed by qPCR for detection, three samples (13 %) were concluded as being uninterpretable due to lack of amplification of internal controls. The pellet sizes of these concentrated samples was up to 100 µl and the turbidity ranged from 5.6-16.0 NTU. Of the remaining 20 samples, one sample was positive for each of the three replicates with threshold cycle values ranging from 32.65-33.59, indicating that contamination of raw drinking water in Norway could occur at around 5%. The sequencing results confirmed the presence of *Toxoplasma* DNA with 99 % identity match to *Toxoplasma* isolates representing different types (e.g. Accession numbers: DQ779189.1, AF487550.1 and LN714508.1).

## 4. Discussion

This study focused upon transferring an LMS-based technique for analyzing environmental water samples for contamination with *T. gondii* oocysts, and for which proof-of-principle had been established, from the research lab to actual use for analyzing water samples. Initial work investigated samples previously analyzed for *Cryptosporidium* and *Giardia* using standard techniques in our lab, with the post-IMS supernatant used as the analytical matrix.

The first finding was that LMS could not be successfully applied to post-IMS supernatants that had not been washed and had been stored at room temperature. It is likely that the buffers used for IMS (and which remain in the post-IMS supernatants) contain media that supports growth of microorganisms such as bacteria and fungi. Indeed, a mucilaginous substance was observed in the material, and preliminary microbiological investigation of some samples revealed the presence of *Pseudomonas* and *Bacillus* species, as well as presence of unidentified fungi. These could well be the source of the mucilaginous material that entrapped the beads and thereby hindered oocyst capture by LMS. *Pseudomonas aeruginosa* and *Bacillus subtilis* are considered to be among the bacteria that secrete biofilms that coat surfaces, as has been discussed recently (Visick et al., 2016). The presence of bacterial slimes has also been reported to hinder IMS for *Cryptosporidium* and *Giardia* when investigating beansprouts for contamination with these parasites, with IMS (as well as other steps in the procedure) considered to have been affected by bacterial exopolysaccharides secreted from *Enterobacteriaceae* (Robertson and Gjerde, 2001).

Although various sample pretreatments (such as detergents, acid, alkali, blocking agent, and bleach) were tested for their ability to digest or remove the mucilaginous substances in these samples, we were unable to identify a suitable treatment that affected the material or improved recovery efficiency by LMS. Although identification of a reagent that can digest or dissolve such substances may improve the situation, it is important that it does not also affect the target organism (in this case, *Toxoplasma* oocysts). Thus, if post-IMS supernatant samples are to be stored for future experiments or analyses, we recommend that they are washed and stored refrigerated to avoid microorganism overgrowth and secretion of substances that may affect analytical techniques; our results from a limited number of samples that were split and stored at room temperature or washed and refrigerated support this recommendation.

Another important step to consider for bringing the LMS technique from the research lab to the water analysis lab was AP treatment of oocysts *in situ*. Overnight incubation at 37°C in a rotating

mixer following addition of AP to the sample spiked with untreated oocysts was found to be satisfactory, but required a neutralization (using sodium hydroxide) and washing procedure prior to LMS. Although, satisfactory recovery results were obtained by LMS for freshly-processed IMS samples using our AP-treatment protocol, it should be noted that the non-specific nature of LMS does mean that other biological materials may co-isolate with *Toxoplasma* oocysts. This was particularly noticeable with larger pellet sizes, which means that microscopy, as the detection method is only appropriate for clean samples. Our results emphasize the importance of coupling LMS with molecular detection techniques rather than relying on microscopy for detection (Harito et al., 2017; Hohweyer et al., 2016). However, it should be noted that even when qPCR was used for detection, inhibitors might affect amplification, highlighting the importance of including an internal control to monitor the reaction. Although we previously speculated that oocyst capture by LMS would result in samples containing few inhibitors (Harito et al., 2017), the results presented in the current work indicate that in the real-life situation this is not necessarily the case. A possible explanation for this could be the non-specific binding characteristics of WGA, which may result in isolation of material that also inhibits PCR. Dilution of the template enabled us to overcome this problem for some samples, but was not always sufficient. For future experiments we recommend that qPCR optimization, particularly to reduce the effect of inhibitors, is investigated more closely. Given that the copy number of the target is likely to be low, dilution is probably not the most appropriate approach for addressing inhibition. We therefore suggest that other approaches, such as use of PCR enhancers (additives) such as T4 gene 32 protein, or using digital-droplet PCR as a detection methodology, as this has been considered to be superior for inhibition-prone samples (e.g., Dingle et al., 2013), would be worthy of investigation.

Likewise, it should be noted that transfer of this method to analysis of fresh produce, as has been done with IMS for *Cryptosporidium* and *Giardia* (ISO, 2016; Utaaker et al., 2015), is likely to be more challenging due to the higher number of contaminants in such samples, particularly those that are likely to bind to WGA.

Following our appraisal and modification of the LMS-approach described by Harito et al. (2017) to bring it into the water-analysis lab, we used it in a pilot project involved 23 non-spiked water sample concentrates (post-IMS supernatants) that had been analyzed for *Cryptosporidium* and *Giardia*. Of the 20 samples with conclusive results, one sample was found to be positive. This suggests that *Toxoplasma* contamination of drinking water samples does occur in Norway,

probably at levels that are similar to those recently reported from Scotland (Wells et al., 2015). Although this pilot study involved only a limited number of samples and we have no information on the infectivity of the *Toxoplasma* detected, our knowledge on the robustness and longevity of *Toxoplasma* oocysts indicates that there is a need to analyze further water samples and to determine the extent of contamination and relevant risk factors.

## 5. Conclusions

- The use of LMS for detection of *T. gondii* in water has been applied to real water samples, and has demonstrated that post-IMS supernatant samples may be suitable for such analyses, provided that they are processed immediately or washed and stored refrigerated after IMS.
- AP treatment of oocysts in these samples can be successfully applied, but a neutralization step must be included before proceeding to LMS.
- qPCR, rather than microscopy, is presently the most appropriate option for detection, particularly for dirty samples, although the use of an internal control is essential to identify false-negative results and to monitor the presence of PCR inhibitors.
- A pilot study indicated that contamination of drinking water sources with *Toxoplasma* oocysts does occur in Norway, and, based on these preliminary results, we recommend that a full-scale study investigating the extent of occurrence of contamination, as well associated risk factors.

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# ERRATA

# Errata

| Page number | Paragraph | Change from | Change to |
|---|---|---|---|
| Vii | 1st; line 7 | My appreciation also for NMBU… | My special appreciation also goes to NMBU… |
| 5 | Figure 2 | tachyzoites invide almost… | tachyzoites invade almost… |
| 15 | 2nd; last line | … 2% sulfuric acid at 48°C … | … 2% sulfuric acid at 4°C… |
| 19 | 2nd; line 21 | … Toxoplasma… | …. *Toxoplasma*... (italic) |
| 22 | 1st; 1st line | … development a quantitative… | … development of a quantitative… |
| 47 | 3rd; line 16 | …FTIC... | …FITC... |
| 51 | Figure 8 | 'Pictures provided by Micronit microfluidics' | should be placed in brackets |
| 55 | 2nd; line 9 | … assess all beads (Table 1)… | … assess all beads (Table 6)… |
| 57 | 3rd; Line 21 | … albumen… | … albumin… |
| 59 | 2nd; line 22 | … 180 mL ATL lysis buffer… | … 180 µL ATL lysis buffer… |
| 63 | 3rd; line 20 | … outlets fractions… | … outlet fractions… |
| 63 | 3rd; line 25 | … several times in water… | … several times in laboratory grade water… |
| Paper I | section 2.3.2 | …. 0.875 µl… | …0.875 mL… |
| Paper II | section 2.2.2.1.1; line 15 | … 180 mL ATL lysis buffer… | … 180 µL ATL lysis buffer… |