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# *VERIFYING THE EFFECTIVENESS OF WATER TREATMENT FOR TOXOPLASMA TENDER REFERENCE: CR/2016/09 (Research conducted between May 2017 – May 2018)*

Final Report - August 2018

**Executive Summary**

The protozoan parasite *Toxoplasma gondii* (*T. gondii*) is a ubiquitous parasite in the environment and is one of the most common parasites to infect warm blooded animals worldwide. Cats are the only definitive host, predominantly young cats which, after being exposed to the parasite for the first time, excrete *T. gondii* oocysts in their faeces and subsequently into the environment (Figure A).

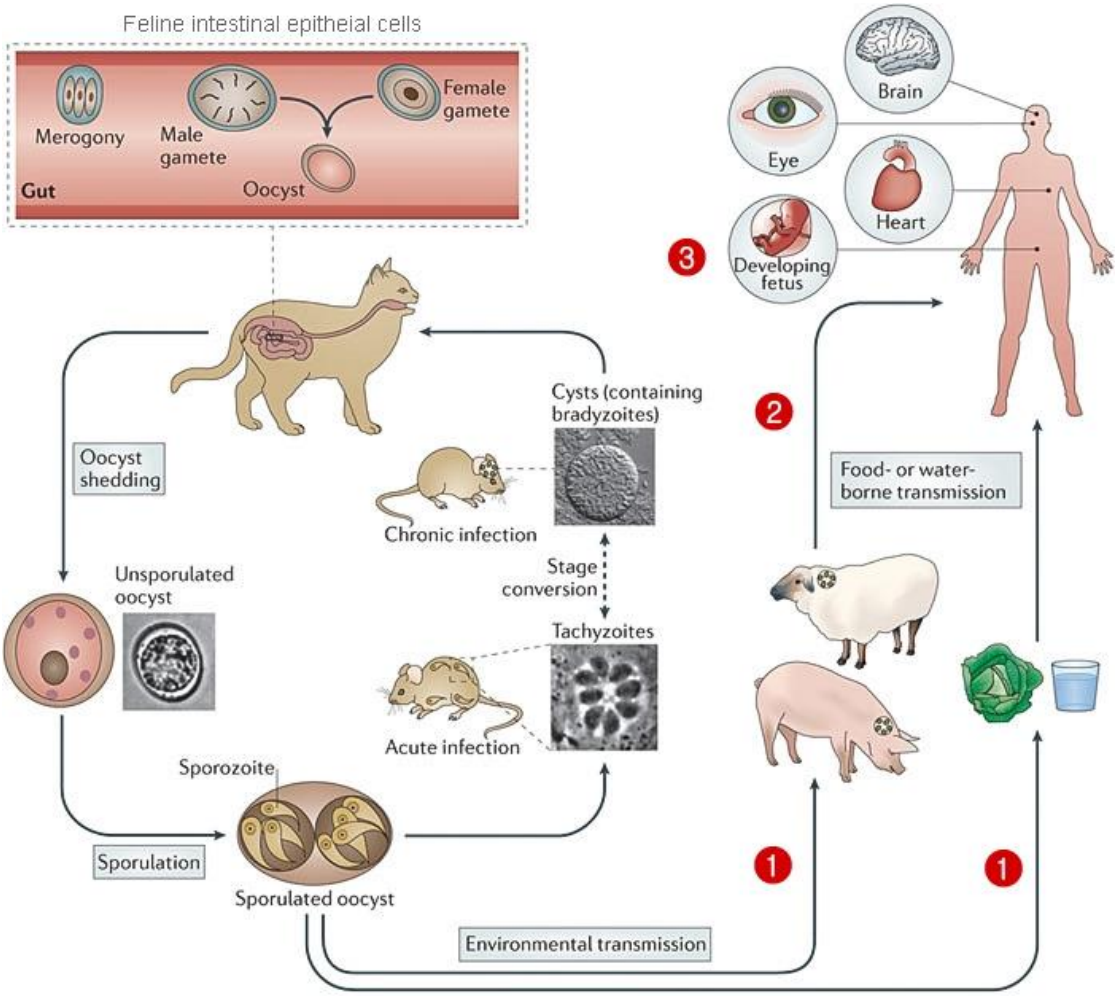



Figure A. Life cycle of *T. gondii*. The three different stages of the parasite (oocysts, tissue cysts and tachyzoites) are transmitted by either: 1) horizontal transmission of oocysts from the environment; 2) horizontal transmission from tissue cysts within intermediate hosts; and 3) vertical transmission of tachyzoites from mother to foetus during pregnancy. Figure adapted from Hunter and Sibley. Nature Reviews Microbiology 2012 Nov; 10:766-778

In 2015, a study by Moredun Research Institute in collaboration with Scottish Water investigated the prevalence of *T. gondii* DNA in drinking water sources in Scotland (Wells *et al*., 2015). The published study described an overall prevalence of 8.8% (124/1411) from 147 water catchments in Scotland. However, as this was the molecular detection of *T. gondii*, we needed to confirm whether the DNA identified in water samples was derived from oocysts.

The overall aim of the tender was to develop/refine a method to detect the presence of *T. gondii* oocysts from drinking water in order to confirm that oocysts are being removed effectively at Scottish Water treatments plants. In addition, this would address whether oocysts are present in Scottish raw and final waters, the effectiveness of individual water treatment plants and whether any oocysts detected were viable.

In order to improve the detection methodology in both raw and final waters, post *Cryptosporidium* IMS samples from Scottish Water were used in all spiking experiments. Nine different concentration methods and two different visualisation methods were tested and optimised using as low as one oocyst. The final methodology for clear and cloudy post *Cryptosporidium* IMS samples was sensitive enough to detect just one oocyst by microscopy. Additional work also demonstrated that a *T. gondii* specific qPCR was capable of detecting a single oocyst scraped from a microscope slide, thereby verifying that what had been visualised was (or was not) *T. gondii*.

It was not possible to visualise dirty *Cryptosporidium* IMS samples (whether raw or final), despite repeated attempts at improving the methodology. Although one method appeared to improve the appearance of dirty final waters, this process compromised the sensitivity, meaning that low numbers of oocysts could not be detected.

The tender originally described examining four catchments previously identified by Wells *et al* (2015) as having *T. gondii* DNA, however, once the methodology was in place, an additional 6 catchments were studied (10 catchments in total). From 201 slides that were screened from post *Cryptosporidium* IMS samples, 27 *T. gondii* like bodies were identified in 80% (8/10) of the 10 catchments studied (Table A). All 27 slides were scraped and the *T. gondii* qPCR completed, resulting in a positive result for one of the samples. This individual

oocyst appeared sporulated, was positive by qPCR and further confirmed as *T. gondii* by DNA sequencing of the qPCR product.

Table A. Identification of *T. gondii* like bodies from catchments and *T. gondii* specific qPCR for confirmation.

| Catchment | Number of slides screened | Number of slides with *T. gondii* like bodies visualised | *% T. gondii* like bodies | Number of slides positive for *T. gondii* by qPCR | *% T. gondii* positive |
|---|---|---|---|---|---|
| A | 26 | 2 | 7.7 | 0 | 0 |
| B | 5 | 0 | 0 | 0 | 0 |
| C | 22 | 1 | 4.5 | 0 | 0 |
| D | 94 | 16 | 17.0 | 0 | 0 |
| E | 4 | 0 | 0 | 0 | 0 |
| F | 16 | 3 | 18.6 | 0 | 0 |
| G | 10 | 1 | 10.0 | 0 | 0 |
| H | 16 | 2 | 12.5 | 1 | 8.3 |
| I | 5 | 1 | 20.0 | 0 | 0 |
| J | 3 | 1 | 33.3 | 0 | 0 |
| **TOTAL** | 201 | 27 | 13.4 | 1 | 0.5 |

In light of this result, additional analysis was performed on raw (dirty) post *Cryptosporidium* IMS samples from the catchment the oocyst was identified in. As these were highly turbid/dirty samples, it was not possible to visualise them by microscopy and each sample was tested for the presence of *T. gondii* DNA using the *T. gondii* specific qPCR. Of the 31 samples which were tested successfully by PCR, 4 (12.9%) were identified as positive *T. gondii* DNA.

Another important part of the study was to examine whether the methodology of filtration at Scottish Water in conjunction with the oocysts concentration method, would recover *T. gondii* DNA. Following three different concentration spikes of *T. gondii* DNA (high – 6645pg, medium – 664.5pg and low (64.45pg), only the high concentration was detectable by qPCR at very low levels (0.008pg), a 830625 fold decrease from the original spike (Table B). These results not only give confidence that in the current study any positives have originated from

*T.gondii* oocysts, but also confirm that the oocysts were the likely source from the work published by Wells *et al* (2015).

Table B. Results of DNA spiking experiment. Low and medium spikes were negative whilst DNA was detected in very low amounts from the high concentration spike.

| | | | Results after filtration, concentration and qPCR | | | |
|---|---|---|---|---|---|---|
| **Spike** | **DNA spike (pg)** | **Approx. oocyst number** | **qPCR CP value** | **Concentration per reaction (pg)** | **Total DNA concentration (pg)** | **Fold dilution from original DNA spike** |
| High | 6645 | 300 | 36.4 | 4.00E-4 | 0.008 | 830625 |
| Medium | 664.5 | 30 | n/a | n/a | 0 | n/a |
| Low | 66.45 | 3 | n/a | n/a | 0 | n/a |

In conclusion the results from screening show that between the periods of 26th September 2017 to 6th February 2018 across 10 different Scottish Water catchments, one *T. gondii* oocyst was identified in a final water sample. An additional 26 *T. gondii* like bodies, which resembled oocysts in their morphology, were not positive by the *T. gondii* specific qPCR. The confirmed *T. gondii* oocyst appeared to be sporulated (the infective stage of the oocyst), although it was not possible to test the viability of this oocyst as the DNA had been extracted for qPCR. From the samples screened, the water treatment process appears to be effective at removing *T. gondii* oocysts, although as only clean/clear post *Cryptosporidium* IMS final water samples could be screened, this may be an underrepresentation as it is dirty samples that are likely to have oocysts present. From the DNA spiking experiment conducted within this study, we can conclude that any PCR positives are likely to be from oocysts, albeit of unknown viability, however, ideally a methodology similar to what is already in place for *Cryptosporidium*, an IMS with a specific monoclonal antibody, would ensure that individual oocysts can be isolated from dirty water samples. As a recommendation, any future work should look at developing such a technique that could be used for the water industry.

**Contents**

# 1 Background

## 1.1 Introduction

The protozoan parasite *Toxoplasma gondii* (*T. gondii*) is a ubiquitous parasite in the environment and is one of the most common parasites to infect warm blooded animals worldwide, with felids being the only definitive host. It is predominantly young cats which, after being exposed to the parasite for the first time, excrete *T. gondii* oocyst in their faeces and subsequently into the environment. Toxoplasmosis is the disease caused by this parasite in susceptible hosts, including humans, where the consequences can be particularly serious in pregnant women and immuno-compromised individuals (Innes, 2010). Studies have confirmed that environmental contamination with *T. gondii* oocysts is widespread (Katzer et al., 2011). Indeed the significance of toxoplasmosis has increased globally, as recent research examining the disease burden of the parasite in the US and The Netherlands (Havealar *et al*., 2012, Hoffman *et al*., 2012), ranked *T. gondii* second when compared to other food-borne pathogens.

There are three infective stages of the parasite; sporozoites (oocysts), bradyzoites (tissue cysts) and tachyzoites. These three stages also equate to the three main transmission routes which are involved in the life cycle: **1)** Horizontal transmission - ingestion of oocysts consumed from the environment; **2)** Horizontal transmission - consumption of bradyzoites (tissue cysts) from infected meat which is either raw or undercooked; **3)** Vertical transmission - *T. gondii* tachyzoites can be passed on to the unborn foetus via the placenta resulting in congenital toxoplasmosis (Figure 1).

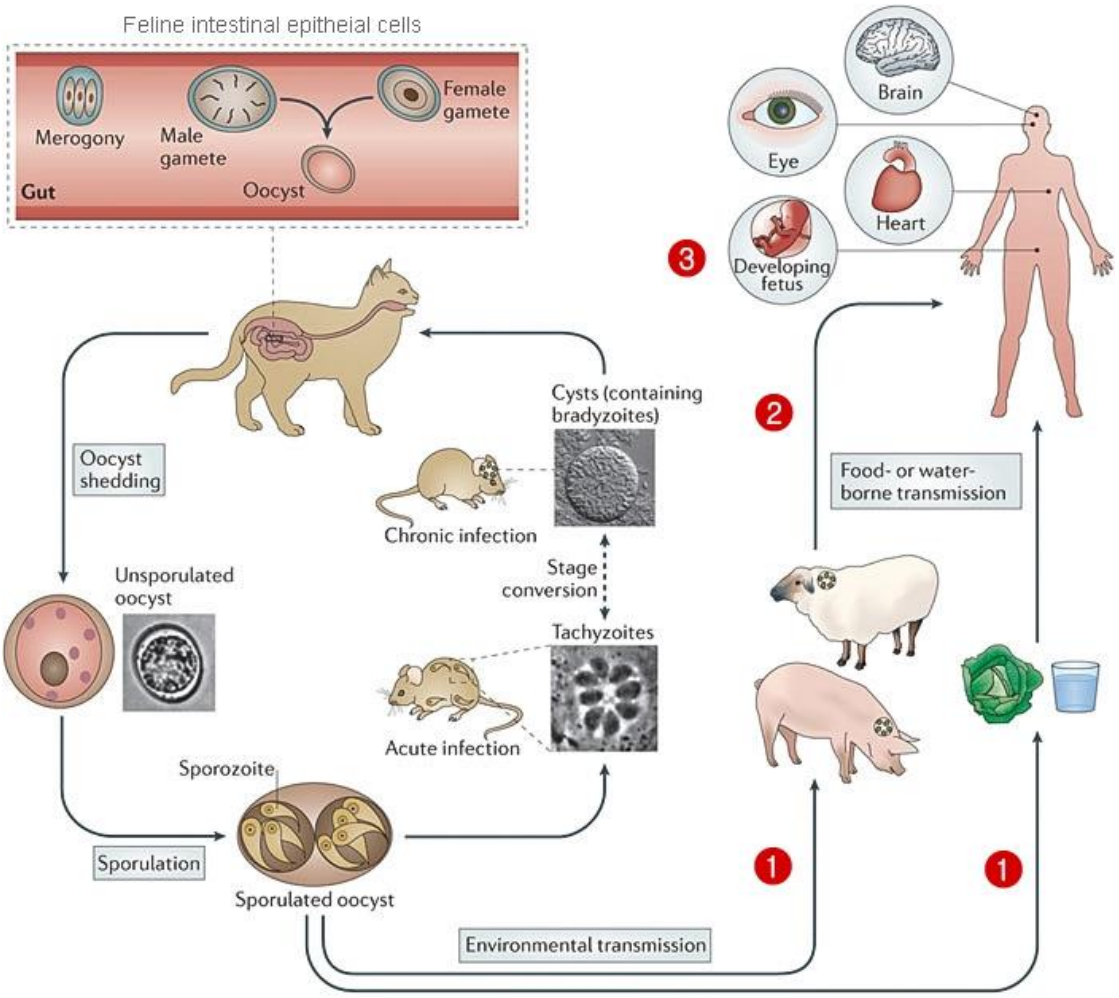



Figure 1. Life cycle of *T. gondii*. The three different stages of the parasite (oocysts, tissue cysts and tachyzoites) are transmitted by either: 1) horizontal transmission of oocysts from the environment; 2) horizontal transmission from tissue cysts within intermediate hosts; and 3) vertical transmission of tachyzoites from mother to foetus during pregnancy. Figure adapted from Hunter and Sibley. Nature Reviews Microbiology 2012 Nov; 10:766-778

The wide scale environmental contamination with the parasite along with the risk of infection of food animals and hence transmission to people, has caused increasing concern among policy makers and government in Europe and UK and there is an urgent need to improve our understanding of the prevalence, genotypes and hence risk posed by *T. gondii.* The waterborne transmission of *T. gondii* is also likely to be more important than previously thought as evidenced by large scale outbreaks of toxoplasmosis caused by contamination of drinking water with *T. gondii* oocysts (Bowie *et al*., 1997).

Currently, Scottish Water tests all of its supplies for the zoonotic parasite *Cryptosporidium,* the frequency of which is based on a risk assessment weighting for each supply. Testing for *Cryptosporidium* oocysts involves filtration (FiltaMax cartridges, IDEXX) of up to 1000 L from each supply, followed by centrifugation and immunomagnetic separation (IMS) to remove *Cryptosporidium* oocysts. In the current study, the eluted post-IMS suspension remaining from this process (approximately 10 - 12ml) was collected for examination of *T. gondii* oocysts. Supplies are not tested for *T. gondii* as there currently is no known risk and there are no accredited methods for analysing *T. gondii* oocysts from drinking water supplies. The microscopic size of *T. gondii* oocysts and the fact that they are not inactivated by conventional chlorination, make this parasite difficult to treat in water supplies. However, filtration systems designed to prevent *Cryptosporidium* oocysts from reaching finished water will be effective at capturing *T. gondii* oocysts, which are slightly larger in size. In the first published study looking at *T. gondii* prevalence in drinking water sources in the UK, Moredun in collaboration with Scottish Water (Wells et al., 2015), has shown that 8.8% of water samples tested (124/1411) from 147 water plants in Scotland tested positive for *T. gondii* DNA, which was detected using molecular techniques. A proportion of these positive samples were taken from finished waters i.e. water suitable for drinking. It is highly likely that the DNA identified in the water samples tested during this project were derived from oocysts, as the only other infectious forms of *T. gondii* (tachyzoites and tissue cysts) are not likely to be in the water in the first instance and if they are then they are also very unlikely to stay intact due to hypotonic tension caused by the osmotic imbalance between the parasite and the water. If the parasite bursts due to hypotonic tension, then it is very unlikely that its DNA would be recovered following the filtration procedure and subsequent processing. We now need to confirm that the DNA identified in water samples was derived from oocysts in the supply and also if those oocysts were viable, i.e. oocysts capable of causing toxoplasmosis. These points, particularly the latter, are extremely important to the water industry and to public health and need to be resolved.

It is highly unlikely that DNA would have survived processing of the water samples designed to recover oocysts using centrifugation, suggesting that the DNA detected was oocyst derived, but this needs to be confirmed. Previous outbreaks of waterborne toxoplasmosis in

humans that have been reported worldwide verify that oocysts can remain viable in water, therefore it is critical that we provide evidence of whether this is the case in Scottish water supplies or not, particularly as experimental studies have shown that oocysts can stay viable in refrigerated water for 5 years (Dubey, 1998).

Bowie, W.R., King, A.S., Werker, D.H., Isaac-Renton, J.L., Bell, A., Eng, S.B., Marion, S.A., 1997. Outbreak of toxoplasmosis associated with municipal drinking water. The Lancet 350, 173-177

Burrells, A., Opsteegh, M., Pollock, K.G., Alexander, C.L., Chatterton, J., Evans, R., Walker, R., McKenzie, C.A., Hill, D., Innes, E.A., Katzer, F., 2016. The prevalence and genotypic analysis of *Toxoplasma gondii* from individuals in Scotland, 2006-2012. Parasites & vectors 9, 324

Dubey, J.P., 1998. *Toxoplasma* gondii oocyst survival under defined temperatures. J Parasitol 84, 862-865.

Havelaar, A.H., Haagsma, J.A., Mangen, M.J., Kemmeren, J.M., Verhoef, L.P., Vijgen, S.M., Wilson, M., Friesema, I.H., Kortbeek, L.M., van Duynhoven, Y.T., van Pelt, W., 2012. Disease burden of foodborne pathogens in the Netherlands, 2009. International Journal of Food Microbiology 156, 231-238.

Hoffmann, S., Batz, M.B., Morris, J.G., Jr., 2012. Annual cost of illness and quality-adjusted life year losses in the United States due to 14 foodborne pathogens. J Food Prot 75, 1292-1302.

Innes, E.A., 2010. A brief history and overview of *Toxoplasma gondii*. Zoonoses Public Health 57, 1-7.

Katzer, F., Brulisauer, F., Collantes-Fernandez, E., Bartley, P.M., Burrells, A., Gunn, G., Maley, S.W., Cousens, C., Innes, E.A., 2011. Increased *Toxoplasma gondii* positivity relative to age in 125 Scottish sheep flocks; evidence of frequent acquired infection. Veterinary research 42, 121.

Wells, B., Shaw, H., Innocent, G., Guido, S., Hotchkiss, E., Parigi, M., Opsteegh, M., Green, J., Gillespie, S., Innes, E.A., Katzer, F., 2015. Molecular detection of *Toxoplasma gondii* in water samples from Scotland and a comparison between the 529bp real-time PCR and ITS1 nested PCR. Water research 87, 175-181.

## 1.2 Study aims and methodology as set out in the original tender (CR2016/09)

As described in the initial tender document, the main aim of this research project was to finalise and use a method to detect the presence of *T. gondii* oocysts in drinking water in order to confirm that oocysts are being removed effectively at Scottish Water treatment plants. The study aimed to:

- To develop / refine a robust method of extracting *T. gondii* oocysts at the low concentrations potentially found in drinking water.
- Use this method to determine the presence of oocysts in Scottish raw and final waters.
- Determine the viability of any oocysts detected via robust and established methodologies.
- Report on the effectiveness of individual water treatment plants and processes at de-activating and removing *T. gondii* oocysts.

It was anticipated that the study would comprise of the following stages:

- Collaborate with Scottish Water to obtain post immunomagnetic separation (IMS) elutions from samples already collected for the purpose of regulatory screening for *Cryptosporidium,* from four water catchments / treatment works previously identified as being of interest with respect to *T. gondii* due to the previous detection of *T.gondii* DNA. There will be no requirement to sample from water sources directly or visit treatment sites. It is anticipated that the number of samples to be analysed will not be less than 60, however the exact number will depend upon the availability of material for analysis.
- Optimise the extraction method for the extraction of *T. gondii* oocysts from water samples and use to extract oocysts from each sample, prior to microscopy. Some adaptation of current techniques to extract *T. gondii* oocysts from faecal material

may be required, in particular to account for the lower concentration of oocysts in the water matrix.

- Use microscopy and fluorescent staining of oocysts to confirm identification and sporulation.
- Directly test oocyst infectivity, probably via bioassay, using PCR and ELISA techniques, to confirm viability of any oocysts extracted.
- Relate the results of the work to the water treatment processes present at the respective water treatment works, within the context of oocyst removal and/or inactivation.

## 1.3 Objectives and milestones

To achieve these outputs the following objectives and milestones were agreed.

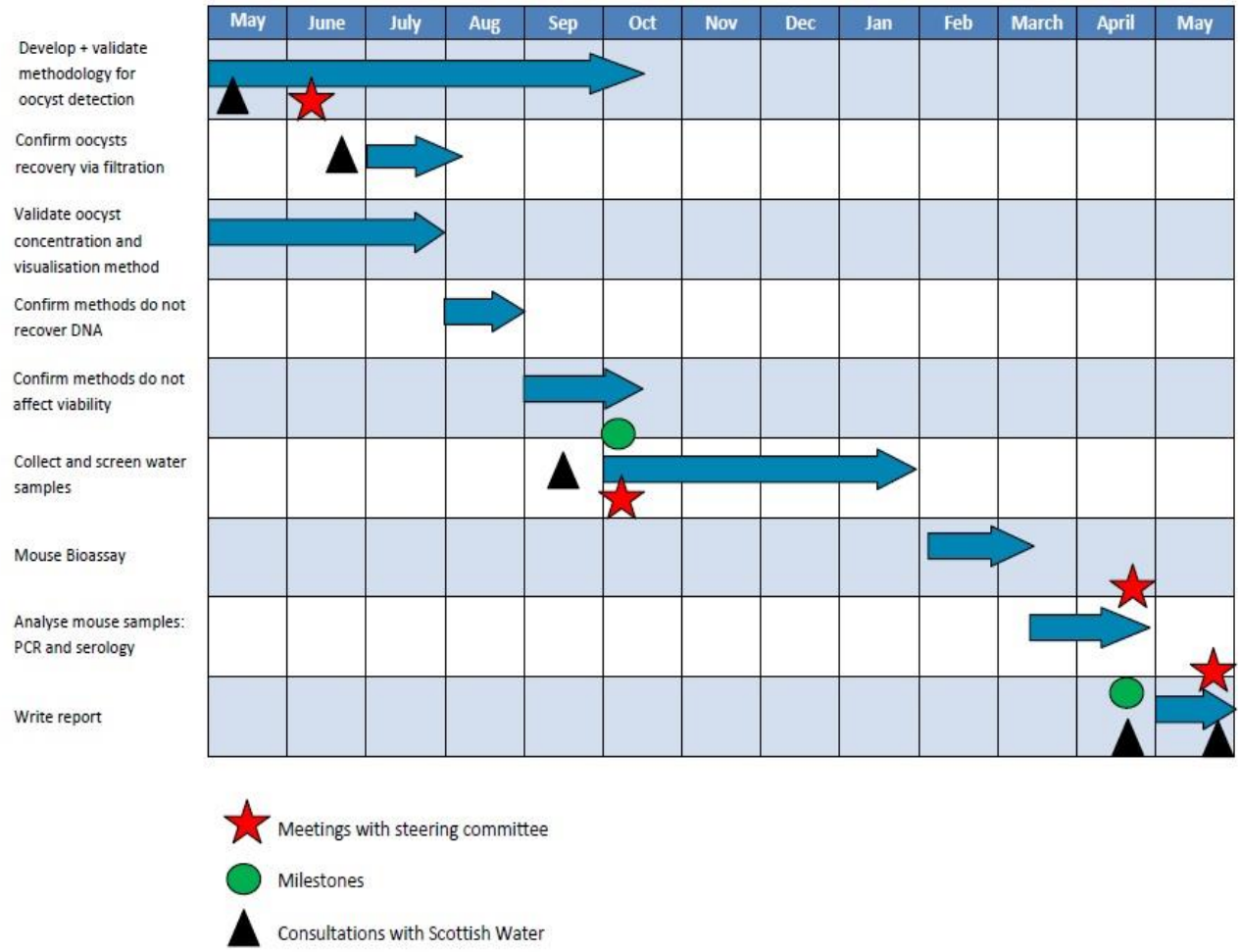



Figure 2. Planned objectives and milestones to enable completion of the project.

The report herein describes how the study met the required aims and methodology of the tender.

# 2 Development and validation of oocyst concentration and visualisation methodology for detection of oocysts in water

The development and validation of various methods for recovering *T. gondii* oocysts from post *Cryptosporidium* immuno-magnetic separation (IMS) water samples from both raw and final water were tested. In collaboration with Scottish Water, post *Cryptosporidium* IMS elution samples were collected (approx. 12 ml each) and individual samples spiked with either 1, 10, 100, or 1000 oocysts, unless otherwise stated. The different methodologies tested for oocyst concentration are described in section 2.1.

Different methodologies were also tested for oocyst visualisation. These methods are described in section 2.2.

Throughout the methodology development, post *Cryptosporidium* IMS water samples, whether raw or final water, are also referred to how they visually appeared. These were described as clean, cloudy or dirty. Even final water samples could appear cloudy/dirty, as the concentration process of 1000 L to 10 ml can also concentrate any particles in the water, making them appear more turbid. Examples of this can be seen in Figure 3.

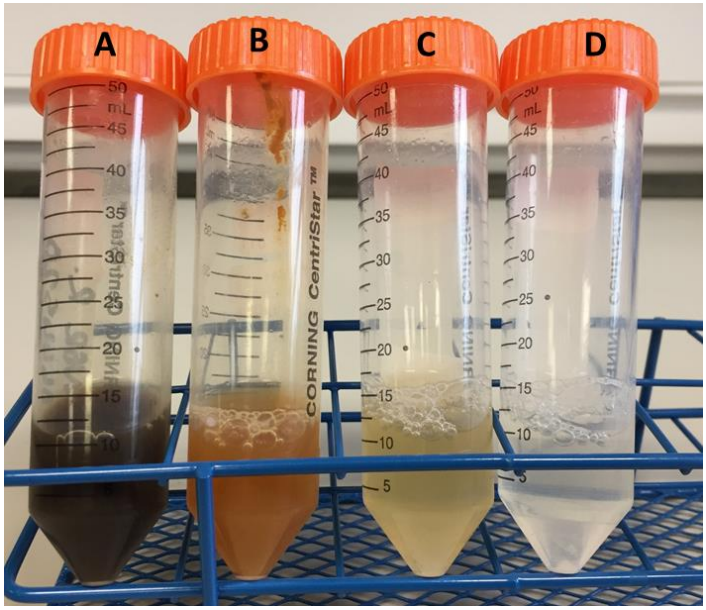



Figure 3. Examples of post *Cryptosporidium* IMS sample state used for testing and developing different methodologies. A = very dirty sample, B = dirty, C = cloudy, D = clear.

## 2.1 Extraction / concentration of oocysts from water

### 2.1.1 Salt flotation (final clean/cloudy and dirty raw samples)

Using a saturated salt solution with a specific gravity of 1.200 for the flotation of parasites is a known parasitological method for isolating oocysts from faecal samples. 500 ml of warm water and approximately 250 grams of salt (until no more salt goes into solution) was dissolved by stirring on a magnetic stirrer. The specific gravity was confirmed using a hydrometer. The spiked water sample was centrifuged at 1500g for 10 minutes to pellet the contents then the supernatant gently removed. Pellet re-suspended in 15 ml saturated salt solution and 5 ml of $dH_2O$ gently layered onto the surface. Sample was centrifuged at 1000 x g for 20 minutes (brake off), oocysts should float to the surface. The meniscus/top layer (approximately 5 – 10 ml) was carefully removed, transferred to a clean 50 ml falcon tube with the addition of up to 50 ml $dH_20$ to wash the oocysts. Sample centrifuged at 1500 x g for 10 minutes (brake on) and the supernatant gently removed. The final pellet/oocysts were re-suspended in 60 – 100 µl $dH_2O$.

**Results**:

As shown in Table 1, following salt flotation, recovery of oocysts was reasonable in clean/cloudy final water samples spiked with low numbers of oocysts, with a 30% recovery rate for samples spiked with 10 oocysts. However the recovery rate was zero for all spiked dirty raw water samples and clean/cloudy final water samples spiked with 1 oocyst. This methodology is not suitable for raw dirty water samples and not sensitive enough for final/cloudy water samples where oocyst numbers are likely to be low.

Table 1. Results of oocyst spiking using salt flotation on post *Cryptosporidium* IMS final clean/cloudy water samples and raw dirty water samples.

| *T. gondii* oocyst spike | Recovery from final clean/cloudy water | % recovery | Recovery from raw dirty water | % recovery |
|---|---|---|---|---|
| 1000 | 200+ | >20 | 38 | 3.8 |
| 100 | 75 | 75 | 3 | 3 |
| 10 | 3 | 30 | 0 | 0 |
| 1 | 0 | 0 | 0 | 0 |

### 2.1.2 Salt flotation plus 0.1% gelatine (raw dirty samples)

Gelatine was added to a final concentration of 0.1% to the saturated salt solution made in 2.1.1. To ensure the gelatine dissolved it was gently heated in a microwave and frequently mixed until all granules of gelatine had dissolved. The salt flotation method was then followed as described in 2.1.1.

N.B. For this experiment three samples were spiked with 100 *T. gondii* oocysts

**Results**:

As highlighted in Table 2, using this methodology no oocysts were recovered from the spiked raw water samples, therefore this method was not suitable for use within this study.

Table 2. Results of oocyst spiking using salt flotation plus 0.1% gelatine on post *Cryptosporidium* IMS raw dirty water samples.

| *T. gondii* oocyst spike | Recovery from raw dirty water | % recovery |
|---|---|---|
| 100 | 0 | 0 |

### 2.1.3 Salt flotation plus 0.05% Tween (raw dirty samples)

0.05% of Tween 20 was added to the saturated salt solution made in 2.1.1 (125 µl Tween 20, 250 ml saturated salt solution). The salt flotation method was then followed as described in 2.1.1.

N.B For this experiment four samples were spiked with 200 and 100 *T. gondii* oocysts. As no oocysts were recovered from the method previously in 2.1.2. using 100 oocysts, a 200 oocyst spike was also included.

**Results:**

As shown in Table 3, due to the low recovery rates, this methodology is not suitable for raw dirty water samples.

Table 3. Results of oocyst spiking using salt flotation plus 0.05% Tween on post *Cryptosporidium* IMS raw dirty water samples.

| *T. gondii* oocyst spike | Recovery from raw dirty water | % recovery |
|---|---|---|
| 200 | 3 | 1.5 |
| 100 | 0 | 0 |

### 2.1.4 Reverse sucrose flotation.

This method was modified from Lelu *et al* 2011 and Ramirez *et al* 2006. Dirty post *Cryptosporidium* IMS samples were spiked with *T. gondii* oocysts. Samples were centrifuged in 50 ml falcon tubes at 1500 x g for 10 minutes and supernatant carefully removed. 5 ml of 2% sulphuric acid ($H_2SO_4$) was added and sample allowed to stand overnight at room temp. Then, 20 ml of $dH_2O$ was added and the sample vortexed for 1 minute, the sample was separated into two 50 ml falcon tubes. Carefully, 20 ml cold (4°C) sucrose solution (s.g 1.2) was layered under the sample and centrifuged at 1500 x g for 20 minutes (brake at deceleration 5). The interphase layer, between the $dH_2O$ (clear) and sucrose (yellow) = approximately 13 ml per 50ml tube, was transferred into another 50 ml falcon tube. The falcon was filled up to 50 ml with $dH_2O$, vortexed briefly to mix and centrifuged at 1500 x g for 20 minutes. The supernatant was carefully removed so not to disturb the pellet/sediment, both the pellets/sediment (approximately 1 ml each) were combined into one 50 ml falcon tube. Falcon tubes were filled up to 50 ml with $dH_2O$, vortexed briefly to mix and centrifuged at 1500 x g for 20 minutes. So as not to disturb the pellet/sediment, the supernatant was carefully removed. The pellet was transferred to either a 1.5ml or 2ml tube and centrifuged at 1700 g for 10 minutes. The supernatant was carefully removed, leaving approximately 60 – 80 µl supernatant plus the pellet/sediment. The pellet/sediment was re-suspended with the remaining supernatant by gently mixing with a 200 µl pipette. The pellet/sediment was then transferred to a slide for visualisation and counting.

Sucrose flotation solution (s.g 1.2): $dH_2O$ (boiled) 80 ml, Sucrose 125g, Phenol red (0.4%) 200 µl, once cooled add 1.5 ml formalin to prevent mould growth and store @ 4°c.

**Results**:

The percentage recovery for raw dirty water was still low (2% - Table 4), but an improvement on results from previous methodologies tested.

Table 4. Results of oocyst spiking using reverse sucrose flotation on post *Cryptosporidium* IMS raw dirty water samples.

| *T. gondii* oocyst spike | Recovery from raw dirty water | % recovery |
|---|---|---|
| 100 | 26 | 26 |
| 50 | 1 | 2 |

Lelu, M., Gilot-Fromont, E., Aubert, D., Richaume, A., Afonso, E., Dupuis, E., Gotteland, C., Marnef, F., Poulle, M.L., Dumetre, A., Thulliez, P., Darde, M.L., Villena, I., 2011. Development of a sensitive method for *Toxoplasma gondii* oocyst extraction in soil. Vet Parasitol 183, 59-67.

Ramirez, N.E., Sreevatsan, S., 2006. Development of a sensitive detection system for *Cryptosporidium* in environmental samples. Vet Parasitol 136, 201-213.

### 2.1.5 Centrifugation (final clean/cloudy and raw dirty samples)

Each 50 ml falcon containing the *T. gondii* oocyst spiked post *Cryptosporidium* IMS sample was filled with $dH_2O$ up to 40 ml and centrifuged at 1700 x g for 10 minutes. The supernatant was gently removed, the pellet re-suspended by vortexing and 12 ml of $dH_2O$ was added. If the sample was not to be processed for visualisation immediately, penicillin/streptomycin was added to make a final concentration of 4% (this reduces biofilm formation/bacterial growth). Centrifugation at 1700 x g was repeated for 10 minutes and the supernatant gently removed. The pellet/sediment was transferred to a slide for visualisation and counting, or used for DNA extraction.

**Results:**

This methodology appears to be the best for recovery of oocysts from final clean/cloudy water samples, detecting as low as one oocyst from spiked final water (Table 5). However this is not a suitable method for analysing raw dirty water samples.

Table 5. Results of oocyst spiking using centrifugation on post *Cryptosporidium* IMS final clean/cloudy water samples and raw dirty water samples.

| *T. gondii* oocyst spike | Recovery from final clean/cloudy water | % recovery | Recovery from raw dirty water | % recovery |
|---|---|---|---|---|
| 1000 | 200+ | >20 | 200+ | >20 |
| 100 | 72 | 72 | 26 | 26 |
| 10 | 6 | 60 | 0 | 0 |
| 1 | 1 | 100 | 0 | 0 |

### 2.1.6 Lectin magnetic separation (raw dirty samples)

This published method by Harito *et al* 2017 captures *T. gondii* oocysts from water using lectin coated magnetic beads. The method was followed as published using, briefly, 100µl Dynabeads (M-280 Streptavadin, Invitrogen), coated with wheat germ agglutinin, (Sigma) were added to the spiked samples in a L10 tube (Dynal) and gently mixed by rotation for 1 hour. Beads were then separated from debris using a magnetic concentrator (MPC), whist in the MPC the supernatant was carefully removed so as not to disturb the beads. The beads were then washed by removing the tube from the MPC and any oocysts which had bound to the beads were dissociated, again using the MPC and GlcNAc (0.5M) mixed with acidified pepsin solution (1%). The supernatant, containing any dissociated oocysts, were transferred onto a slide for visualisation using UV microscopy.

**Results:**

No oocysts were observed from the dissociated samples. However during the stage which separates the beads from the debris, a large amount of debris appeared to be hampering the separation of the beads from the supernatant. The supernatant from this stage was kept, concentrated by centrifugation and viewed under the microscope. Oocysts were observed, indicating that due to the amount of debris in the dirty raw water samples, the magnetic beads and magnets were not successful in isolating any oocysts. In this study, this method would not be suitable for analysing dirty raw water samples.

Harito, J.B., Campbell, A.T., Tysnes, K.R., Dubey, J.P., Robertson, L.J., 2017. Lectin-magnetic separation (LMS) for isolation of *Toxoplasma* gondii oocysts from concentrated water samples prior to detection by microscopy or qPCR. Water Res 114, 228-236.

### 2.1.7 Acid flocculation (raw dirty samples)

The *T. gondii* spiked post *Cryptosporidium* water sample was transferred to a 500ml glass cylinder and topped up to 300 ml with tap water. Then 3 ml of 2% $H_2SO_4$ was added to the cylinder and stirred for 5 minutes on a magnetic stirrer. The sample was left to settle for 15 minutes, and the clear layer removed using a 50ml pipette, avoiding any debris on the surface. The clear layer was transferred to a 250 ml centrifuge bottle and centrifuged at

1000 x g for 20 minutes (maximum acceleration with brake on), the supernatant was carefully removed, pellet re-suspend 6 ml of $dH_20$ and transferred to a 50 ml centrifuge tube. The 250 ml tube was rinsed with a further 3-6 ml of $dH_20$, washings were added to the 50 ml tube which was then centrifuged at 1962 x g for 5 minutes (maximum acceleration and deceleration). The supernatant was carefully removed and the pellet re-suspended in 50 – 100 µl $dH_2O$. The pellet/sediment was then transferred to a slide for visualisation and counting by UV microscopy.

N.B For this experiment samples were only spiked with 50 and 100 *T. gondii* oocysts

**Results:**

No oocysts were observed using this methodology.

### 2.1.8 Flotac device (raw dirty samples)

The mini flotac device has been reported to be a useful tool for identifying oocysts from cat faeces (Djokic *et al* 2014). In order to accommodate greater volumes the larger flotac device was obtained (personal communication Laura Rinaldi, Naples University, Italy). Spiked samples (12 ml total) were centrifuged at 1700 x g for 10 minutes, the supernatant carefully removed and the pellet vortexted to re-suspend. 10 ml of saturated salt solution (see section 2.1.1) was added, the samples mixed by vortexing and 5 ml added into each of the two chambers on the device. Samples were centrifuged for 5 minutes at 220 x g. The flotac device was carefully removed from the centrifuge and the disc on the flotac device rotated to the reading pane. The flotac was carefully placed into the supplied microscope adaptor and visualised using a UV microscope.

**Results:**

The amount of debris from the dirty samples meant that the Flotac slide could not be visualised clearly under the microscope. In addition, during the centrifugation stage the Flotac device was prone to leaking. Overall this would not be a suitable method for raw dirty samples.

Djokic, V., Blaga, R., Rinaldi, L., Le Roux, D., Ducry, T., Maurelli, M.P., Perret, C., Djakovic, O.D., Cringoli, G., Boireau, P., 2014. Mini-FLOTAC for counting *Toxoplasma gondii* oocysts from cat feces - Comparison with cell counting plates. Experimental Parasitology 147, 67-71.

### 2.1.9 Sulphuric acid and citric acid lysis buffer (raw and final dirty samples)

12 ml post *Cryptosporidium* IMS sample dirty final and raw water samples were spiked with 100 and 50 *T. gondii* oocysts. Samples were centrifuged at 1700 x g for 10 minutes. The supernatant was gently removed and the pellet re-suspended by vortexing, then 2 ml 2% sulphuric acid was added and the sample left overnight at room temperature. The sample was vortexed vigorously then 40 ml $dH_2O$ was added, followed by centrifugation at 1700 x g for 10 minutes. The supernatant was gently removed so as not to disturb the pellet and 1.5 ml Flocculation Lysis Buffer was added and the sample vortexed vigorously. The sample was incubated at room temperature for 1 hour, vortexing every 15 minutes (as described by Kourenti *et al* 2003). After 1 hour, 40 ml $dH_2O$ was added to the sample followed by centrifugation for 20 minutes at 1500 x g, the supernatant was then carefully removed, ensuring approximately 3 ml of liquid/pellet remained in the tube. The pellet was re-suspend by vortexing and washed by adding approximately 40 – 45 ml $dH_2O$. The sample was centrifuged at 1500 x g for 20 minutes and should be less dirty than previously. Supernatant was carefully removed, leaving behind approximately 2 ml which was gently mixed with a 1 ml pipette and transferred to a 2ml Eppendorf tube. Finally the sample was centrifuged at 5000 x g for 5 minutes, supernatant carefully removed leaving behind approximately 60 – 120 µl of pellet/water (depending on pellet size). This was gently re-suspended by pipetting and, depending how dirty the sample was, the pellet was transferred to one or two slide wells for visualisation by UV microscopy.

**Results:**

The process cleared two of the dirty final water samples but not the dirty raw water as shown in Figure 4. Therefore only the spiked dirty water sample could be screened. However from Table 6 it is clear that the methodology had effected the sensitivity for detecting low numbers of oocysts.

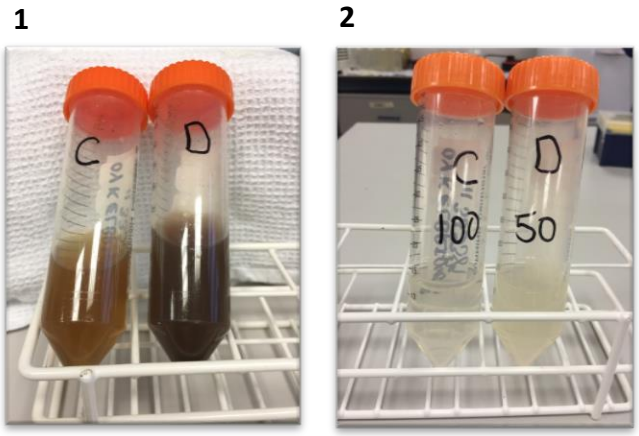



Figure 4. Result of acid lysis buffer for cleaning dirty final water samples. 1 = before acid flocculation buffer, 2 = after acid flocculation buffer. C = final dirty sample spiked with 100 *T. gondii* oocysts; D = final dirty water sample spiked with 50 *T. gondii* oocysts.

Table 6. Results of oocyst spiking whilst incorporating the acid lysis methodology on post *Cryptosporidium* IMS final dirty water samples raw dirty water samples.

| *T. gondii* oocyst spike | Recovery from final dirty water | % recovery | Recovery from raw dirty water | % recovery |
|---|---|---|---|---|
| 100 | 45 | 45 | 0 | 0 |
| 50 | 2 | 4 | 0 | 0 |

## 2.2 Visualisation of oocysts

### 2.2.1 Auto-fluorescence (final clean/cloudy and dirty raw samples)

The methodology for visualising *T. gondii* oocysts by auto-fluorescence is described by Lindquist *et al* 2003. Briefly, the entire processed water sample pellet/sediment (approximately 50 µl) derived from the methods described in sections 2.1.1 to 2.1.9 were pipetted onto a slide well, 2 to 4 slide wells may be required depending on the sample state. The sample was air dried on a slide in a 37°C incubator (approximately 20 minutes) and once dry, fixed onto the slide using 25 µl methanol and allowed to air dry. The cover slip was applied with mountant and sealed with nail varnish and allowed to fully dry in the dark. Slides were examined using an Olympus BX30 microscope with an appropriate filter cube

(filter cube WU = dichroic mirror DM400, excitation filter BP330-385, barrier filter BA420) and oocysts were identified by their spherical structure, measuring approximately 11 to 13 µm.

**Results:**

This simple method worked well for clean/cloudy spiked water samples, as each oocyst could be seen, determined whether un-sporulated or sporulated and counted. An example of what was observed is shown in Figure 5, where un-sporulated oocysts are visible in image A and C and sporulated oocysts in image B, C and D (these images are from final clean/cloudy post *Cryptosporidium* IMS spiked samples). N.B It was only possible to visualise oocysts from the raw dirty samples spiked with 100 oocysts (as described in section 2.1.5).

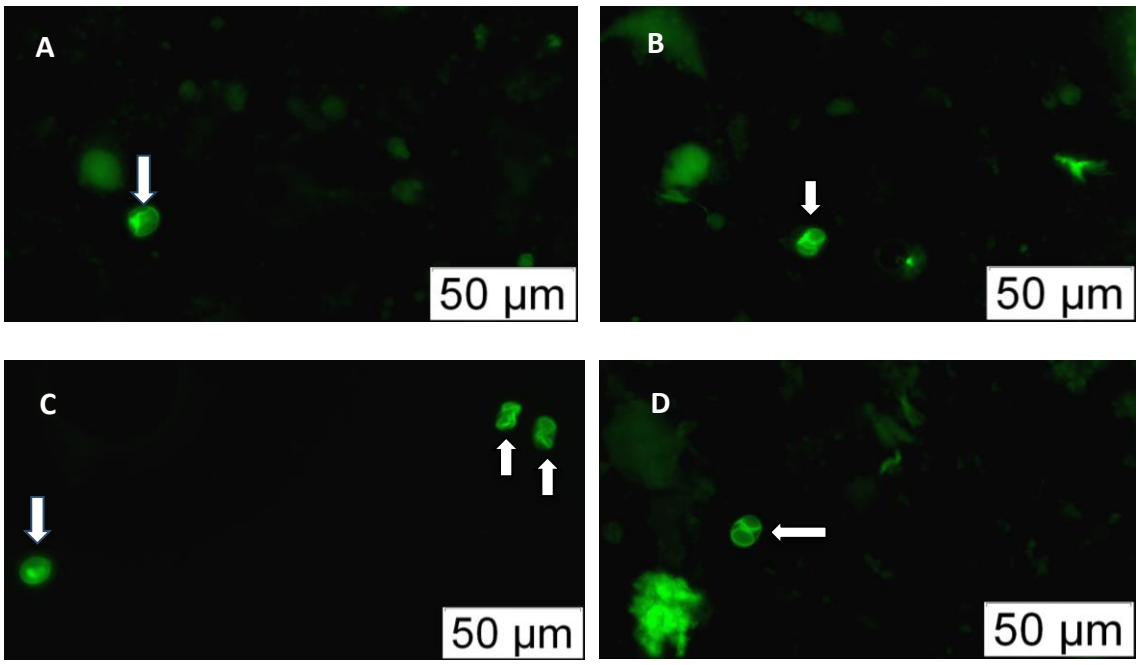



Figure 5. Post *Cryptosporidium* IMS final water sample spiked with sporulated and un-sporulated oocysts and visualised by auto-fluorescence. A = un-sporulated oocyst, B = sporulated oocyst, C = un-sporulated and sporulated oocysts, D = sporulated oocyst.

Lindquist, H.D., Bennett, J.W., Hester, J.D., Ware, M.W., Dubey, J.P., Everson, W.V., 2003. Autofluorescence of *Toxoplasma gondii* and related coccidian oocysts. J Parasitol 89, 865-867.

### 2.2.2 *T. gondii* specific monoclonal antibody (mAb) staining (TgOWP3)

All of the final processed water sample pellet/sediment (approximately 50 µl) derived from the methods described in sections 2.1.1 to 2.1.9 were transferred to a slide well, 2 to 4 slide wells may be required depending on the sample state. Sample was air dried onto slide in a 37°C incubator (approximately 20 minutes) and once dry, fixed onto the slide using 25 µl methanol and allowed to air dry. The sample was blocked with 40 µl 2%FCS/PBS and incubated at 37°C in a humid box for 10 minutes, then washed three times with 30 µl PBS. 25 µl of primary antibody (TgOWP3 IG12 mAb, as described in Possenti *et al* 2010 – obtained from Furio Spano, personal communication) was added to the slide at a dilution of 1:800 and incubated in a humid box at 37°C for 30 minutes.

The slide was washed three times with 30 µl PBS then a small droplet of the secondary antibody (Alexa fluor 488 goat anti mouse IgG – Invitrogen) applied to each well and the slide incubated in a humid box at a 37°C for 30 minutes. The slide was washed three times with PBS as previously described, cover slip carefully applied using mountant and the coverslip/slide sealed using clear nail varnish. The slide was dried in the dark and then examined using an Olympus BX30 microscope as described previously (2.2.1).

**Results:**

Although initial testing of *T. gondii* oocysts spiked into laboratory grade $dH_2O$ worked well, when tested using the post *Cryptosporidium* IMS water samples the TgOWP mAb also stained any algae/debris within these samples. This made identification of oocysts difficult and therefore use of this mAb was not suitable for use in this study.

Possenti, A., Cherchi, S., Bertuccini, L., Pozio, E., Dubey, J.P., Spano, F., 2010. Molecular characterisation of a novel family of cysteine-rich proteins of *Toxoplasma gondii* and ultrastructural evidence of oocyst wall localisation. Int J Parasitol 40, 1639-1649.

### 2.2.3 Removal of oocysts from slides.

To remove oocysts for further downstream processing e.g. for future mouse bioassays, or for DNA extraction and qPCR, slides were scraped and processed as follows; method adapted from Claire Alexander (personal communication), Scottish Parasite Diagnostic and Reference Laboratory, Glasgow. Coverslip from the slide was removed using nail varnish remover and gently lifted off using a scalpel blade, and the Teflon coated area of the slide surrounding the well dried with a small piece of paper towel. 30 µl of buffer T1 (Macherey-Nagel nucleospin tissue, isolation of genomic DNA kit) was pipetted onto the well and the surface scraped using a 1 µl bacterial inoculation loop. The slide was tilted at an angle and the 30 µl buffer T1 removed from the slide and aliquoted into a sterile Eppendorf. The slide was rotated 90$^{o}$ and the process repeated using 30 µl of fresh buffer T1 and scraped using the same inoculation loop. Once the slide had been rotated and scraped through 360$^{o}$, the end of the inoculation loop was removed and kept within the Eppendorf by pressing on the inside rim of the tube. The lid was closed and DNA was extracted as described in Appendix 9.3 or used for mouse bioassay as described in section 3 (N.B in cell scraping for use in the mouse bioassay, buffer T1 was replaced with sterile PBS).

## 2.3 Outcome of oocyst concentration and visualisation

Early into the development of the concentration methodologies, it became apparent that dirty raw and dirty final water samples were problematic. The methodology development mainly focused on trying to "clean up" these dirty samples to enable them to be screened by microscopy, as well as ensuring that the method remained sensitive enough to detect low numbers of oocysts. Centrifugation of clear and cloudy spiked post *Cryptosporidium* IMS samples proved to be a successful quick and straight forward method for concentrating oocysts, with as low as one oocyst being detected using this process (2.1.5).

Overall, auto fluorescence (2.2.1) provided the best results for visualisation as the *T. gondii* oocyst wall protein monoclonal antibody (TgOWP3) bound to additional material in the *T. gondii* spiked post *Cryptosporidium* IMS samples making it difficult to visualise the oocysts.

## 2.4 Conclusion

In conclusion the best methodology to use for this study will be to screen final clean and cloudy post *Cryptosporidium* IMS water samples using centrifugation (as described in 2.1.5) and auto-fluorescence (as described in 2.2.1). However, depending on the results from the catchments studied, dirty final water samples could be treated with sulphuric acid and citric acid (as described in lysis in 2.1.9) in an attempt to clean them enough to be used for microscopy, however this would have an impact on sensitivity.

# 3 Sensitivity of mouse bioassay and whether visualisation and recovery of oocysts from slides affect viability.

## 3.1 Sensitivity of mouse bioassay and confirmation that the methods used for oocyst recovery and visualisation do not affect the viability

As the proposed plan involved a mouse bioassay to test the viability of any *T. gondii* oocysts identified, it was important to determine whether the concentration and visualisation process had any effect on oocyst viability. Therefore, water samples spiked with a known number of oocysts (Table 7) were processed using the final method decided upon for concentration (centrifugation 2.1.5) and visualisation (auto-fluorescence 2.2.1). In addition, prior to testing water samples from Scottish Water, we must first confirm that infection in mice is possible with low numbers of oocysts and is therefore sensitive enough to detect potentially low numbers of oocysts that may be present within water samples.

## 3.2 Mouse bioassay

Prior to the start of the mouse bioassay, ethical approval was sought from the experiments committee at Moredun Research Institute. Within this committee was a representative from the Home Office. Following approval, twenty-four female Swiss Webster mice were used to test the sensitivity of the assay, inoculating with a titrated dose of 100, 20, 10, 5 and 1 Type II oocysts (SP-1, see Appendix 9.1) (groups 1 – 5, Table 7). Five mice were used to ensure that when present at low numbers, centrifugation and visualisation did not compromise oocyst viability (group 6, Table 7). Five spiked water samples were titrated to contain between 1 and 200 oocysts and prepared as described in section 2.1.5 and 2.2.1.

Once oocysts had been visualised and counted, slides were scraped using the methodology described in section 2.2.3 and tested for viability by mouse bioassay.

Table 7. Experimental groups for mouse bioassay to test sensitivity and viability of methodology. Groups 1- 6 are to test the sensitivity of the assay, group 6 was to test whether the methodology had an effect on viability.

| Group No. | No. of mice | Number of *T. gondii* oocysts | Purpose |
|---|---|---|---|
| 1 | 4 | 100 | Sensitivity of dose |
| 2 | 4 | 20 | " |
| 3 | 4 | 10 | " |
| 4 | 4 | 5 | " |
| 5 | 7 | 1 | " |
| 6 | 5 | 19, 14, 11, 10, 7 | Test whether visualisation & scraping affect viability |

Each mouse was orally inoculated with the appropriate number of oocysts. Mice were assessed twice a day and each mouse scored against a Home Office approved scoring system specifically for *T. gondii* in mice (see Appendix 9.2). Any animal reaching a maximum score in either category, or if an animal has a total score of 4 for two consecutive days, was euthanised using a Schedule 1 procedure. All remaining animals were euthanised using a Schedule 1 procedure at the end of 4 weeks. Post mortems were completed on mice, collecting blood, brain, heart and lung from each mouse into separate sterile containers. DNA was extracted from brain, heart and lung as described in Appendix 9.4 and a *T. gondii* ITS1 specific PCR was completed as described in Appendix 9.5.

## 3.3 Results of mouse bioassay sensitivity and viability of oocysts following centrifugation / visualisation and recovery from slides.

The sensitivity of the bioassay was not as expected, particularly as previous studies have shown that infection in pigs and mice with as low as one, 2.5 and 5 oocysts is possible (Dubey *et al* 1996, Isaac-Renton *et al* 1998, Muller *et al* 2017). However, 2 mice in the 100 oocyst group showed clinical signs of *T.gondii* infection, these two mice were also positive

for *T. gondii* by PCR and ELISA (group 1, Table 8). None of the remaining mice in any of the groups showed signs of *T. gondii* infection and were negative by PCR and ELISA.

Table 8. Results of mouse bioassay for sensitivity and viability of methodology.

Groups 1- 6 are to test the sensitivity of the assay, group 6 was to test whether the methodology had an effect on viability.

| Group No. | Number of *T. gondii* oocysts | No. PCR positives (Brain, Heart, Lung) | ELISA | Observations in mice |
|---|---|---|---|---|
| 1 | 100 | 2/4 | 2/4 | 1 mouse euthanised due to signs *of T. gondii* infection (d13).<br>1 mouse showed mild signs of *T. gondii* infection (d12 - d15). |
| 2 | 20 | 0/4 | 0/4 | All mice appeared normal |
| 4 | 10 | 0/4 | 0/4 | All mice appeared normal |
| 5 | 5 | 0/4 | 0/4 | All mice appeared normal |
| 6 | 1 | 0/7 | 0/7 | All mice appeared normal |
| 7 | 19, 14, 11, 10, 7 | 0/5 | 0/5 | All mice appeared normal |

Dubey, J.P., Andrews, C.D., Lind, P., Kwok, O.C., Thulliez, P., Lunney, J.K., 1996. Antibody responses measured by various serologic tests in pigs orally inoculated with low numbers of *Toxoplasma gondii* oocysts. Am J Vet Res 57, 1733-1737.

Isaac-Renton, J., Bowie, W.R., King, A., Irwin, G.S., Ong, C.S., Fung, C.P., Shokeir, M.O., Dubey, J.P., 1998. Detection of *Toxoplasma gondii* oocysts in drinking water. Appl Environ Microbiol 64, 2278-2280.

Muller, J., Aguado-Martinez, A., Ortega-Mora, L.M., Moreno-Gonzalo, J., Ferre, I., Hulverson, M.A., Choi, R., McCloskey, M.C., Barrett, L.K., Maly, D.J., Ojo, K.K., Van Voorhis, W., Hemphill, A., 2017. Development of a murine vertical transmission model for *Toxoplasma gondii* oocyst infection and studies on the efficacy of bumped kinase inhibitor (BKI)-1294 and the naphthoquinone buparvaquone against congenital toxoplasmosis. J Antimicrob Chemother 72, 2334-2341.

## 3.4 Conclusions

As previously reported in the literature, we could not confirm that, in our laboratory, the mouse bioassay could detect low numbers of oocysts and the assay was not as sensitive as expected. This raised the question as to whether the oocysts we obtained were still viable. The oocysts were obtained from a source in Spain (Universidad Complutense de Madrid, Spain; batch produced from cats in autumn 2016) and the ratio of sporulated / un-sporulated oocysts was high (>95%). When the oocysts were used for an animal study in both sheep and mice in Spain, they were known to be infective and viable (however the infective dose was not known). It is possible that due to storage oocysts may have become less viable. Because of the lack of sensitivity data for the mouse bioassay, it is not possible to draw a conclusion on whether the methodology (centrifugation and auto-fluorescence) had an effect on oocyst viability.

# 4 Additional objective - Sensitivity of the *T. gondii* 529bp qPCR for detecting low numbers of oocysts from visualised and scraped from slides.

As the bioassay was not successful for detecting low numbers of oocysts, it was discussed within the steering group how it would be important to confirm that any low numbers of oocysts or *Toxoplasma* like bodes visualised under the microscope from water samples were indeed *Toxoplasma*. Therefore a spiking experiment was set up to determine how sensitive the *T. gondii* 529bp qPCR was for detecting low numbers of oocysts from visualised and scraped slides.

## 4.1 Oocyst spiking, visualisation and DNA extraction

Spiked cloudy final water samples from catchments out with the study were titrated to contain 10, 5 and 1 oocysts and the methodology described in 2.1.5 and 2.2.1 was used for concentration and visualisation. Following visualisation of the spiked samples, 9, 3 and 1 oocysts were observed (Table 9). Slides were then scraped as described in 2.2.3 and DNA extracted from these samples (as described in Appendix 9.3)

To ensure DNA contamination was not present, an extraction control was included throughout the process.

## 4.2 529bp qPCR

The method for the 529pb qPCR is fully described in Appendix 9.6. In brief, DNA from scraped slides (see 4.1) and the extraction control DNA was added in sextuplicate to the wells of a 96 well qPCR plate. The same plate contained 5 titrated DNA standards of known concentration, these were used as a reference to determine the DNA concentration from the scraped slides.

## 4.3 Result of 529bp sensitivity testing from scraped slides

The qPCR was sensitive enough to detect *T. gondii* DNA from scraped slides containing 9, 3 and 1 oocyst. Each sample replicate was also positive at least 5 times, indicating a high degree of reproducibility, as shown in Table 9. In addition, the qPCR from the DNA extracted from the scraped slides gave curves that are in line with what would be expected for a positive result as shown in Figure 6.

Table 9. Sensitivity of *T. gondii* 529bp qPCR on scraped slides containing low numbers of oocysts. *The higher the qPCR CP value = less DNA present.

| No. oocysts in original spike | No. oocysts after visualisation | qPCR positive | Number of replicates positive | qPCR CP value* |
|---|---|---|---|---|
| 10 | 9 | Yes | 6/6 | 30.6 |
| 5 | 3 | Yes | 5/6 | 35.3 |
| 1 | 1 | Yes | 6/6 | 36.0 |

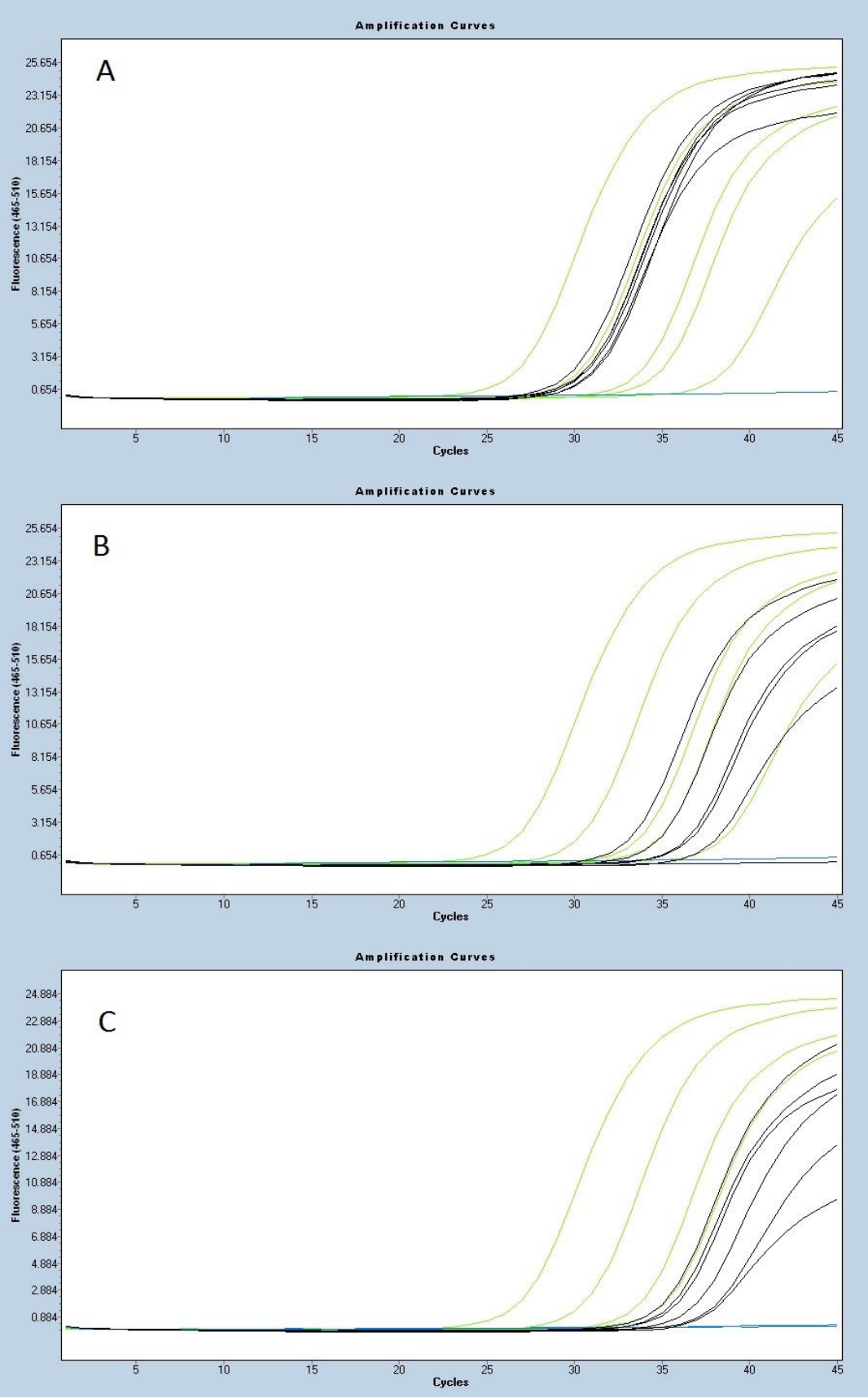



Figure 6. qPCR amplification curves for low numbers of oocysts scraped from slides.
A= 9 oocysts, B= 3 oocysts, C= 1 oocyst. The greater the cycle number (cp value) the less DNA present. Light green line = standard curve, black line = *T. gondii* DNA, blue line = negative control.

## 4.4 Conclusions from 529pb qPCR for detecting low numbers of oocysts from visualised and scraped slides

The concentration and visualisation process did not appear to affect detection of *T. gondii* DNA by qPCR. As shown in section 4.3, the *T. gondii* specific qPCR has been shown to be sensitive enough to detect low numbers of oocysts (as low as one oocyst) from slides that have been scraped using the methodology described in 2.2.3. As the mouse bioassay proved not to be as successful as originally hoped, this technique will now play a vital role in the project for confirming whether *T. gondii* like bodies visualised by microscopy are *Toxoplasma* oocysts or not. The only drawback to using qPCR to confirm whether a visualised sample is *T. gondii* positive is that it is not possible to determine whether the oocyst is viable or not, however, as only the sporulated stage of the oocyst has the potential to be viable, it can be concluded that any non-sporulated oocysts visualised will be non-viable. If a sample is identified as positive by qPCR it will also be sent for DNA sequencing, this will further confirm whether it is *T. gondii* DNA.

# 5 Confirm methods do not recover DNA

The work prior to this study (Wells *et al* 2015) identified *T. gondii* DNA from drinking water catchments, however it is important to confirm that the usual methods of filtration at Scottish Water in conjunction with the concentration methodology which will be used for this study (as described in 2.1.5), do not recover *T. gondii* DNA. This part of the project involved spiking known concentrations of *T. gondii* DNA in 1000 litres of water at the Scottish Water Microbiology Laboratory and then testing the resulting post *Cryptosporidium* IMS solution to determine whether *T. gondii* DNA has passed through the system and can be detected by our concentration methodology and subsequent qPCR.

## 5.1 *T. gondii* spiking, concentration and qPCR.

Three different concentrations of *T. gondii* DNA were used, classed as high (6645pg), medium (664.5pg) and low (66.45pg). These concentrations correlate to an equivalent number of oocysts (1 oocyst = approximately 22.15pg) as shown in Table 10.

Table 10. *T. gondii* DNA concentrations for spiking water samples.

| Spike | DNA spike (pg) | Approx. oocyst number* |
|---|---|---|
| High | 6645 | 300 |
| Medium | 664.5 | 30 |
| Low | 66.45 | 3 |

*one oocyst = approximately 22.15pg

Each sample was spiked into the rig at Scottish Water which enables the DNA to be spiked into 1000 litres of water. Spiked samples were then processed in an identical way to all water samples that come into the Scottish Water Microbiology Laboratory for routine *Cryptosporidium* testing. The resulting 12 ml post *Cryptosporidium* IMS solution was then further concentrated at Moredun Research Institute by centrifugation using the methodology described in 2.1.5. DNA was extracted from the pellet (as described in Appendix 9.3), along with extraction controls to monitor for any contamination. DNA was then tested using the *T. gondii* specific 529bp qPCR (Appendix 9.6).

## 5.2 Result of DNA spiking experiment

From the three different concentrations of *T. gondii* DNA (low, medium and high), only DNA from the high concentration was detectable at very low levels following filtration and concentration, however no DNA was detected from the low and medium spike (Table 11). Compared to the original high concentration spike of 6645pg the amount detected by qPCR was only 0.008pg, which is a 830625 fold dilution from the amount of DNA that originally went into the system.

Table 11. Results of DNA the spiking experiment. Low and medium spikes were negative whilst DNA was detected in very low amounts form the high concentration spike.

| | | | Results after filtration, concentration and qPCR | | | |
|---|---|---|---|---|---|---|
| **Spike** | **DNA spike (pg)** | **Approx. oocyst number** | **qPCR CP value** | **Concentration per reaction (pg)** | **Total DNA concentration (pg)** | **Fold dilution from original DNA spike** |
| High | 6645 | 300 | 36.4 | 4.00E-4 | 0.008 | 830625 |
| Medium | 664.5 | 30 | n/a | n/a | 0 | n/a |
| Low | 66.45 | 3 | n/a | n/a | 0 | n/a |

## 5.3 Conclusions from DNA spiking

The high concentration DNA spike (6645pg) was the only sample where *T. gondii* DNA could be detected. As shown in the results, there was a 830625 fold decrease in DNA concentration compared to the original (Table 11). Although the majority of DNA does not pass though the filtration and IMS concentration, very small amounts are still detectable. However, as shown in Table 11, this high DNA concentration equated to DNA from 300 oocysts. It is less likely that DNA from 300 oocysts would be captured at one sample point unless a cat was defecating directly into the water catchment close to the sample point.

In addition, when using this data to analyse some of the unpublished raw data by Beth Wells (Wells *et al* 2015), the qPCR detected only very low DNA concentrations. If the result of this was from DNA alone (other than oocysts) then using the 830625 fold change (Table 11) would mean that the amount of DNA entering the catchment (as much as 54.9µg – Table 12) would be unrealistic. This further confirms that a much more likely explanation is that the DNA detected in the original study came from oocysts, and as shown in Table 12 the numbers of oocysts are far more realistic, ranging from approximately 3 to 1 oocysts. Nine further samples, which had been shown to have the highest concentration of DNA, were also analysed to determine what this result would indicate if the original DNA source had been from oocysts, or, in light of the DNA spiking experiment, estimate how much DNA would have had to be in the original sample to give the result obtained in the qPCR. To do the latter, the fold decrease (830625) shown in Table 11 was used as a multiplication factor.

Table 12. Estimated DNA concentration in original water samples

| Sample ID | Location | Total DNA concentration (pg) as detected by qPCR | Number of oocysts* | Fold increase (x 830625) = pg DNA required in an original sample to give qPCR result | Equivalent number of oocysts |
|---|---|---|---|---|---|
| 1241 | B | 66.20 | 2.99 | 54987375 (54.9µg) | 2,482,500 |
| 1246 | C | 59.40 | 2.68 | 49339125 (49.3µg) | 2,227,500 |
| 1251 | H | 53.60 | 2.42 | 44521500 (44.5µg) | 2,010,000 |
| 1247 | C | 52.47 | 2.37 | 43582894 (43.6µg) | 1,967,625 |
| 1252 | H | 36.80 | 1.66 | 30567000 (30.6µg) | 1,380,000 |
| 1243 | J | 22.83 | 1.03 | 18963169 (19.06µg) | 856,125 |
| 1227 | I | 21.55 | 0.97 | 18398344 (18.46µg) | 830,625 |
| 1249 | H | 21.01 | 0.95 | 17451431 (17.56µg) | 787,874 |
| 1250 | H | 18.12 | 0.82 | 15050925 (15.16µg) | 679,500 |

*one oocyst = approximately 22.15pg

As can clearly be seen from Table 12, it is much more reasonable to assume that the DNA, which was detected in these samples, was from *T. gondii* oocysts rather than DNA that was in suspension within the catchment, as the concentrations of DNA were too high. These results show that in the previous study *T. gondii* oocysts were likely to be present at very low levels.

## 6 Finalised processing methodology for water sample screening

The final methodology to be used in the study for concentrating, visualising and confirming that any oocyst like bodies were *T. gondii* oocyst were as follows:

Concentration – centrifugation, see section 2.1.5

Visualisation - microscopy using auto-fluorescence – see section 2.2.1

Confirmation of *T. gondii* from slides where *T. gondii* like bodies are observed – cell scraping of slide (see section 2.2.3) followed by DNA extraction (see Appendix 9.3), followed by *T. gondii* specific qPCR (see Appendix 9.6).

# 7 Collection and microscopy screening of water samples: 24 September 2017 – 06 February 2018

## 7.1 Introduction

201 water samples from 10 catchments were screened, which is more than was suggested in the initial proposal (it was recommended that raw and finished water (no less than 60 samples) from 4 catchment areas was to be screened). However, as it was not possible to screen very dirty samples form raw and final water, it was agreed within the steering group that a larger number of catchments and samples would be screened. Using the results generated by Wells *et al* 2015, 10 catchments previously identified as having the greatest number of samples containing *T. gondii* DNA present in the water were selected (catchments named A- J).

Following the results from validating the concentration and visualisation methodology, a meeting was held with the steering committee and the following plan was agreed for clean and cloudy samples (Figure 7) (dirty samples could still not be effectively processed, as oocysts could not be visualised from spiked samples). The 12 ml post *Cryptosporidium* IMS samples from clean and cloudy samples would be treated as described in section 2.1.5, the final pellet was to be re-suspended on 12 ml $dH_20$ with the addition of 4% penicillin/streptomycin solution. Initially each sample would be screened from the entire 12 ml sample, however, if oocyst like bodies were being frequently identified by microscopy then confirmed by qPCR that they were *T. gondii*, subsequent samples would be divided into two; 6mls would be used for visualisation and qPCR with the remaining 6mls used for mouse bioassay (Figure 7).

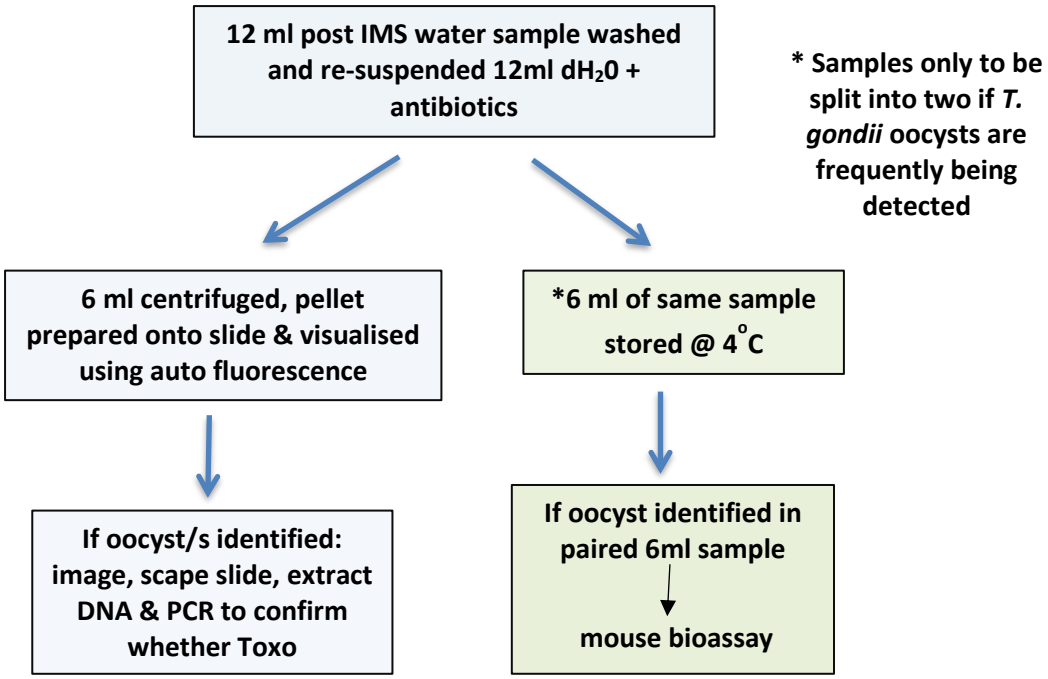



Figure 7. Plan for screening final clean and cloudy water samples from post *Cryptosporidium* IMS water samples.

## 7.2 Catchments and samples to be screened by microscopy

Raw data from the previous study by Wells *et al* (2014) was made available and analysed to identify the top ten catchments where the greatest number of samples had been positive for *T. gondii* DNA, these 10 catchments have been anonymised and are named A – J. Although the original proposal suggested focusing on between 3 – 4 catchments, as agreed by the steering committee, increasing the number of catchments would increase the sensitivity of the study. In hindsight, as only clean and cloudy water samples could be visualised, this increase in catchment number would be beneficial to the overall number of samples available for screening, as if only 3 – 4 catchments were to be used this would have greatly reduced the number of samples available for screening.

Each week from the beginning of October 2017 until the beginning of February 2018, post *Cryptosporidium* IMS samples were collected from Scottish Water (beginning of February collection included to ensure all samples from the end of January were available). All samples were logged (n = 511) and those which were clean enough to be screened (n = 201)

were processed as described in section 6. Using the method described in section 2.1.9 an additional 13 samples that initially appeared too dirty to visualise were able to be screened.

The number of post *Cryptosporidium* samples that were collected and those which were clean enough for microscopy are highlighted in Table 13. The majority of these samples were final water samples (95.0% - Table 14), with only 3.0% of raw water samples screened by microscopy. A full description of the samples screened are detailed in Appendix 9.7.

Table 13. Catchments and number of samples obtained and screened by microscopy from each area.

| Catchment | Number obtained | Number screened | % total screened |
|---|---|---|---|
| A | 53 | 26 | 49.1 |
| B | 64 | 5 | 7.8 |
| C | 23 | 22 | 91.3 |
| D | 128 | 94 | 72.7 |
| E | 33 | 4 | 12.1 |
| F | 36 | 16 | 44.4 |
| G | 37 | 10 | 27.0 |
| H | 64 | 16 | 18.8 |
| I | 64 | 5 | 6.3 |
| J | 9 | 3 | 42.9 |
| **TOTAL** | **511** | **201** | **39.3** |

Table 14. Number of raw and final water samples screened by microscopy.

| Water type | Number screened | % screened |
|---|---|---|
| Final water | 191 | 95.0 |
| Raw water | 6 | 3.0 |
| Unknown | 4 | 2.0 |
| **Total** | **201** | **39.3** |

## 7.3 Screening results

Of the 201 samples that were screened by microscopy *T. gondii* like bodies (TLB's) were identified in 27 samples, with TLB's visualised in 8 catchments (Table 15 and Figure 8). An example of TLB's identified can be seen in Figure 9. However, following slide scraping, DNA extraction and *T. gondii* specific qPCR only 1 sample was found to be qPCR positive from the H catchment (Table 15 and Table 16). The PCR product from this one positive sample was sent for sequencing which further confirmed that it was *T. gondii* that had been amplified. The positive result had a high Cq value (36.8) and the final concentration calculated was very low (1.000 pg), equating to less than one oocyst (Table 16). The oocyst was identified from a final water sample that was processed for routine *Cryptosporidium* testing at Scottish Water on 28th January 2018 (Table 16). An image of the oocyst as visualised by microscopy can be seen in Figure 10. The oocyst has the appearance of a sporulated oocyst, however it does not look like a typical fresh sporulated oocyst (as can be seen in Figure 5 B & D). It is likely the oocyst has been in the environment for some time and has therefore been exposed to harsh environmental conditions. It is therefore not surprising that its morphology differs to an oocyst stored in optimum conditions in the laboratory. As throughout the study, visualised *T. gondii* like bodies were not confirmed by qPCR to be Toxoplasma oocysts, the plan to complete mouse bioassays to determine whether any oocysts were viable was not completed. Therefore it is not possible to conclude whether this oocyst was viable.

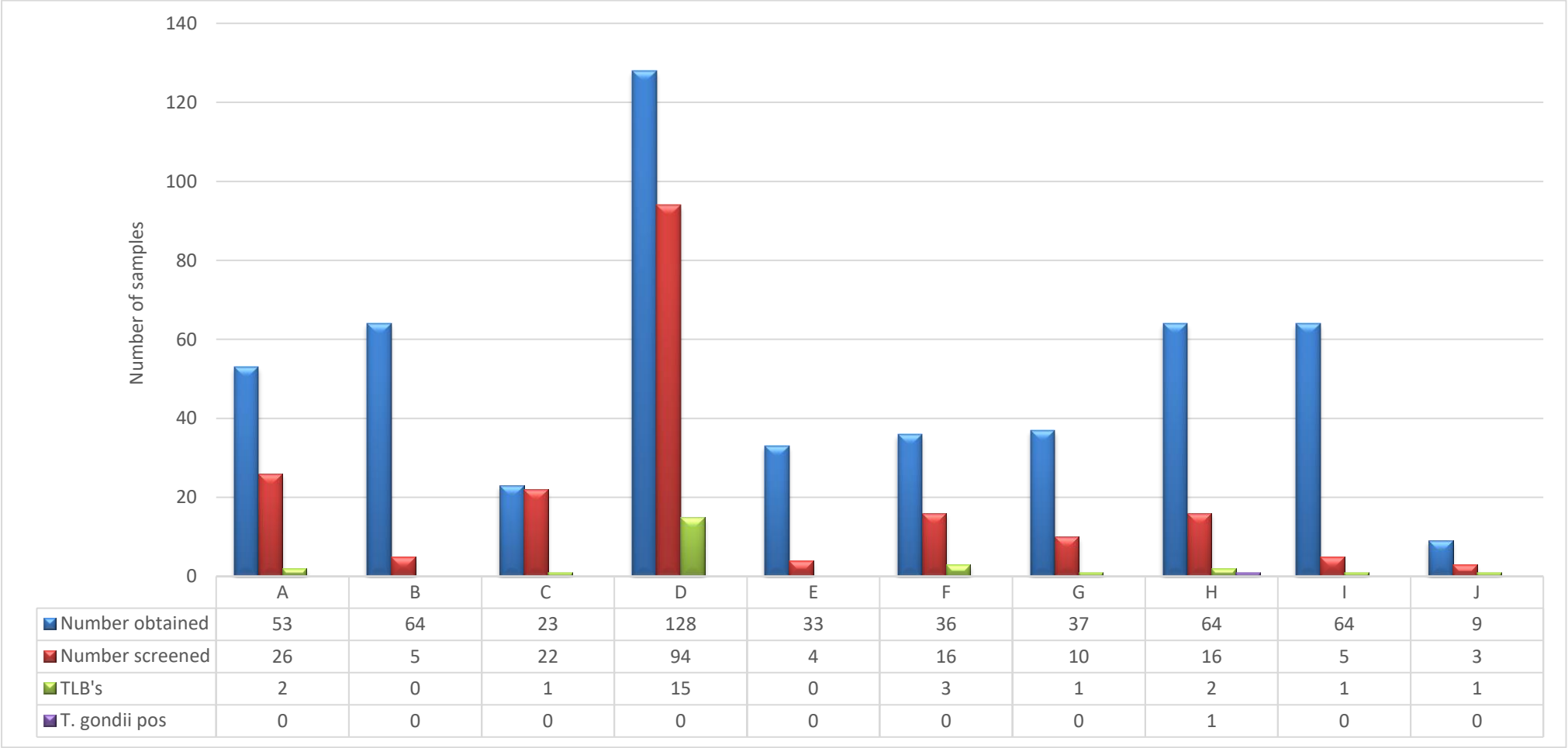
Table 15. Identification of *T. gondii* like bodies from catchments and *T. gondii* specific qPCR for confirmation.

| Catchment | Number of slides screened | Number of slides with *T. gondii* like bodies visualised | *% T. gondii* like bodies | Number of slides positive for *T. gondii* by qPCR | *% T. gondii* positive |
|---|---|---|---|---|---|
| A | 26 | 2 | 7.7 | 0 | 0 |
| B | 5 | 0 | 0 | 0 | 0 |
| C | 22 | 1 | 4.5 | 0 | 0 |
| D | 94 | 16 | 17.0 | 0 | 0 |
| E | 4 | 0 | 0 | 0 | 0 |
| F | 16 | 3 | 18.6 | 0 | 0 |
| G | 10 | 1 | 10.0 | 0 | 0 |
| H | 16 | 2 | 12.5 | 1 | 8.3 |
| I | 5 | 1 | 20.0 | 0 | 0 |
| J | 3 | 1 | 33.3 | 0 | 0 |
| **TOTAL** | 201 | 27 | 13.4 | 1 | 0 |

Table 16. Details of visualised and confirmed *T. gondii* positive sample

| Catchment | Collection date | Colour | Final / Raw water | qPCR Cp value | Concentration per reaction (pg) | Total DNA concentration (pg) | Equivalent number of oocysts* |
|---|---|---|---|---|---|---|---|
| H<br>ID No. = 469 | 28 Jan 2018 | Slightly cloudy | Final | 36.8 | 5.02E-2 | 1.000 | 0.045 |

*one oocyst = approximately 22.15pg




| | A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|---|
| Number obtained | 53 | 64 | 23 | 128 | 33 | 36 | 37 | 64 | 64 | 9 |
| Number screened | 26 | 5 | 22 | 94 | 4 | 16 | 10 | 16 | 5 | 3 |
| TLB's | 2 | 0 | 1 | 15 | 0 | 3 | 1 | 2 | 1 | 1 |
| T. gondii pos | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |

Figure 8. Catchments, samples obtained and samples screened in relation to *T. gondii* like bodies (TLB's) identified.

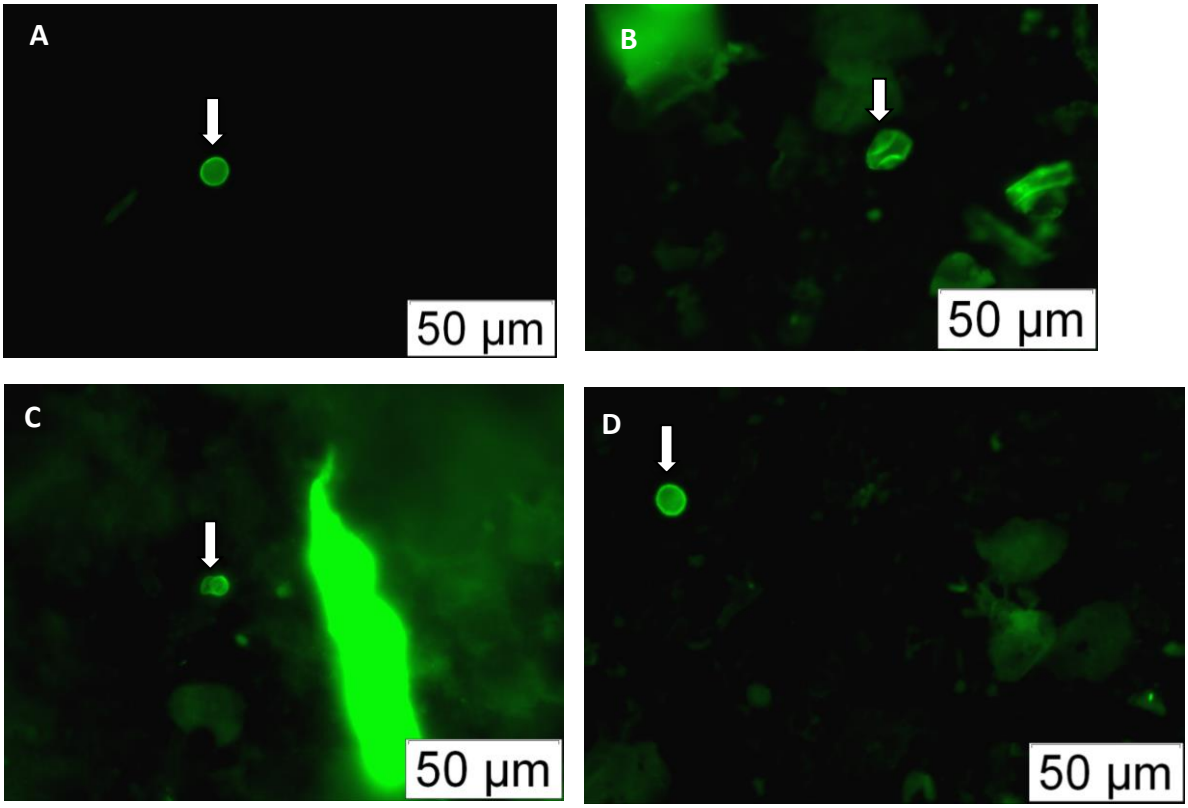



Figure 9. Example of *T. gondii* like bodies (TLB's) identified from different catchments. A = possibly non-sporulated – catchment F from 21 November 2017, B = possibly old non-sporulated – catchment D from 03 November 2017, C = possibly sporulated – catchment D from 12 October 2017, D = possibly non-sporulated – catchment F from 24 October 2017. After slide scraping and qPCR none of these TLB's were *T. gondii* positive.

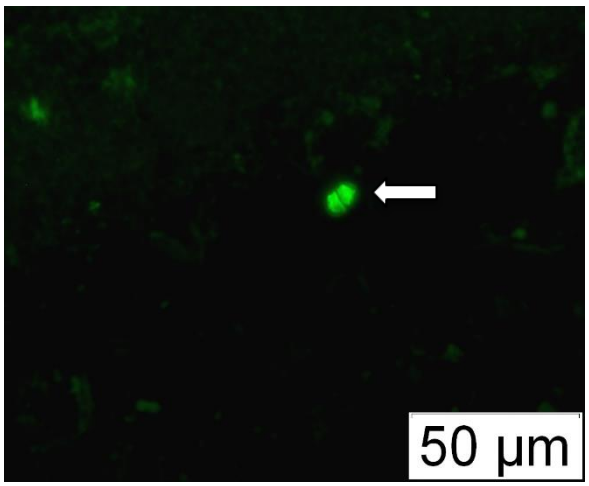



Figure 10. TLB identified and confirmed as *T. gondii* by qPCR. Oocyst appears to be sporulated but also appears ragged/damaged.

In relation to the number of TLB's observed across the 4 month time frame, the majority of TLB's were identified at the start of the screening period, with most TLB's identified in October as shown in Table 17.

| Month | Total number of slides screened | TLB's visualised | % total TLB's | 95% CI | |
|---|---|---|---|---|---|
| | | | | Lower | Upper |
| October | 52 | 11 | 21.2 | 11.1 | 34.7 |
| November | 64 | 8 | 12.5 | 55.5 | 23.2 |
| December | 41 | 5 | 12.2 | 4.1 | 26.2 |
| January | 44 | 3 | 6.8 | 1.4 | 18.7 |

Table 17. Number of TLB's identified in relation to month

## 7.4 Additional objective - analysis on catchment H samples - DNA extraction and *T. gondii* specific qPCR

Following the results in section 7.3, additional analysis to screen raw water samples from catchment H was undertaken. As raw water samples had a high turbidity it was not possible to visualise these samples, therefore, any analysis would need to focus on the detection of *T. gondii* DNA.

DNA was extracted from 41 raw water samples and the *T. gondii* specific qPCR was completed using the methodology described in Appendix 9.3 and Appendix 9.6. Of the 41 samples, 10 could not be assessed due to the presence of PCR inhibitors and were removed from analysis, whilst 4 were positive for *T. gondii* DNA (Table 18). Therefore, 12.9% (4/31) of samples tested by qPCR from catchment H were positive (the full data set of these results can be seen in Appendix 9.8). The matched raw water sample collected on the same day (28 Jan 2018) as the final water sample in which a *T. gondii* oocyst was observed was negative by qPCR (as shown in Appendix 9.8)

Table 18. *T. gondii* qPCR positive raw water samples from the H catchment.

| ID No. | Catchment | Collection date | Colour | Final / Raw water | qPCR Cq value | Total DNA concentration (pg) | Equivalent number of oocysts* |
|---|---|---|---|---|---|---|---|
| 157 | H | 12 Nov 2017 | Dirty | Raw | 35.9 | 22.0 | 0.99 |
| 199 | H | 19 Nov 2017 | Dirty | Raw | 36.4 | 13.9 | 0.63 |
| 238 | H | 24 Nov 2017 | Very dirty | Raw | 37.2 | 0.43 | 0.02 |
| 361 | H | 24 Dec 2017 | Dirty | Raw | 35.9 | 28.4 | 1.28 |

*one oocyst = approximately 22.15pg

## 8 Discussion and conclusions

What is clear from the initial work carried out in the methodology, it that the 529bp qPCR is a reliable sensitive method for screening water samples, either alone or in combination with microscopy. 27 slides containing *T. gondii* like bodies were identified by microscopy from the 201 samples which were able to be screened, only one was confirmed by qPCR to be a *T. gondii* oocyst. The oocyst appeared to be sporulated and had been collected from a final water sample supplied by catchment H on 28th January 2018. The overall morphology of the oocyst was consistent to those exposed to harsh environmental conditions. As mouse bioassay was not possible for the reasons explained in section 7.3, it was not possible to verify whether this oocyst was viable. However given that a *T. gondii* oocyst must first sporulate to become infective, it is likely that, at one point, this oocyst was viable.

When examining raw water for catchment H by qPCR detection alone, *T. gondii* DNA was detected in 9.8% of samples (n = 4) (Table 18) (these samples were too dirty to screen for oocysts by microscopy). Detection of parasite DNA in raw water samples was not carried out for the other nine catchments, as this would have simply been repeating the work carried out by Wells *et al* (2015), and the main objective of this study was to determine the effectiveness of water treatment in removing oocysts and whether they could be visualised in post *Cryptosporidium* IMS samples. However, as one oocyst had been positively identified from this catchment, it was thought beneficial to the study to screen raw water samples for *T. gondii* DNA.

Interestingly, *T. gondii* DNA was not detected from the matched raw water sample from catchment H on 28th January 2018. This is not surprising as maximum oocysts numbers are

likely to be as low as 3 oocysts, as shown from the analysis of the results from the original study (Table 12). From the DNA analysis of these additional raw water samples 12.9% were identified, again the approximate number of oocysts calculated from the sample DNA concentration was low and ranged from 1.28 to 0.02 oocysts (Table 18), however just one oocyst has been shown to cause infection in animal models (Dubey *et al* 1996). The remaining 26 slides, in which structures similar in size and morphology to *T. gondii* oocysts were observed (*T. gondii* like bodies), were not positively identified as the parasite. These structures appeared to be more frequently found early on in the study.

The cause of these bodies was not clear, however after discussions with the steering group it was thought that it may be other oocysts with a similar shape and size to *T. gondii* such as *Isospora* or *Eimeria*. Another explanation could be that the structures are algae or yeast which were perhaps able to bud off from a longer chain, an example of this is shown in Figure 11. However, it should be noted that these *T. gondii* like bodies were found in final water and had therefore managed to get through the barriers already in place.

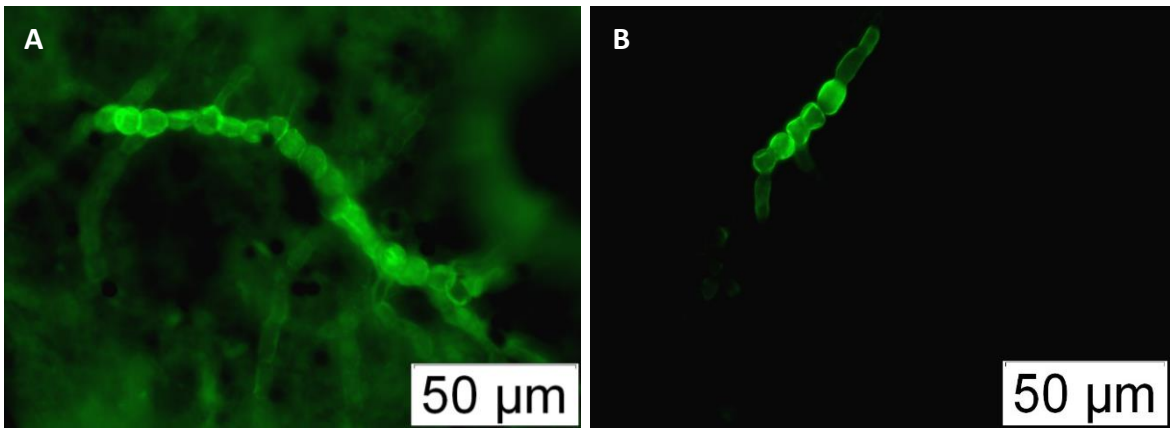



Figure 11. Long chains of possibly yeast or algae observed by microscopy. These could have the potential to bud off and appear similar to in size and shape to *T. gondii* oocysts. A = visualised slide, ID no. 128, catchment D, 15 Oct 2017; B = visualised slide, ID no. 217, catchment D, 21 Nov 2017.

As the majority of final water samples which could be screened by microscopy were either clean or cloudy, there is possibly an underestimation to the number of oocysts which may be present. Although attempts were made to clean and concentrate oocysts from dirty/turbid water samples, the methodology still requires improvement. Ideally, a method similar to that used for concentrating *Cryptosporidium* oocysts, using immunomagnetic

separation (IMS) along with a specific monoclonal antibody should be developed, which would allow oocysts to be isolated from dirty raw and final water samples.

Another factor which must be considered, is how high rainfall events may affect results. The screening period of October to January was chosen as in the previous study (Wells *et al* 2015), high rainfall events observed in October and November 2013 were associated with a greater number of DNA positive results from water samples collected within the same timeframe. However, in 2017, according to MET office statistics the main high rainfall event occurred earlier in the year in June to August, with this period recorded as Scotland's 5th wettest summer on record (https://www.metoffice.gov.uk/news/releases/2017/a-wet-summer-comes-to-a-close). Therefore, selecting October to January for the current study may not have been the best period to complete the screening. At wetter times of the year there is more surface run off, with this there is a greater chance of any environmental oocysts being washed into rivers, lochs and reservoirs and in turn an increased chance of oocysts entering water catchments. However, it is impossible to precisely predict high rainfall events. The caveat in selecting months which have high rainfall events and increased surface run-off would be that these samples would have an increased turbidity and would therefore be difficult to screen by microscopy. To visualise these samples by microscopy a successful methodology would still need to be established.

In summary the results from this study show after visualising 201 samples for *T. gondii* oocysts between the periods of 26th September 2017 to 6th February 2018 across 10 different Scottish water catchments only one *T. gondii* oocyst from one catchment was identified. Therefore, it can be concluded that although the water treatment process appears to be effective at removing oocysts across the remaining nine catchments there may also be an underrepresentation, as the most turbid/dirty final samples could not be screened for oocysts and it is the dirty samples that are likely to have oocysts present. In addition, from the previous study, high rainfall looks to play an important part for surface run off of oocysts, in hindsight this study was not completed when rainfall for 2017 was at its greatest.

Recommendations which would enhance any future studies would be to develop a suitable IMS technique using a *T. gondii* specific monoclonal antibody, similar to what is already in place for the *Cryptosporidium* IMS methodology, this would ensure that *T. gondii* oocysts

could be isolated from dirty/turbid water samples. Examine samples from different catchments over a full year of sampling would resolve the issue of trying to predict high rainfall events. Develop a sensitive oocyst viability assay, ideally one which moves away from using animal models. Finally it is not known how *T. gondii* oocysts spread within the environment and understanding this would help to predict how they behave and how to prevent them entering the water supply.

Dubey, J.P., Andrews, C.D., Lind, P., Kwok, O.C., Thulliez, P., Lunney, J.K., 1996. Antibody responses measured by various serologic tests in pigs orally inoculated with low numbers of *Toxoplasma gondii* oocysts. Am J Vet Res 57, 1733-1737.

# 9 Investigation carried out by Scottish Water on Catchment H following the results

The water treatment works for Catchment H is a membrane filtration plant, theoretically capable of removing particles and suspended solids down to 1µm in diameter. Raw water enters this works and the first stage is to pass through the membrane filters. Filtered water is then passed over a Limestone Contactor to re-mineralise the water, prior to disinfection with Sodium Hypochlorite. Final water is stored in a service reservoir at the works, from where the *Cryptosporidium* sample is collected. In the current study, it was the post *Cryptosporidium* IMS sample that was then used for microscopic examination of *T. gondii* oocysts.

Following isolation and identification of the sporulated *T. gondii* oocyst from Catchment H, Scottish Water undertook a site specific investigation into environmental conditions, water quality and water treatment works performance at the time of and prior to the period this sample was collected. In the days prior to this sample being collected, there was some rainfall and snow melt, which had slightly increased the Dissolved Organic Carbon (DOC) levels in the raw water source for this treatment works, however, on the day of sampling DOC had returned to typical levels. Membrane pressure and filter turbidity measurements taken at the water treatment works showed raw water quality had not deteriorated significantly to cause a breach of the filters.

Bacteriological and *Cryptosporidium* samples are collected from this site once per week for raw and final water and the sample containing the *T. gondii* oocyst was one of the weekly *Cryptosporidium* samples. There was no evidence of increased biological loading at the time of the failure and historical data does not show a seasonal pattern or progressive long term increase of biological (bacterial or cryptosporidial) loading. Bacterial flow cytometry testing is conducted on all microbiology samples. Bacterial flow cytometry sample results show a significant total cell count reduction through the treatment process, especially across the membrane filters. Additionally *Cryptosporidium* oocysts have not been detected in the final water from Catchment H, confirming this membrane plant is working very effectively. Scottish Water was not able to determine the source of this *T. gondii* oocyst or its route through the water treatment process.

# 10 Appendix

## 10.1 Details of oocysts used for spiking experiments and for mouse bioassay

*SALUVET INNOVA, S.L.*
*AVDA. PUERTA DE HIERRO, S/N*
*28040 MADRID, SPAIN*
*CIF B-87296497*

Customer: **Alison Burrells, MRI, Scotland**
Concept: *Toxoplasma gondii* oocysts supply
Number: 01/2017 Date: June 14, 2017

**Description**

*Toxoplasma gondii* oocysts of SP-1 isolate obtained from cats infected in October 2016.

**Budget**

| Ref. number | Concept | Cost (€) |
|---|---|---|
| OQ TOXO | *Toxoplasma gondii* oocysts (100.000 oocysts, isolate SP-1, 2016 batch) | 800 |
| | TOTAL | 800 |

**Observations**

Shipping costs not included.
This information is confidential.

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## 10.2 Mouse bioassay scoring system

**Animal Scoring System:**

| Category | Description | Score |
|---|---|---|
| **A** | Sleek/glossy coat | 0 |
| Coat condition | Ruffled coat | 1 |
| (max score 3) | Stary coat | 2 |
| | Stiff stary coat | 3 |
| **B** | Bright and active | 0 |
| Demeanour | Hunched | 1 |
| Scoring cumulative | A reluctance to move | 1 |
| (max score 3) | Tottering gait | 1 |

Animals will be euthanised using a Schedule 1 procedure if an animal reaches a maximum score in either category (A or B) or if an animal has a total score of 4 for two consecutive days.

## 10.3 Method for extraction of DNA from *T. gondii* oocysts using the Macherey-Nagel nucleospin tissue, isolation of genomic DNA kit

After the addition of buffer T1 used for slide scraping or 200µl for pelleted oocysts, the remaining reagents of the Macherey-Nagel kit (product number: 740952.250) were used as follows:

- 10 freeze thaw cycles in liquid nitrogen (vortex to mix after 5 freeze thaws)
- Add 25 µl proteinase K and vortex to mix
- Incubate for at least 1 – 3 hours or overnight at 56$^{o}$C (prefer to leave very dirty samples overnight).
- Vortex the samples and incubate at 95$^{o}$C for 10 minutes.
- Vortex the samples
- Add 200 µl Buffer B3, vortex vigorously and incubate at 70$^{o}$C for 10 minutes. Vortex briefly to mix the samples.
- Centrifuge at 11,000 x g for 5 minutes to remove insoluble particles and transfer the supernatant to a new Eppendorf.
- Add 210 µl EtOH (100%) and vortex vigorously.
- Place spin column in a collection tube and add the whole sample.
- Centrifuge for 1 minute at 11,000 x g. Discard the flow through and replace column into collection tube (repeat centrifugation if some of the samples is retained in the column).
- Add 500 µl Buffer BW and centrifuge at 1 minute. Discard flow through and replace column into collection tube.
- Add 600 µl Buffer B5 and centrifuge at 11,000 x g for 1 minute. Discard flow through and replace column into collection tube.
- Centrifuge column at 11,000 x g for 1 minute to dry membrane.
- Place column into a 1.5 ml Eppendorf and add 100 µl ultra-pure water. Centrifuge at 11,000 x g for 1 minute to elute DNA. Store samples at -20$^{o}$C for long term storage or 4$^{o}$C for immediate use.

Proceed to the Toxo 529bp qPCR for detection of *T. gondii* parasite DNA, alternatively store DNA at -20$^{o}$C.

N.B an extraction control should also be included and processed alongside each batch of test samples.

## 10.4 DNA extraction from mouse tissues following bioassay

DNA was extracted from approximately 1 g of homogenised tissue. Briefly, tissue was placed into a CK28 precellys tube (tubes prefilled with ceramic beads with a diameter of 2.8 mm - Peqlab, Sarisbury Green, Hampshire, UK), containing 1000µl Nuclei Lysis Solution (Promega, UK), and the tissue homogenised using a Precellys® 24 homogeniser (Peqlab) using two cycles of 50 seconds at 380 x g (6500rpm). 400 µl of homogenate was further processes with 900 µl Nuclei Lysis Solution (Promega), and incubated overnight in a water bath at 55°C (the remaining unused tissue homogenate was stored at -20°C for future use). Once removed the lysate was allowed to cool to room temperature, 300µl Protein Precipitation Solution (Promega) was added and mixed for 20 seconds using a vortex, then incubated on ice for 5 minutes. Following incubation the mixture was centrifuged at 13,000 x g for 5 minutes and the resulting supernatant transferred to a 2ml Eppendorf tube, containing 900 µl isopropanol, mixed by inversion and incubated at -20°C overnight. The DNA was pelleted by centrifugation at 13,000 x g for 5 minutes, supernatant removed and DNA pellet washed with 600 µl 70% ethanol. Centrifugation was repeated for 2 minutes at 13,000 x g, supernatant removed and the DNA pellet allowed to briefly air dry. The final pellet was re-suspended in 200 µl distilled $H_2O$. The DNA was stored at 4°C for immediate use or at -20°C for longer term storage.

## 10.5 ITS1 PCR method for detection of *T. gondii* DNA

Standard 20 µl PCR reactions contained 2 µl 10x custom PCR mix – SM0005 (45 mM Tris-HCl. 11 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 0.113 mg/ml BSA, 4.4 µM EDTA and 1.0 mM dATP, dATC, dGTP, dTTP – ABgene, Epsom, Surrey, UK), 0.75 units BioTaq (Bioline, London, UK), and 2 µl DNA. All reactions were made to a final volume of 20 µl with sterile $dH_2O$. First round reactions used 5 µM of primers NN1-ext-F and NN1-ext-R see Table 19.

Cycling conditions were 5 minutes at 95°C, followed by 35 cycles of 1 minute at 95°C, 1 minute at 55°C, 1 minute at 72°C, and a final extension period of 5 minutes at 72°C. To reduce unused primers from the primary PCR, first round PCR products were diluted 1:5 with $dH_2O$. The conditions for each second round reaction were identical to the first, except 2 µl of diluted first round product was used instead of DNA, and 5 µM of primers Toxo_NP-1_F and Toxo_NP-2_R (see Table 19) were used.

All PCR products were visualised following electrophoresis in 2% agarose gels incorporating Biotum Gel Red™ (Cambridge Bioscience Ltd, Cambridge, UK).

Table 19. Primers used for the ITS1 PCR.

| Primers 5' - 3' (external forward, external reverse internal forward, internal reverse') | | Nested PCR Product Size (bp) |
|---|---|---|
| NN1_ext_F | tca acc ttt gaa tcc caa | 227 |
| NN2_ext_R | cga gcc aag aca tcc att | |
| Toxo_NP-1_F | gtg ata gta tcg aaa ggt at | |
| Toxo_NP-1_F | act ctc tct caa atg ttc ct | |


| Primers 5' - 3' (external forward, external reverse internal forward, internal reverse') | | Nested PCR Product Size (bp) |
|---|---|---|
| NN1_ext_F | tca acc ttt gaa tcc caa | 227 |
| NN2_ext_R | cga gcc aag aca tcc att | |
| Toxo_NP-1_F | gtg ata gta tcg aaa ggt at | |
| Toxo_NP-1_F | act ctc tct caa atg ttc ct | |

## 10.6 529bp qPCR method for detecting *T. gondii* DNA

As described in Wells *et al* 2015.

Initial PCR reactions were performed in single wells and the amplification mixture consisted of: 1.2 µl LightCycler Probes Master (2X) (Roche, 4707494001); 8 µg/µl BSA; 0.7 µM Tox-9F and 0.7 µM Tox-11R primers, 0.1 µM Tox-TP1 probe and 0.2 µM CIAC probe; 7.5 ag/µl CIAC and 5 µl DNA template to give a final reaction volume of 20 µl. The PCR amplification was performed in a LightCycler 480 (Roche) thermal cycler with an initial incubation at 95 °C for 10 minutes, followed by 45 cycles at: 95 °C 10 s, 58 °C 20 s and 72 °C 20 s, with a final cooling step at 40 °C 5 s. Fluorescence was measured in the 465–510 nm channel (Tox probe) and 533–580 nm (CIAC probe) after each amplification cycle. Samples recorded as positive had Ct values of 35 cycles or less and showed a smooth exponential curve when compared to the standards curves. Samples which were positive for *T. gondii* were repeated in duplicate and the mean qPCR result recorded.

Standards

*T. gondii* tachyzoite genomic DNA standards (a ten-times dilution series ranging from 400 pg/µl to 400 fg/µl, and 200, 100 and 50 fg/µl) were included on each run to enable calculation of a standard curve for determination of DNA concentration in samples. All samples showing a smooth exponential amplification curve when compared to the standards were scored as positive and all samples without amplification of *T. gondii* DNA, but which were CIAC positive, were scored negative.

PCR controls

PCR standards were used as *T gondii* positive controls and *T. gondii* negative controls were included in each row of the 96 well plate using water instead of DNA template.

Wells, B., Shaw, H., Innocent, G., Guido, S., Hotchkiss, E., Parigi, M., Opsteegh, M., Green, J., Gillespie, S., Innes, E.A., Katzer, F., 2015. Molecular detection of *Toxoplasma* gondii in water samples from Scotland and a comparison between the 529bp real-time PCR and ITS1 nested PCR. Water research 87, 175-181.

## 10.7 All samples screened by microscopy

| ID | Catchment | Lims No | Filter Type | Date | State | Colour |
|---|---|---|---|---|---|---|
| 120 | D | 1 | g | 24 September 2017 | FNC | Slightly cloudy |
| 119 | D | 1 | g | 25 September 2017 | FNC | clear |
| 13 | D | 4 | g | 01 October 2017 | FNC | Slightly cloudy |
| 22 | H | 3 | g | 01 October 2017 | FNC | Clear |
| 24 | D | 1 | g | 01 October 2017 | FNC | Slightly cloudy |
| 117 | D | 2 | g | 01 October 2017 | FNC | clear |
| 113 | D | 7 | g | 02 October 2017 | FNC | Clear |
| 53 | C | 35 | g | 04 October 2017 | FNC | Cloudy |
| 112 | D | 2 | g | 04 October 2017 | FNC | Clear |
| 4 | D | 3 | g | 05 October 2017 | FNC | Clear |
| 10 | D | 23 | g | 06 October 2017 | FNC | Clear |
| 47 | I | 20 | g | 06 October 2017 | FNC | Dirty |
| 8 | D | 1 | g | 08 October 2017 | FNC | Clear |
| 12 | D | 5 | g | 08 October 2017 | FNC | Clear |
| 21 | H | 7 | g | 08 October 2017 | FNC | Cloudy |
| 1 | D | 4 | g | 09 October 2017 | FNC | Clear |
| 3 | D | 6 | g | 10 October 2017 | FNC | Clear |
| 33 | A | 31 | g | 10 October 2017 | FNC | Slightly cloudy |
| 54 | C | 39 | g | 10 October 2017 | FNC | Very cloudy |
| 2 | D | 3 | g | 11 October 2017 | FNC | Clear |
| 34 | A | 44 | g | 11 October 2017 | FNC | Slightly cloudy |
| 116 | D | 1 | g | 12 October 2017 | FNC | clear |
| 9 | D | 8 | g | 15 October 2017 | FNC | Clear |
| 15 | H | 6 | g | 15 October 2017 | FNC | Clear |
| 11 | D | 2 | g | 15 October 2017 | FNC | Clear |
| 128 | D | 3 | g | 15 October 2017 | FNC | Slightly cloudy |
| 78 | F | 26 | g | 17 October 2017 | FNC | Clear |
| 81 | E | 2 | g | 17 October 2017 | FNC | Slightly cloudy |
| 125 | D | 4 | g | 17 October 2017 | FNC | Slightly cloudy |
| 83 | G | 6 | g | 17 October 2017 | FNC | Slightly cloudy |
| 76 | A | 19 | g | 18 October 2017 | FNC | Slightly cloudy |
| 84 | C | 14 | g | 18 October 2017 | FNC | Slightly cloudy |
| 70 | D | 34 | g | 18 October 2017 | FNC | Clear |
| 126 | D | 2 | g | 19 October 2017 | FNC | clear |
| 115 | D | 9 | g | 20 October 2017 | FNC | Slightly cloudy |
| 71 | D | 18 | g | 20 October 2017 | FNC | Clear |
| 122 | D | 3 | g | 23 October 2017 | FNC | Slightly cloudy |
| 85 | B | 19 | g | 24 October 2017 | FNC | cloudy |
| 106 | E | ? | g | 24 October 2017 | g | cloudy |
| 111 | D | 8 | g | 24 October 2017 | FNC | Clear |
| 105 | F | 40 | g | 24 October 2017 | FNC | Clear |
| 86 | B | 13 | g | 25 October 2017 | FNC | cloudy |
| 110 | C | 19 | g | 26 October 2017 | g | Slightly cloudy |

| ID | Catchment | Lims No | Filter Type | Date | State | Colour |
|---|---|---|---|---|---|---|
| 124 | D | 1 | g | 26 October 2017 | FNC | Slightly cloudy |
| 103 | D | 18 | g | 27 October 2017 | FNC | Slightly cloudy |
| 102 | D | 4 | g | 29 October 2017 | FNC | Slightly cloudy |
| 92 | H | 8 | g | 29 October 2017 | FNC | Slightly cloudy |
| 121 | D | 2 | g | 29 October 2017 | FNC | clear |
| 114 | D | 3 | g | 30 October 2017 | FNC | Clear |
| 144 | A | 24 | g | 31 October 2017 | FNC | Slightly cloudy |
| 123 | D | 7 | g | 31 October 2017 | FNC | clear |
| 145 | F | 42 | g | 31 October 2017 | FNC | Clear |
| 142 | A | 36 | g | 01 November 2017 | FNC | Slightly cloudy |
| 182 | D | 4 | g | 01 November 2017 | FNC | Slightly cloudy |
| 153 | C | 27 | g | 02 November 2017 | FNC | Slightly cloudy |
| 185 | D | 4 | g | 02 November 2017 | FNC | Slightly cloudy |
| 127 | D | 21 | g | 03 November 2017 | FNC | clear |
| 118 | D | 7 | g | 05 November 2017 | FNC | clear |
| 137 | H | 2 | g | 05 November 2017 | FNC | Dirty |
| 187 | D | 1 | g | 05 November 2017 | FNC | Clear |
| 180 | D | 3 | g | 06 November 2017 | FNC | Clear |
| 162 | F | 21 | g | 07 November 2017 | FNC | Clear |
| 164 | A | 33 | g | 07 November 2017 | FNC | Slightly cloudy |
| 175 | C | 43 | g | 07 November 2017 | FNC | Slightly cloudy |
| 181 | D | 3 | g | 07 November 2017 | FNC | clear |
| 161 | F | 35 | g | 08 November 2017 | FNC | Clear |
| 166 | A | 43 | g | 08 November 2017 | FNC | Clear |
| 173 | B | 29 | g | 08 November 2017 | FNC | cloudy |
| 174 | C | 41 | g | 08 November 2017 | FNC | Slightly cloudy |
| 178 | D | 4 | g | 08 November 2017 | FNC | Clear |
| 179 | D | 5 | g | 09 November 2017 | FNC | Clear |
| 170 | D | 21 | g | 10 November 2017 | FNC | Clear |
| 154 | H | 5 | g | 12 November 2017 | FNC | Clear |
| 184 | D | 4 | g | 12 November 2017 | FNC | Slightly cloudy |
| 171 | D | 6 | g | 12 November 2017 | FNC | Clear |
| 169 | E | 2 | g | 13 November 2017 | FNC | cloudy |
| 183 | D | 3 | g | 13 November 2017 | FNC | Slightly cloudy |
| 189 | D | 4 | g | 14 November 2017 | FNC | Slightly cloudy |
| 203 | A | 42 | g | 14 November 2017 | FNC | cloudy |
| 206 | C | 48 | g | 14 November 2017 | FNC | Clear |
| 208 | C | 45 | g | 14 November 2017 | FNC | cloudy |
| 209 | F | 38 | g | 14 November 2017 | FNC | Clear |
| 211 | G | 6 | g | 14 November 2017 | FNC | Slightly cloudy |
| 188 | D | 4 | g | 15 November 2017 | FNC | Slightly cloudy |
| 204 | A | 28 | g | 15 November 2017 | FNC | cloudy |
| 207 | C | 39 | g | 15 November 2017 | FNC | cloudy |
| 195 | I | 10 | c | 15 November 2017 | RWC | Slightly cloudy |
| 219 | D | 4 | g | 16 November 2017 | FNC | Slightly cloudy |

| ID | Catchment | Lims No | Filter Type | Date | State | Colour |
|---|---|---|---|---|---|---|
| 202 | H | 24 | g | 19 November 2017 | FNC | Clear |
| 216 | D | 2 | g | 19 November 2017 | RWC | Slightly cloudy |
| 214 | D | 1 | g | 20 November 2017 | FNC | Clear |
| 343 | D | 7 | g | 20 November 2017 | FNC | Slightly cloudy |
| 235 | A | 46 | g | 21 November 2017 | FNC | cloudy |
| 242 | J | 36 | g | 21 November 2017 | FNC | cloudy |
| 245 | G | 8 | g | 21 November 2017 | FNC | cloudy |
| 334 | D | 6 | g | 21 November 2017 | FNC | Slightly cloudy |
| 241 | J | 12 | c | 21 November 2017 | RWC | cloudy |
| 244 | F | 50 | g | 21 November 2017 | FNC | Clear |
| 217 | D | 5 | g | 21 November 2017 | FNC | Slightly cloudy |
| 234 | A | 26 | c | 22 November 2017 | FNC | cloudy |
| 240 | C | 28 | g | 22 November 2017 | FNC | Slightly cloudy |
| 342 | D | 6 | g | 22 November 2017 | FNC | Slightly cloudy |
| 239 | C | 21 | g | 23 November 2017 | FNC | Slightly cloudy |
| 346 | D | 2 | g | 23 November 2017 | FNC | Clear |
| 336 | D | 2 | g | 27 November 2017 | FNC | Slightly cloudy |
| 258 | A | 46 | g | 28 November 2017 | FNC | Slightly cloudy |
| 262 | I | 18 | c | 28 November 2017 | RWC | cloudy |
| 266 | F | ? | g | 28 November 2017 | FNC | Clear |
| 339 | D | 3 | g | 28 November 2017 | FNC | cloudy |
| 246a | B | 24 | g | 28 November 2017 | FNC | cloudy |
| 248 | B | 18 | g | 29 November 2017 | FNC | cloudy |
| 257 | A | 23 | g | 29 November 2017 | FNC | Slightly cloudy |
| 261 | I | 3 | c | 29 November 2017 | RWC | cloudy |
| 333 | D | 5 | g | 29 November 2017 | FNC | Slightly cloudy |
| 256 | G | 4 | g | 30 November 2017 | FNC | Slightly cloudy |
| 273 | D | 5 | g | 30 November 2017 | FNC | Slightly cloudy |
| 255 | G | 11 | g | 01 December 2017 | ? | Slightly cloudy |
| 263 | D | 22 | g | 01 December 2017 | FNC | Clear |
| 253 | H | 8 | g | 03 December 2017 | FNC | Clear |
| 270 | D | 5 | g | 03 December 2017 | FNC | Slightly cloudy |
| 269 | D | 1 | g | 04 December 2017 | FNC | Slightly cloudy |
| 267 | D | 18 | g | 05 December 2017 | FNC | Slightly cloudy |
| 294 | G | 9 | g | 05 December 2017 | FNC | Slightly cloudy |
| 295 | F | 39 | g | 05 December 2017 | FNC | Clear |
| 298 | J | 29 | g | 05 December 2017 | FNC | cloudy |
| 268 | D | 12 | g | 06 December 2017 | FNC | Clear |
| 299 | C | 27 | g | 06 December 2017 | FNC | cloudy |
| 279 | E | 6 | g | 07 December 2017 | FNC | cloudy |
| 290 | A | 25 | g | 07 December 2017 | FNC | Slightly cloudy |
| 330 | D | 15 | g | 07 December 2017 | FNC | Clear |
| 272 | D | 24 | g | 08 December 2017 | FNC | Slightly cloudy |
| 275 | H | 2 | g | 10 December 2017 | FNC | Slightly cloudy |
| 337 | D | 2 | g | 11 December 2017 | FNC | Slightly cloudy |

| ID | Catchment | Lims No | Filter Type | Date | State | Colour |
|---|---|---|---|---|---|---|
| 364 | H | 3 | g | 11 December 2017 | FNC | Slightly cloudy |
| 271 | D | 3 | g | 11 December 2017 | FNC | Clear |
| 300 | A | 40 | g | 12 December 2017 | FNC | Slightly cloudy |
| 332 | D | 13 | g | 12 December 2017 | FNC | Slightly cloudy |
| 301 | A | 26 | g | 13 December 2017 | FNC | Slightly cloudy |
| 323 | G | 9 | g | 13 December 2017 | FNC | Slightly cloudy |
| 328 | C | 27 | g | 13 December 2017 | FNC | cloudy |
| 338 | D | 7 | g | 13 December 2017 | FNC | Slightly cloudy |
| 322 | G | 6 | g | 14 December 2017 | FNC | Slightly cloudy |
| 344 | D | 6 | g | 14 December 2017 | FNC | Slightly cloudy |
| 303 | A | 22 | g | 14 December 2017 | FNC | Slightly cloudy |
| 315 | D | 17 | g | 15 December 2017 | FNC | Slightly cloudy |
| 304 | A | 6 | g | 15 December 2017 | FNC | Slightly cloudy |
| 316 | D | 3 | g | 17 December 2017 | FNC | Clear |
| 319 | H | 6 | g | 17 December 2017 | FNC | Slightly cloudy |
| 340 | D | 3 | g | 17 December 2017 | FNC | Clear |
| 331 | D | 6 | g | 18 December 2017 | FNC | Clear |
| 369 | F | 29 | g | 19 December 2017 | FNC | Slightly cloudy |
| 377 | A | 43 | g | 19 December 2017 | FNC | cloudy |
| 378 | A | 28 | g | 20 December 2017 | FNC | cloudy |
| 379 | C | 26 | g | 20 December 2017 | FNC | v. Cloudy |
| 380 | C | 19 | g | 21 December 2017 | FNC | v. Cloudy |
| 368 | F | 11 | g | 28 December 2017 | FNC | Clear |
| 365 | H | 4 | g | 31 December 2017 | FNC | cloudy |
| 385 | I | 28 | g | 04 January 2018 | FNC | v. Dirty |
| 388 | F | 24 | g | 04 January 2018 | FNC | Clear |
| 393 | G | 27 | g | 04 January 2018 | FNC | cloudy |
| 382 | D | 15 | g | 05 January 2018 | FNC | Clear |
| 383 | D | 13 | g | 07 January 2018 | FNC | cloudy |
| 390 | H | 8 | g | 07 January 2018 | FNC | cloudy |
| 406 | D | 4 | g | 07 January 2018 | FNC | Slightly cloudy |
| 410 | A | 45 | g | 09 January 2018 | FNC | Slightly cloudy |
| 435 | F | 15 | g | 09 January 2018 | FNC | Clear |
| 408 | A | 32 | g | 10 January 2018 | FNC | Slightly cloudy |
| 436 | C | 29 | g | 10 January 2018 | FNC | cloudy |
| 398 | D | 15 | g | 10 January 2018 | FNC | Slightly cloudy |
| 438 | C | 16 | g | 11 January 2018 | FNC | Slightly cloudy |
| 446 | D | 5 | g | 11 January 2018 | FNC | cloudy |
| 401 | D | 11 | g | 14 January 2018 | RWC | Slightly cloudy |
| 426 | H | ? | g | 14 January 2018 | FNC | Slightly cloudy |
| 447 | D | 5 | g | 14 January 2018 | FNC | clear |
| 444 | D | 3 | g | 15 January 2018 | FNC | Slightly cloudy |
| 432 | F | 23 | g | 16 January 2018 | FNC | Clear |
| 442 | D | 17 | g | 16 January 2018 | FNC | Slightly cloudy |
| 437 | C | 30 | g | 17 January 2018 | FNC | Slightly cloudy |

| ID | Catchment | Lims No | Filter Type | Date | State | Colour |
|---|---|---|---|---|---|---|
| 448 | D | 18 | g | 17 January 2018 | FNC | Slightly cloudy |
| 453 | C | 16 | g | 18 January 2018 | FNC | cloudy |
| 454 | A | 18 | g | 18 January 2018 | FNC | cloudy |
| 459 | D | 5 | g | 18 January 2018 | FNC | Slightly cloudy |
| 445 | D | 12 | g | 19 January 2018 | FNC | Slightly cloudy |
| 460 | D | 5 | g | 21 January 2018 | FNC | Slightly cloudy |
| 443 | D | 3 | g | 22 January 2018 | FNC | Slightly cloudy |
| 456 | D | 13 | g | 23 January 2018 | FNC | clear |
| 475 | A | 39 | g | 23 January 2018 | FNC | cloudy |
| 481 | F | 23 | g | 23 January 2018 | FNC | Clear |
| 486 | C | 31 | g | 24 January 2018 | FNC | Slightly cloudy |
| 458 | D | 4 | g | 24 January 2018 | FNC | clear |
| 461 | D | 19 | g | 26 January 2018 | FNC | clear |
| 455 | D | 8 | g | 28 January 2018 | FNC | clear |
| 469 | H | 4 | g | 28 January 2018 | FNC | Slightly cloudy |
| 488 | A | 40 | g | 30 January 2018 | FNC | Slightly cloudy |
| 507 | F | 27 | g | 30 January 2018 | FNC | clear |
| 490 | A | 32 | g | 31 January 2018 | FNC | Slightly cloudy |
| 509 | C | 37 | g | 31 January 2018 | FNC | cloudy |
| 497 | D | 10 | g | 04 February 2018 | FNC | Slightly cloudy |
| 498 | G | 4 | g | 05 February 2018 | FNC | Slightly cloudy |
| 491 | H | ? |  | ?? January 2018 |  | Slightly cloudy |
| 335 | D | 11246200 | g | ?? November 2017 | FNC | Slightly cloudy |

Key:

Screened slide

*T. gondii* like body observed

*T. gondii* like body observed and positive by qPCR & sequencing

## 10.8 All samples analysed by qPCR only – catchment H

| Tube No | Source | Lims No | Filter Type | Date | State | Colour |
|---|---|---|---|---|---|---|
| 14 | H | 1 | c | 01 October 2017 | RKS | Dirty |
| 16 | H | 2 | c | 13 October 2017 | RKS | Dirty |
| 17 | H | 5 | c | 10 October 2017 | RKS | Dirty |
| 18 | H | 6 | c | 03 October 2017 | RKS | Dirty |
| 19 | H | 1 | c | 15 October 2017 | RWC | Dirty |
| 20 | H | 5 | c | 06 October 2017 | RKS | Dirty |
| 72 | H | 1 | c | 22 October 2017 | RKS | Dirty |
| 74 | H | 6 | c | 20 October 2017 | RKS | Very dirty |
| 90 | H | 2 | c | 24 October 2017 | RKS | Dirty |
| 93 | H | 6 | c | 27 October 2017 | RKS | Dirty |
| 134 | H | 4 | c | 31 October 2017 | RKS | v. Dirty |
| 135 | H | 1 | c | 05 November 2017 | RKS | v. Dirty |
| 136 | H | 2 | c | 03 November 2017 | RKS | v. Dirty |
| 155 | H | 1 | c | 10 November 2017 | RKS | Dirty |
| 157 | H | 1 | c | 12 November 2017 | RKS | Dirty |
| 199 | H | 5 | c | 19 November 2017 | RWC | Dirty |
| 200 | H | 7 | c | 14 November 2017 | RKS | v. Dirty |
| 201 | H | 2 | c | 17 November 2017 | RKS | Dirty |
| 236 | H | 1 | c | 26 November 2017 | RKS | Dirty |
| 238 | H | 2 | c | 24 November 2017 | RKS | v. Dirty |
| 250 | H | 1 | c | 03 December 2017 | RKS | v. Dirty |
| 251 | H | 1 | c | 01 December 2017 | RKS | v. Dirty |
| 252 | H | 8 | c | 28 November 2017 | RKS | Dirty |
| 276 | H | 1 | c | 08 December 2017 | RKS | Dirty |
| 278 | H | 19 | c | 05 December 2017 | RKS | Dirty |
| 318 | H | 3 | c | 15 December 2017 | RKS | Dirty |
| 320 | H | 4 | c | 12 December 2017 | RKS | Dirty |
| 361 | H | 1 | c | 24 December 2017 | RWC | Dirty |
| 362 | H | 3 | c | 19 December 2017 | RKS | Dirty |
| 363 | H | 2 | c | 22 December 2017 | RKS/RWC? | Dirty |
| 424 | H | ? | c | 16 January 2018 | RKS | Dirty |
| 425 | H | ? | c | 15 January 2018 | RKS | Dirty |
| 427 | H | ? | c | 09 January 2018 | RKS | Dirty |
| 449 | H | 2 | c | 21 January 2018 | RKS | Dirty |
| 450 | H | 1 | c | 19 January 2018 | RKS | Dirty |
| 470 | H | 9 | c | 23 January 2018 | RKS | Dirty |
| 471 | H | 1 | c | 28 January 2018 | RKS | Dirty |
| 472 | H | 6 | c | 26 January 2018 | RKS | Dirty |
| 492 | H | 1 | c | 04 February 2018 | RKS | Dirty |
| 493 | H | 7 | c | 30 January 2018 | RKS | Dirty |
| 494 | H | 2 | c | 02 February 2018 | RKD | v. Dirty |

Key:


Tested by qPCR


qPCR inhibition


Positive for *T. gondii* by qPCR only