Use of the Polymerase Chain Reaction (PCR) for the Analysis and Enumeration of *Cryptosporidium* Oocysts in Drinking Water

Final Report

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Report Title	**Use of the Polymerase Chain Reaction (PCR) for the Analysis and Enumeration of *Cryptosporidium* Oocysts in Drinking Water**
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Glossary

Accuracy

Describes how close the result is to the true value

Amplicon

A DNA fragment generated through an amplification process e.g. PCR.

Amplification efficiency (E)

The ratio representing the increase in amplicons per PCR cycle.

AWWA

American Water Works Association

Blue Book

In this report, this refers to the standard method published in The Microbiology of Drinking Water (2010) - Part 14 - Methods for the isolation, identification and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts.

bp

Base pairs – individual units of two nucleotides which make up the DNA double helix.

Calibration curve

The result of a set of serially diluted DNA samples of known concentration. The calibration curve is used to calculate the efficiency, linear range and reproducibility of a PCR and allows for the quantification of an unknown amount of the target DNA in a sample.

Conventional PCR

Amplification of a specific section (sequence) of a DNA template using sets of forward and reverse primers and a DNA polymerase enzyme. The reaction is carried out in a Peltier heated block with holes to accommodate the PCR reaction tubes. These are endpoint assays in that products of the reaction (the amplicons) need to be visualised, usually by gel electrophoresis. Contrast with real-time PCR (see below).

Cq value

Quantification cycle value - the number of cycles required for the fluorescent signal to cross the threshold during the exponential amplification of a real-time PCR.

CRU

Cryptosporidium Reference Unit

DAPI

4’,6-diamidino-2-phenylindole

Digital PCR (dPCR)

A PCR method where the sample is diluted and split into thousands of individual reactions called partitions, resulting in individual fluorescence measurements allowing for statistical analysis and highly sensitive, precise and accurate quantification of the target DNA.

DNA

Deoxyribonucleic acid

DNA extraction

The process by which DNA is released from cells, purified and isolated for use in molecular assays.

DNA microarray

A collection of microscopic DNA spots, each with a different known sequence, which are immobilised on a solid surface and used as probes to hybridise to specific targets.

DNA template or target

DNA extracted from a sample and used as the starting point for amplification by PCR.

Droplet Digital PCR (ddPCR)

A type of dPCR that creates the sample partitions as thousands of water-oil emulsion droplets within which the PCR takes place and the fluorescence amplitude is read for all droplets in a droplet flow cytometer.

DWI

Drinking Water Inspectorate

FITC

Fluorescein isothiocyanate

Fluorescence in situ hybridization (FISH)

A method where fluorescent probes bind to a specific sequence in the nucleic acid of an organism, allowing for detection or visualisation of a specific target using fluorescence microscopy.

Genomic DNA (gDNA)

A whole DNA preparation extracted from an organism, cell or sample; contains copies of all genes present in that organism, cell or sample.

GenBank

A publicly-accessible nucleotide sequence database, produced and maintained at the National Center for Biotechnology Information https://www.ncbi.nlm.nih.gov/genbank/

Genotype

In the context of *Cryptosporidium*, a genotype is the name given to a genetically distinct group of organisms that are yet to be described as an individual species. Genotypes are often named after the first identified host, e.g. *Cryptosporidium* skunk genotype.

Genotyping

The use of molecular methods for characterising an organism. In the context of *Cryptosporidium,* genotyping is used for the process of identifying species and genotypes.

High resolution melt (HRM) analysis

A real-time PCR analysis method that uses the specific melting temperature of a DNA sequence to detect variations between different sequences. The intercalating dye is released at different temperatures depending on the specific DNA sequence as the temperature slowly increases at the end of the PCR.

Immunofluorescence microscopy (IFM)

A type of microscopy that detects fluorescently-labelled antibodies used to specifically stain organisms of interest in a sample.

Immunomagnetic separation (IMS)

The process in which paramagnetic beads coated with anti-*Cryptosporidium* antibodies capture and concentrate oocysts from a sample; the oocyst-bead complex is immobilised on a magnet so that the other debris present is washed away.

Inhibition

The lack of PCR amplification caused by the presence of inhibitors that can directly interact with the DNA or PCR components to reduce or stop the reaction.

Internal amplification control (IAC)

A non-target sequence of DNA added, with specific primers and probes, to each sample and amplified simultaneously in the PCR to identify false negatives due to PCR inhibition.

ISO

International Organisation for Standardization

Limit of detection (LOD)

The concentration of target organisms at which at least a specified proportion, usually 95%, of samples are positive.

Limit of quantification (LOQ)

The concentration at which a quantitative assay can accurately quantify the amount of target organisms within a sample.

Linearity

Describes how well experimentally defined data correlate with the values from the calibration curve dilution series.

Method 1623

The USEPA's standard method for the detection and enumeration of *Cryptosporidium* and *Giardia* in water by filtration/IMS/FA.

MIQE

Guidelines for the minimum information for publication of quantitative real-time PCR experiments

Multiplex PCR

The simultaneous detection of multiple targets in a single PCR assay.

NCBI

National Center for Biotechnology Information

Nested PCR

A conventional PCR assay using two sets of PCR primers, the first set amplify a longer segment of DNA that the second set is "nested" within to amplify a shorter product from the generated template. Nested PCRs tend to further increase the amount of amplified DNA often resulting in a more sensitive assay, and through the use of two sets of primers can also increase the specificity of an assay.

Next generation sequencing

A term used to describe various technologies used for high-throughput parallel sequencing.

Oligonucleotide

A short strand of synthetically produced DNA or RNA.

Oocyst

The environmentally-robust life-cycle stage of *Cryptosporidium* shed from an infected host that contains the four infective sporozoites which excyst following ingestion of the oocyst to initiate a new infection.

Pan-genus

Including all species within a genus.

Plasmid DNA (pDNA)

Extracted from bacteria into which a specific sequence of DNA has been inserted (cloned) in order to prepare large numbers of pure copies of that sequence.

PCR

Polymerase Chain Reaction; a method of amplifying DNA so that there is sufficient to detect or further analyse.

Precision

The degree to which an instrument or process will repeat the same value.

Primers

Short stretches of single-stranded synthetic DNA (oligonucleotides) that bind to the template in the initiation of PCR amplification.

Propidium monoazide

A photo-reactive dye that binds to double stranded DNA, and following activation inhibits PCR amplification. It is used in oocyst viability assays where pre-treatment only allows the dye to penetrate and bind to DNA within non-viable cells which can then no longer be amplified meaning only DNA from viable cells can be detected by PCR.

Quantitative PCR (qPCR)

A real-time PCR that incorporates a calibration curve to determine the concentration of target organism in an unknown sample

Real-time PCR

Amplification of a specific section (sequence) of a DNA template using primers and DNA polymerase enzyme. The reaction is carried out in the presence of an excitation source (lamp or laser), a fluorophore and a fluorescence detector. Two types of fluorophore are common: non-specific dyes that intercalate with any double-stranded DNA or sequence-specific probes. Results are collected in real-time usually in the form of an on-screen graph which is visible throughout the reaction as it progresses. A Cq value is reported for each positive template. A lower Cq value indicates a higher starting amount of DNA template and in a well-designed and optimised assay this relationship is linear.

Recovery rates

The percentage of oocysts detected following processing of a sample spiked with a known number of oocysts.

Repeatability

A measure of how close results are to each other when repeated by the same analyst, in the same laboratory, on the same equipment.

Reproducibility

A measure of how close the results are to each other when tested by different analysts, in different laboratories, on different equipment.

rRNA

Ribosomal ribonucleic acid

Slide genotyping

The whole process of characterising *Cryptosporidium* species or genotypes from water samples using molecular methods, from removing coverslips from microscope slides, disrupting oocysts, extracting sporozoite DNA through to detecting amplicons by PCR and characterising the species or genotype, usually by sequencing.

ssu rRNA gene

The gene (DNA sequence) encoding small subunit ribosomal RNA.

Subtyping

The process of identifying genetically distinct groups of organisms within an individual *Cryptosporidium* species.

Taqman® MGB probe

A dual-labelled hydrolysis probe that binds to the minor groove of DNA – the use of these probes allows for shorter probes to be used and increases the specificity of real-time PCR assays.

UKWIR

United Kingdom Water Industry Research

UKWIR18S

The real-time PCR assay, targeting the 18s gene, developed during UKWIR project (UKWIR, 2020) and further developed as a quantitative assay in this project.

USEPA

United States Environmental Protection Agency

Viability

A measure of whether a *Cryptosporidium* oocyst shows metabolic activity or integrity. A viable oocyst may be infective or non-infective, but a non-viable oocyst will be non-infective.

Water monitoring slides

Microscope slides generated using the Blue Book standard method for testing water for the presence of *Cryptosporidium* oocysts.

WRF

The Water Research Foundation

Executive Summary

Introduction

To ensure that public water supplies in England and Wales are free of any micro-organism or parasites at a concentration or value which would constitute a potential danger to human health, they are scrutinised by risk assessment and regular monitoring. The protozoan parasite *Cryptosporidium* presents one of the main waterborne public health risks due to its resistance to standard disinfection and ability to cause large-scale outbreaks. The standard method, currently used in the UK water industry, for detection and enumeration of *Cryptosporidium* is based on high volume filtration, elution of oocysts from the filter, concentration by centrifugation, retrieval by immune-magnetic separation (IMS), and detection and enumeration using a microscope following immunofluorescence staining. This method is time consuming, expensive, has low specificity, with a number of other contaminants ending up on the microscope slide, and has multiple opportunities for loss of oocysts. Molecular methods such as PCR are more open and amenable to streamlining through automation, improving workflow and standardising procedures. This report investigates whether there is sufficient DNA for detection and enumeration using the Polymerase Chain Reaction (PCR) for the analysis and enumeration of *Cryptosporidium* oocysts in drinking water.

PCR as an emerging technology in the water industry

Literature reviews in April 2020 revealed that PCR was an emerging technology in the water industry for microbial detection and enumeration. In the UK, qPCR has been established as a standard method for detection and quantification of *Legionella* spp. / *L. pneumophila* and in the United States for Enterovirus, Norovirus and Enterococci. For parasite detection, technologies based on PCR are not currently used routinely in the water industry and there are no published standard methods. For *Cryptosporidium*, the focus has been on using PCR for genotyping which is mostly outsourced to specialist laboratories.

Perceptions of the use of PCR within the UK water industry were explored through surveys to the water testing laboratories and water supply companies between May and August 2020. They indicated that although PCR was not being widely used, this technology might be considered if clear operational and scientific advantages over the current microscopy based standard method were demonstrated.

Selection of a PCR-based method

For the purposes of this feasibility study, quantitative real-time PCR (qPCR) was explored for the detection and enumeration of *Cryptosporidium* in drinking water. Alternative options were identified (e.g. digital PCR) but required more specialist equipment. Two PCR assays were shortlisted from a literature review and attribute assessment. A real-time PCR developed previously for *Cryptosporidium* slide genotyping in an UKWIR-funded project (UKWIR, 2020) was selected as most suitable for adaption as a qPCR.

Development and evaluation of a qPCR

Examination of the impact of pre-analytical processes, the incorporation of internal amplification control DNA in the PCR mix, and whether genomic, plasmid or synthetic DNA provided the most suitable reference material for the generation of calibration curves was undertaken. Synthetic DNA was selected for its more ready availability as a quantified standard, and for the robustness it provided in use.

Although the qPCR indicated reduced reliability of quantification at very low numbers of oocysts, it was sufficiently robust to proceed with a comparison with the standard method by testing 10 L tap water spiked with 10 and 100 oocysts in a Phase 1 trial.

Detection (positive/negative) by qPCR was reliable at both low and high numbers of oocysts. For samples spiked with 100 oocysts there was no statistically significant difference in median oocyst counts between the two methods. However, for samples spiked with 10 oocysts the median number detected by qPCR was significantly less than by the standard method. Additionally, the variance was greater by qPCR than the standard method, and considerably lower or higher counts could be generated.

The qPCR provided mostly practical advantages over the standard method including a faster time to result, reduced consumables costs, easier interpretation of results, as well as providing material (amplicons) ready for genotyping.

Recommendations and future work

Although this work indicated some potential limitations with the use of PCR-based methods for enumeration, the recovery efficiency of oocysts from tap water is inherently variable. Quantification by PCR is dependent on the amount of DNA recovered from the sample, which may be improved by revising upstream sample processing (IMS and DNA extraction) to reduce losses and variability, improve the measurement of uncertainty and provide a more robust and reliable approach. An IMS-free method may well be suitable for PCR-based enumeration and would have additional time and cost-saving benefits. Alternative DNA extraction techniques may increase yield. Further improvement in the enumeration of low numbers of oocysts may be gained from different PCR-based methods, such as digital PCR or other newly developed technologies, but would require substantial investment.

It also needs to be borne in mind that the detection of DNA in a sample by PCR does not necessarily indicate the presence of intact oocysts, an issue that needs to be considered if public health or operational decisions are required.

This project demonstrated that a qPCR can be developed for the detection and to some extent enumeration of *Cryptosporidium* oocysts in drinking water. Although the qPCR method is not at a point where recommendations can be made to the industry about its adoption, areas for further development have been identified and further discussion with the water industry and stakeholders is warranted.

Conclusions

PCR technology is currently not widely utilised in the water industry.

Detection of *Cryptosporidium* oocysts by qPCR was reliable at low numbers, but enumeration was less reliable and highly variable.

There is potential for use of PCR-based methods in *Cryptosporidium* analysis, if improvements in sample preparation and the technology for enumeration are explored.

Introduction

To ensure that public water supplies in England and Wales are free of any micro-organism or parasites at a concentration or value which would constitute a potential danger to human health, they are scrutinised by risk assessment and regular monitoring, thus supporting provision of wholesome water to consumers (The Water Supply (Water Quality) Regulations 2016 (as amended); The Water Supply (Water Quality) Regulations 2018). The microbiological parameters for drinking water quality are measured at various stages (Table 1), using methods described in the Standing Committee of Analysts (SCA) Blue Books within the series Methods for the Examination of Waters and Associated Materials, available at http://standingcommitteeofanalysts.co.uk/.

For public supplies, the water supply company is responsible for sampling and testing, and raw water monitoring samples will also be tested. For private supplies, the Local Authority is responsible for sampling. All samplers and test laboratories must be UKAS accredited against ISO 17025. The test methods are based on bacterial culture and colony counts or most probable number (MPN) estimation (Table 1).

Although the bulk of the drinking water samples and tests are for defined water quality parameters, operational investigations may generate additional samples, and these may be tested for other microorganisms as required (Table 1). For example, heterotrophic bacterial counts can provide early indication of pollution; taste and odour and other aesthetic complaints might be investigated for actinomycetes, yeasts and fungi. In outbreaks, samples might be tested for the causative pathogen e.g. *Campylobacter* or *Cryptosporidium*. Again, the methods for bacteria are mostly based on culture, and for parasites on microscopy.

Laboratories testing drinking water are also likely to be involved in testing associated materials, sewage effluents and sludges, and recreational and environmental waters for which there are defined microbiological parameters and methods (Table 1). The emphasis on bacterial culture and colony counting is clear – although for *Legionella* spp., tested for in environmental and recreational waters, there is an alternative Standard Method based on quantitative polymerase chain reaction (qPCR). A similar trend is seen in the International Standards Organisation (ISO) standard methods for water testing, where the only molecular-based Standard Method is also for *Legionella* spp. (ISO/TS 12869:2019(en)).

The protozoan *Cryptosporidium* presents one of the main waterborne public health risks due to its resistance to standard disinfection and ability to cause large-scale outbreaks. The Standard Method for detection and enumeration is based on direct detection of oocysts by microscopy, as this parasite is hard to culture in the laboratory. Large volumes of water are first sampled through filter modules, the captured oocysts are eluted from the filter, concentrated by centrifugation, recovered from the sample matrix by immunomagnetic separation (IMS), and detected and counted using immunofluorescence microscopy (IFM). While developments have enabled semi-automation of much of this process, detection using IFM involves fixing the prepared sample on a microscope slide, staining using multiple manual procedures, mounting and sealing coverslips over the stained material, and enumeration of oocysts by lengthy, time consuming, expensive, and highly-skilled microscopy. The end-point is an oocyst count from the slide; losses will be incurred during sample processing and staining, and no additional information is provided about whether the oocysts were alive or dead at the time of sampling, or whether they are of a species infectious for humans. Occasionally organisms visually mimicking *Cryptosporidium* are detected and differentiation can be difficult (Anon, 2010). PCR-based methods might provide an alternative with potential benefits to the water industry, for

Table 1. The Standing Committee of Analysts Blue Book methods for the examination of waters and associated materials for microbiological quality parameters

Sample type	Microbiological parameters	Standard method for detection	Standard method for enumeration
Water leaving water treatment works	*Escherichia coli*	Culture	Colony count or MPN
	Coliform bacteria	Culture	Colony count or MPN
	Clostridium perfringens	Culture	Colony count
Water leaving service reservoirs	*E. coli*	Culture	Colony count or MPN
	Coliform bacteria	Culture	Colony count or MPN
Water sampled at customers' taps	*E. coli*	Culture	Colony count or MPN
	Enterococci	Culture	Colony count or MPN
Additional tests	Heterotrophic bacterial counts	Culture	Colony count or MPN
	Mesophilic *Aeromonas* spp.	Culture	Colony count
	Yersinia	Culture	Colony count
	Vibrio spp.	Culture	Colony count
	Campylobacter	Culture	Colony count
	Actinomycetes	Culture	Colony count
	Cryptosporidium	Concentration by IMS	Microscopy count
	Giardia	Concentration by IMS	Microscopy count
Environmental and recreational waters	*Legionella* spp. / *L. pneumophila*	Culture; qPCR	Colony count; qPCR
	Pseudomonas aeruginosa	Culture	Colony count or MPN

example streamlining the analytical process, as DNA extraction and PCR are amenable to automation; providing a shorter time to result; improved specificity of detection; and generation of amplicons that could be further processed for species identification (Chalmers *et al*., 2010).

Although few Standard Methods used in the water industry are based on PCR, such methods have been used in research projects and are evolving continuously (Efstratiou *et al*., 2017).

To explore the potential use of PCR methods for the analysis and enumeration of *Cryptosporidium* oocysts in drinking water this project was undertaken to meet the following objectives:

1. Literature review summary of current published research into the use of PCR methods that would be suitable for the analysis and enumeration of *Cryptosporidium* in drinking water.
2. A review of the use of PCR within the water industry at the moment, and how the industry may adopt the technology into their routine sample analysis.
3. Develop a PCR based method based on findings of the literature review and practical investigations.

4. Duplicate analysis of spiked *Cryptosporidium* samples, using the current Blue Book method for *Cryptosporidium* analysis, alongside the method of PCR deemed suitable.
5. Collation and review of the results of the spiked sample analysis, to determine the benefits of the use of PCR against the current blue book method.
6. Detail any recommendations for the industry for the use of PCR in routine analysis of *Cryptosporidium*, and recommendations for any future areas of research that may be required from the results of this study.

Objective 1 – Literature Review

Literature review summary of current published research into the use of PCR methods that would be suitable for the analysis and enumeration of *Cryptosporidium* in drinking water.

Aim

To identify and summarise the contemporary use of PCR-based methods for detection and enumeration generally applicable for water and environmental samples, and identify those that can be used to analyse and enumerate *Cryptosporidium* in drinking water.

Methods

Database searches were undertaken using PubMed (all fields), Scopus (title, abstract, keywords) and Google (first 100 results). Google Scholar was not used; although it is a powerful search engine for scientific literature it is less useful for getting overview of a topic with limited options to combine search terms and restrictions. The selection of results is not transparent and is dependent upon Google's frequently changing algorithms (e.g. may be impacted by things such as locations, etc). In addition, the websites of DWI, UKWIR, AWWA, and WRF were searched for published reports. The search terms were based on two questions:

1. What is the status of PCR-based methods for detection and enumeration in the water industry?
   - drinking AND water AND PCR AND detection AND (enumerat* OR quanti*)

2. What PCR-based methods have been, or could be, applied for detection and quantification of *Cryptosporidium* in water?
   - Cryptosporidium AND PCR AND detection AND (enumerat* OR quanti*)

The searches were from 1st January 2014 to 6th April 2020, and papers/reports from previous literature reviews from two WRF projects (Refs: 4099 and 4284) (Di Giovanni *et al*., 2010, 2014) and the recently completed UKWIR project (*Cryptosporidium*: Enhancing the water industry's capability to respond, Ref: 20/DW/06/22)(UKWIR, 2020) were included to cover earlier years.

The searches were not limited by geography but were restricted to papers published in English.

The results were imported into Mendeley (https://www.elsevier.com/solutions/mendeley) and de-duplicated. The merged lists were exported into Covidence (https://www.covidence.org) for screening independently by title and abstract by two scientists. For the first question, subsequent synthesis was based on title and abstract alone, supplemented by full text reading of reviews or reports where no abstract was available. For the second question, results were merged back into Mendeley and screening proceeded to full text using a structured data capture form (Appendix 1) and subsequent synthesis. MS Excel was used to keep track of the numbers of articles and exclusions which were recorded using Prisma flow-diagrams (Moher *et al*., 2009).

For the question "What is the status of PCR-based methods for detection and enumeration in the water industry?" the exclusion criteria for screening by title and abstract were:

- Do not use PCR-based methods or technologies for primary detection
- Not applied to actual water samples (e.g. only reported use of spiked samples)

The type of article, country where the work was done, and the organisms sought were recorded. Where possible, involvement or application in the water industry was identified from author affiliations and recorded.

For the question "What PCR-based methods have been, or could be, applied for detection and quantification of *Cryptosporidium* in water?" The exclusion criteria for screening by title and abstract were:

- Do not describe PCR-based methods or technologies
- Do not describe PCR specifically for *Cryptosporidium*

And the exclusion criteria for full text were:

- Full text not available
- Reviews
- Do not use or describe PCR-based methods or technologies for *Cryptosporidium*
- Describing methods that are part of a clinical multi-organism testing panel
- Do not describe methods that are compatible with quantitation
- Do not describe a method for pan-genus detection (inclusivity/exclusivity)
- Do not produce an amplicon that can be used for species identification

To investigate potential inclusivity and exclusivity, primer sets of likely pan-genus assays were aligned against sequences of known *Cryptosporidium* species retrieved from GenBank (https://www.ncbi.nlm.nih.gov/nuccore/). Sequence polymorphisms within the primer regions that may stop or reduce the efficiency of amplification in those species were identified.

Although the aim of this question was to identify methods for the detection and enumeration of *Cryptosporidium* in water, the last exclusion criterion was included, as the subsequent genotyping of *Cryptosporidium* oocysts in water has been beneficial in many investigations that inform operational management activities or water quality incidents and outbreaks. Therefore, it was considered that in some circumstances the detection and enumeration of the *Cryptosporidium* in water would lead to the request for genotyping. As the entire water sample would be tested for detection, the resulting amplicons would likely be the only material remaining and should therefore be amenable to genotyping. Using sequences retrieved from GenBank and aligned in BioEdit, the amplified fragments were assessed for their ability to differentiate species.

Results and Discussion

What is the status of PCR-based methods for detection and enumeration in the water industry?

A total of 77 records were eligible for inclusion by title and abstract (Figure 1). The full list of record references for this section are in Appendix 2.

Five articles were Standard Methods published by the USEPA, one for Enterovirus and Norovirus by reverse transcriptase qPCR (Fout *et al*., 2016), two for Enterococci by qPCR (USEPA, 2015a, 2015b) and two for characterisation of human faecal pollution by qPCR (USEPA, 2019a, 2019b). Interestingly, the Blue Book method for *Legionella* spp. by qPCR was not picked up by the literature search, but these are now located on the Standing Committee of Analysts website which was not included in the search.

One article reported data modelling to estimate probabilities of recreational exposure to Entero- and parechovirus surface water using previously acquired RT-PCR data (Lodder *et al*., 2015).

Six articles were reviews, three of which considered bacteria, two viruses and one *Cryptosporidium*.

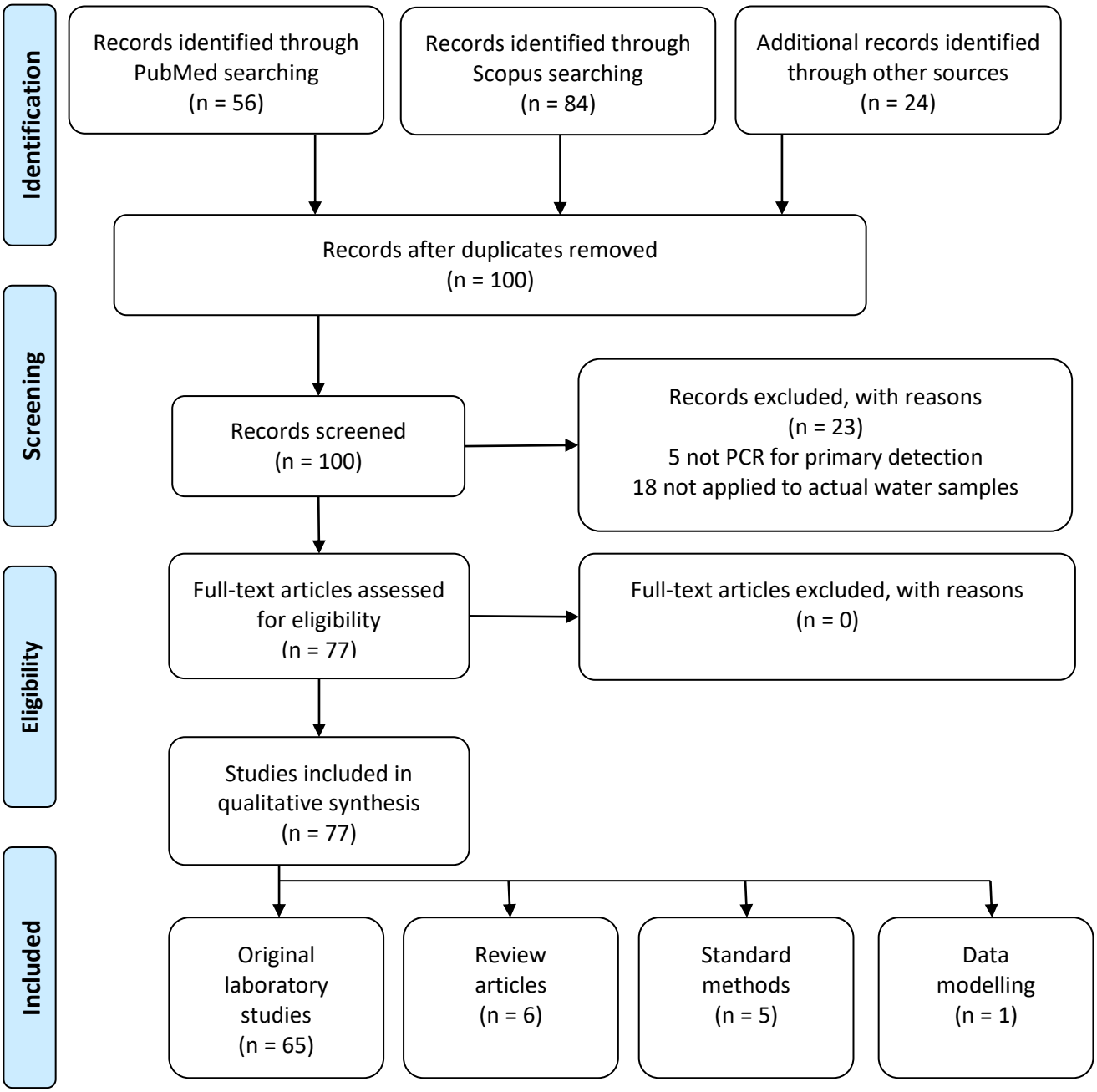

Figure 1. The PRISMA flow diagram (Moher *et al*., 2009) for search terms - drinking AND water AND PCR AND detection AND (enumerat* OR quanti*)

Of the bacteria-focussed reviews, one included nucleic acid-based methods for detection and enumeration of waterborne bacteria; polymerase chain reaction (PCR), digital droplet PCR (ddPCR), real-time or quantitative PCR (qPCR), multiplex PCR, DNA microarray, next-generation sequencing and fluorescence *in situ* hybridization (FISH) (Deshmukh *et al*., 2016). They identified the disadvantages of culture-based methods, citing lack of discrimination between target and background organisms, false-positive counts, laborious protocols, long time-to-results, and failure to detect viable but non-cultivable (VBNC) bacteria. The advantages of nucleic acid-based methods were identified as short time-to-results, efficient laboratory processes and can be independent of culture. Underpinning this is efficient DNA extraction, and amplification which may be reduced by the presence of humic substances and colloidal matter in water samples. Additionally, most nucleic acid-based methods

cannot distinguish between viable and non-viable cells or extraneous DNA persisting in the sample which may lead to overestimation of target. To overcome this, propidium monoazide (PMA) and ethidium monoazide may be employed, especially PMA which has greater efficiency (Nocker *et al.*, 2006). PMA is a photoreactive dye that permeates only membrane-compromised cells, and preferentially binds to double stranded DNA allowing selective amplification of DNA from live cells. Efficient DNA extraction, measures to mitigate inhibition, appropriate primer design and annealing temperature were identified as the key critical factors in overcoming limitations of nucleic-acid based methods.

Another article reviewed methods specifically for *E. coli* in water (Nurliyana *et al*., 2018), and additionally identified that nucleic acid-based tests require high cost equipment and skilled laboratory staff and transport of samples from site to laboratory. They concluded that further development of portable or in-line biosensor devices, especially to improve sensitivity, would facilitate improved water testing.

A third bacterial article reviewed the diagnosis of *Helicobacter pylori* including detection in free-living amoeba in wastewater and drinking water including PMA-qPCR (Skrebinska *et al*., 2018). The article also included a report of the first evidence for survival of *H. pylori* inside free-living amebae in these samples, supporting the hypothesis that free-living amoebae could play a role in the transmission of *H. pylori* to humans.

Two reviews considered viruses. One focussed on viruses in groundwater, particularly their occurrence, fate and behaviour, and the risks posed to public health, but did not explore the use of any molecular-based methods (UKWIR, 2018). Another article reviewed the use of human polyomavirus as a human-specific viral marker / source tracking tool in water and through water treatment processes (Rachmadi *et al*., 2016). Concentration of virus particles from the sample was considered a critical step prior to any detection method, and although culture was traditionally used for viral detection, and provide the gold standard for viral infectivity, PCR-based methods have been widely adopted for testing water samples. This is partly because of the cumbersome nature of cell culture, but also because cell lines are not available for many viruses and their development may lag behind that of PCR-based tests for emerging viruses.

One review was of *Cryptosporidium* species in animals and potential for impacting water sources in the UK, but did not describe the use of PCR-based methods (UKWIR, 2017).

The other 65 articles were of individual original research studies using PCR to test water or water-related samples, conducted in 26 countries and one across Europe (Table 2).

Most studies were undertaken by academic or research institutes; the involvement or participation of water utilities or laboratories was sometimes unclear but the exception rather than the rule (Table 2). It is possible that publication bias underestimated the work undertaken using PCR in water testing laboratories; this was explored through surveys undertaken with water supply companies and water testing laboratories (see Objective 2).

It was clear from the titles and abstracts that there was an emphasis on the published use of PCR for testing for bacteria (35 studies) or viruses (22 studies), with protozoa or parasites investigated by PCR in eight studies (Table 3). This emphasis is perhaps not surprising given that the inclusion of PCR as a Standard Method has focussed initially on bacteria (in the UK *Legionella* spp.; in the US *Enterococci*) and enteric viruses (in the US Enterovirus and Norovirus), that the feasibility of virus testing is greatly improved by PCR, and that parasites and protozoa are less frequently tested for generally and *Cryptosporidium* sampling is particularly expensive. Of the eight studies investigating protozoa or

parasites, three included *Cryptosporidium* (Aquavalens, 2018; Kimble *et al*., 2015; Stokdyk *et al*., 2020) and are discussed further below.

Table 2. Location of research studies identified as using PCR to test water or related samples (references available in Appendix 2).

Country	Number of studies	Studies with water utility / laboratory involvement
Australia	2	2
Austria	3	1*
Bangladesh	2	0
Brazil	3	2*
Canada	4	1 and 1*
China	6	2*
Egypt	1	1*
France	2	1*
Germany	2	1*
India	2	0
Iran	2	0
Israel	1	0
Italy	1	0
Japan	2	0
Netherlands	1	1*
Nepal	3	0
Norway	1	0
Poland	1	0
Portugal	1	1
South Africa	2	0
Sweden	1	1*
Switzerland	2	1*
Taiwan	2	0
UK	2	0
Uruguay	1	0
USA	11	2 and 1*
Pan-Europe	1	1
Netherlands and USA	1	0
RCA & Ecuador	1	1*

* = unclear whether a water utility / laboratory was involved

Table 3. Organisms investigated by PCR in water and related samples (note that some studies tested for more than one type) (references available in Appendix 2).

Included organisms	Number of studies
Bacteria	35
Viruses	22
Protozoa or Parasites	8
Fungi	3
Cyanobacteria	2
Phage or source tracking tools	5
Resistance genes	3
General screen using 16S or eDNA primers	2

This first part of the literature review identified that the status of PCR for detection and enumeration in the water industry is an emerging technology. Both the Blue Book and ISO Standard Method for *Legionella* spp. / *L. pneumophila* by qPCR were published in 2019 but detection and enumeration by culture remains the gold standard.

Key overarching issues that arise for the use of PCR for detection and enumeration in the water industry are:

1. PCR will detect DNA from viable and non-viable organisms. Additional steps are required where the current standard for detection or enumeration is of viable organisms. Culture-independent options used in academic studies include the use of PMA-PCR but this has not been included in the Standard Methods using PCR.
2. For *Cryptosporidium* the current standard is oocyst detection and enumeration; viability or infectivity are not currently measured so this is not an issue for equivalence with current methods.
3. The use of real-time or qPCR for *Cryptosporidium* would align with technologies most likely to be familiar to those laboratories using PCR.
4. Interpretation of qPCR detections will require robust validation of methods, encompassing inclusivity and exclusivity of primers and probes, verification of the calibration function of the quantitative PCR phase, verification of the PCR limit of quantification, verification of the PCR limit of detection, robustness, and finally, measurement of uncertainty of the whole method.

What PCR-based methods have been, or could be, applied for detection and quantification of Cryptosporidium in water?

A total of 73 records were identified in the literature search for this question, of which 13 were initially excluded from the title and abstract, and 60 were taken forward to assess the full text for eligibility (Figure 2). The full table of record references, exclusion reason results and structured data collection for the included papers for this section are in Appendix 3. Below, the reasoning behind the exclusion criteria is briefly discussed, and the numbers excluded by each criterion, in explanation of why some of the methods currently being used may not be suitable within the aims of this project.

Full text screening identified five articles that did not use or describe PCR-based methods for *Cryptosporidium*. Four were review articles, two of which looked at methods of detecting *Cryptosporidium* in clinical samples (Destura et al., 2015; Pomari et al., 2019), one in clinical and environmental samples particularly from developing countries (Adeyemo et al., 2018), and one was part of the final report of the large EU-funded Aquavalens project on improving methods for the detection of pathogens in drinking water (http://aquavalens.org/) (Aquavalens, 2018). This project made some progress with PCR-based methods across viruses, bacteria and parasites. As part of the Aquavalens project, the *Cryptosporidium* Reference Unit had explored the possibility of developing a genus-specific qPCR, but the project focussed on the development and evaluation of *C. hominis* and *C. parvum*-specific assays (Deliverable 4.3, Chalmers *et al*., 2015). The possibility of developing a genus-specific single-tube nested real-time PCR for *Cryptosporidium* targeting the small sub-unit ribosomal RNA (ssu rRNA) gene had been investigated to some extent, but this approach would not be compatible with quantitation due to the unknown amount of template amplified by a primary PCR prior to the secondary PCR that would be used for detection. During that investigation potential problems were identified with many of the published ssu rRNA gene primers including mismatches between the PCR primers and different species of *Cryptosporidium*, potentially reducing the pan-genus application potential, and non-specific matches with other genera that could increase the possibility of non-specific amplification (Chalmers *et al*., 2015).

Five of the excluded articles described the use of commercial or single-unit multiplex assays that target detection of many different organisms in a single assay for clinical diagnostics. These multi-pathogen panel assays are gaining popularity in the clinical setting, but in their current form would generally not be suited for detection and enumeration of *Cryptosporidium* in water as that part of the assay cannot be separated from other targets that are not relevant and / or not compatible with the pre-testing processes (e.g. IMS specific for *Cryptosporidium* would remove other targets from the sample prior to testing making the multiplex nature of the assay redundant).

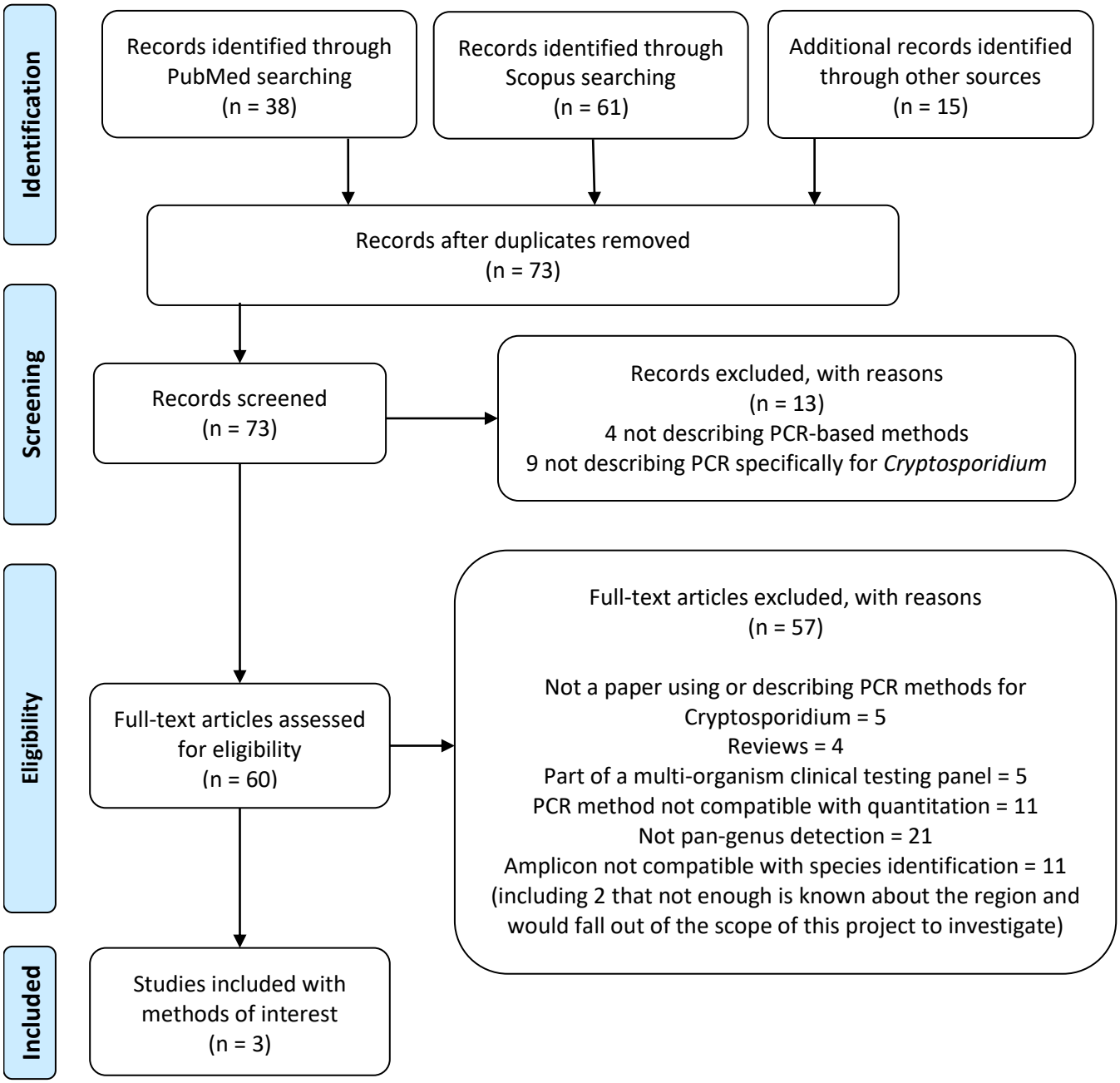

Figure 2. The PRISMA flow diagram (Moher *et al*., 2009) for search terms - Cryptosporidium AND PCR AND detection AND (enumerat* OR quanti*)

The literature search also returned 11 articles that while describing PCR-based methods or technologies specifically for *Cryptosporidium*, were not compatible with quantitation in their current

format. Nine of these described nested PCR assays; as described above nested PCR is not suited to quantitation of oocyst numbers due to the initial amplification of the DNA in a primary PCR before amplifying a shorter fragment for detection in a secondary PCR. The other two papers described multiplex PCRs for multiple pathogens, including *Cryptosporidium*, that were then detected by microarray (Srinivasan *et al*., 2017; Thanthrige-Don *et al*., 2018). Although the format of these assays is not currently compatible with quantitation it may be possible to identify useful primer sets that could be used in qPCR format through *in silico* investigation.

Of the remaining papers that described approaches which were already in a format to enumerate *Cryptosporidium* or were compatible with development for quantitation (e.g. a real-time PCR assay being used for detection only as a standard curve was not included), 21 described the use of primer sets that were not considered to be pan-genus, presenting problems for inclusivity. This is despite some of the papers claiming the assays will amplify all *Cryptosporidium* species; the genetic loci investigated by these assays are shown in Table 4. Some of these loci may well be present in all *Cryptosporidium* spp. and could be useful for pan-genus detection with different primers, the obvious example being the ssu rRNA gene. The use of primer sets that are able to amplify all species of *Cryptosporidium* is vital when testing water samples as the sources of contamination could be from human, livestock, wildlife or companion animals (Xiao *et al.*, 2001; Chalmers *et al.*, 2010; Ruecker *et al.* 2013). Some studies have taken an approach to target human-pathogenic species only, however while this has obvious public health information regarding the sample in hand, it does little to assist in catchment management that can have a wider more sustained public health benefit. Furthermore, defining human-pathogenic species can be hard. The current methods for testing and enumerating *Cryptosporidium* oocysts in water using microscopy are also pan-genus and to investigate/demonstrate equivalence for detection and enumeration any molecular assay should aim for this too.

Even though some of the 14 remaining articles described assays for the detection and enumeration of any *Cryptosporidium* species, 11 produced amplicons that cannot be used for genotyping as they would not differentiate all currently known species and genotypes. Two described a method targeting the *actin* gene (Yang *et al*., 2014; Jacobson *et al*., 2016), but showed that the while the sequence could differentiate between many species, some of the fairly closely related species were indistinguishable. For example, *C. hominis*, *C. cuniculus*, *C. tyzzeri*, and *C. erinacei* were identical in this region, and as both *C. hominis* and *C. cuniculus* have caused waterborne outbreaks of human cryptosporidiosis (Chalmers *et al*., 2019), it is important that these species can be differentiated.

The majority of the articles excluded for their lack of discrimination between different species targeted the ssu rRNA gene (n=9), of which one also described use of the *actin* gene, and tended to produce a very short PCR amplicon, reducing the amount of usable genetic information, or a fragment that differentiated between most species but not between *C. xiaoi* and *C. bovis*. These two species are very closely related and are only distinguishable by one or two nucleotides. The identification of either species gives very specific information as the host ranges (sheep and cattle, respectively) don't overlap and both have been identified during investigation of UK water supplies (Drinking Water Inspectorate, 2014; CRU data).

Two of the excluded papers reported on methods that produced amplicons of which there is little knowledge across the wide range of *Cryptosporidium* species (Operario *et al*., 2015; Jenkins *et al*., 2016). Operario *et al*. (2015) targeted a little investigated region of the ssu rRNA gene meaning the database resource is not available for genotyping, and Jenkins *et al.* (2016) described *Cryspovirus*, a virus infecting at least some *Cryptosporidium* species. *Cryspovirus* contains double stranded RNA that is extracted with the *Cryptosporidium* DNA and has been used to differentiate between isolates of *C. parvum* (Leoni *et al*., 2003). However, little is known about the virus variation between most

Cryptosporidium species, or reliability of detection. Generating the information required for either of these two methods to be considered for the detection, enumeration and identification of *Cryptosporidium* is not within the scope of this project.

Table 4. A summary of articles which described primer sets presenting problems for inclusivity of detection of *Cryptosporidium* spp.

Genetic locus (CryptoDB Gene ID in *C. parvum* IOWA II)	**Number of articles**	**Original references describing the primer sets and potential problems for *Cryptosporidium* spp. inclusivity**
cowp	7	Guy *et al*., 2003 Primer mismatches in some species e.g. *C. muris, C. andersoni*, *C. felis*.
ssu rRNA	5	Jothikumar *et al*., 2008 Lots of reverse primer mismatches in many species and genotypes and almost no variation between primers making genotyping impossible. Gao *et al*., 2014 Stated their assay was specific to *C. parvum* only and didn't amplify a selection of other *Cryptosporidium* species, although our alignment suggests that all species (and other apicomplexans) should amplify with these primers. de Araújo *et al*., 2018 Primer set are genus specific, but the probe is designed for *C. hominis* or *C. parvum*, although it actually also matches a few of the more closely related species.
DnaJ domain containing protein (cgd8_450)	6	Laxer *et al*., 1991; Fontaine & Guillot, 2002; Verweij *et al*., 2004; Mejia *et al*., 2013 Primers and probes have some mismatches between different *Cryptosporidium* spp. such as *C. ubiquitum*, *C. muris* and *C. andersoni*, but limited data is available to know the extent across all the species.
Swi2/Snf2 ATpase, Rad16 ortholog (cgd4_140)	1	Sim *et al*., 2017 The primers show some mismatches, e.g. in *C. ubiquitum*. This gene has other, more conserved areas between species and may allow for a pan-genus assay, but data are lacking and generation is outside of the scope of this project.
hsp70	2	Garcés-Sanchez *et al*., 2009 Primers designed to be specific for *C. hominis*, *C. parvum* and closely related human pathogenic species.

Three papers were considered to describe a sufficiently inclusive, pan-genus PCR-based method potentially compatible with quantitation that produced an amplicon suitable for species identification (Agulló-Barceló *et al*., 2014; Di Giovanni *et al*., 2014; UKWIR, 2020). Unsurprisingly, due to the vast amount of data available at the ssu rRNA gene for all *Cryptosporidium* species, all targeted this locus by real-time PCR. All three of the articles also described other methods that are not considered here as they did not meet the inclusion criteria: Agulló-Barcelló *et al*. (2014) described a short ssu rRNA

gene assay (Stroup *et al*., 2006), Di Giovanni *et al*. (2014) described an *hsp70* assay (LeChevallier *et al*., 2003), and UKWIR (2020) described two other ssu rRNA gene assays ("ALC2" based on Li *et al*., 2015; "UMRT" from Zahedi *et al*, 2018), none of which would enable differentiation of all *Cryptosporidium* species. The qPCR methods used by Di Giovanni *et al*. (2014) and in UKWIR (2020) were both based on previously described primers by Johnson *et al*. (1995) that amplify a 435 bp fragment encompassing key polymorphic regions of the ssu rRNA gene. These primers were incorporated in to real-time PCRs using a high resolution melt (HRM) analysis (Di Giovanni *et al*., 2010) or a TaqMan probe (UKWIR, 2020). Both assays were developed specifically for genotyping *Cryptosporidium* spp. from slides prepared by the USEPA Method 1622/23 and Blue Book standard methods. The other method of interest as described by Agulló-Barcelló *et al*. (2014) used primers described by Xiao *et al*. (2001) that amplify a larger fragment (~830 bp) that is particularly useful for genotyping; the *Cryptosporidium* Reference Unit have historically investigated this region by conventional nested PCR and DNA sequencing. However, two issues arise with the assay as described by Agulló-Barcelló *et al*. Firstly, PMA pre-treatment was used for assessing viability; this step can be omitted. Secondly, the size of the fragment is generally considered too large for efficient qPCR and has, in previous research, shown limited success (CRU data). However, different reagents and master mixes can have a big impact on the dynamics of a qPCR and Agulló-Barcelló *et al*. presented an R^2 correlation coefficient of 0.99 and limit of detection of at least 1 oocyst, suggesting that this assay may also be worth exploring further.

This second literature review, completed under Objective 1, identified three feasible options for detection and quantification of *Cryptosporidium* by qPCR that could be used in this project. One (Agulló-Barcello *et al*., 2014) would need further development and evaluation within our laboratory, while the other two (Di Giovanni *et al*., 2014; UKWIR, 2020) have been used previously by the Cryptosporidium Reference Unit and are known to work with the Unit's reagents and platforms (SensiMix™ II Probe Kit, Bioline and Rotor-Gene Q, Qiagen). The probe-base version of this assay (UKWIR, 2020) has been adopted at the Cryptosporidium Reference Unit for slide genotyping and has also been adopted for the development of a food-testing assay by collaborators in the EFSA-funded IMPACT project.

Based on previous experience, the UKWIR assay was selected to assess the potential for detection and enumeration of *Cryptosporidium* oocysts in drinking water, but further developmental work was required before method evaluation. This included the production and addition of a standard curve to enable quantification, as the assays are currently designed only for detection and genotyping. Additionally, the inclusion of an internal amplification control was required to monitor for PCR inhibition from components in the sample. The performance of the assay was re-assessed following adjustments.

The key consideration were the qPCR's ability to detect and enumerate *Cryptosporidium* oocysts, particularly low numbers, which have been recovered directly from water samples and processed through the Blue Book method of immunomagnetic separation to the point prior to dissociation.

Objectives 3 and 4 aimed to determine and verify the inclusivity and exclusivity of the primers and probes, the calibration function of the quantitative PCR phase, and determine the limit of detection, limit of quantification, accuracy and uncertainly of measurement of the whole method, to establish the surety of a negative result, and finally compare detection and enumeration with the current Blue Book methods of microscopy.

Objective 2 – Industry Survey

A review of the use of PCR within the water industry, and how the industry may adopt the technology into their routine sample analysis.

Aim

To understand the current use and perceptions of the use of PCR-based methods generally, and specifically for *Cryptosporidium*, within the UK water industry.

Methods

Two separate online questionnaires were designed; one for drinking water testing laboratories and the other for drinking water supply companies in England, Wales, Scotland and Northern Ireland. Each survey was sent to individuals with appropriate status to answer on behalf of their company or laboratory. This approach was chosen so that the research team could collect scientific, capability and capacity opinions and perceptions from the testing laboratories and acceptability, interpretation and implementation opinions and perceptions from the supply companies.

The 14 UKAS-accredited water testing laboratories in the UK were approached directly by email from the CRU using a secure database. Twenty three water supply companies in England and Wales and the drinking water quality regulators in Scotland and Northern Ireland were contacted.

The surveys were designed using SmartSurvey© (https://www.smartsurvey.co.uk) and had up to 22 questions, depending on the survey and choice of answers to each. Each survey was trialled by individuals at the CRU and DCWW before being sent out to targeted respondents. This tested the question logic and ensured that respondents would be able to correctly navigate through the questionnaires. The recipients of the trial runs at DCWW were not those individuals to whom the questionnaire was administered during main survey, to avoid pre-empting their responses, and unwanted bias.

The final (Word) versions of each survey and list of receiving laboratories and companies are in Appendices 4 and 5.

The key dates for the survey development, administration and reminders are shown in Table 5.

Table 5. Survey key dates

Activity	By whom	When
Design surveys	CRU	April / May 2020
Initial road test of surveys	CRU	01/05/2020
Beta test by DCWW	DCWW / CRU	13/05/2020
Main surveys distributed	CRU / DWI	14/05/2020
Survey reminders (if needed)	CRU / DWI	01/06/2020
Second reminder	CRU / DWI	08/06/2020
Individual reminders (n)	CRU (3) / DWI (5)	16/06/2020
Collate and analyse data	CRU	23/07/2020
Close surveys	CRU	10/08/2020

Responses to both surveys were analysed to understand the current usage and planned uptake of PCR technology, the perceived benefits and barriers to its implementation, and the present level of experience with, and available facilities for PCR applications in the industry.

Additionally, since the completion of the surveys in 2020, the COVID-19 pandemic initiated monitoring wastewaters for viral nucleic acid using qPCR, increasing awareness and public health applications of qPCR as a technology. This was explored at a meeting of the UK Water Quality Network in July 2021 and is summarised below in page 36. Participants were asked ahead of the meeting:

1. Is any more PCR testing being done in water laboratories now? (for any pathogen)

2. If so, what for and what were the drivers for this?

Results

Survey One: drinking water testing laboratories

All 14 water testing laboratories that were invited to complete this survey, did so.

Survey questions and responses:

1. What is the name of the water testing laboratory you are answering this survey on behalf of? *
2. What is your name? *
3. Please add your contact details (email address preferred) *
4. What is your position? *

The first four questions recorded information about the person completing the survey on behalf of their laboratory. Responses to Question 4 were:

Microbiology Senior Analyst (Looking after the Cryptosporidium Laboratory)
Microbiology Manager
Technical Development Manager
Principal Scientist Microbiology
Team Leader & Technical Manager
Microbiology & Cryptosporidium Technical Manager
Senior Technical Analyst
Team Leader Microbiology
Scientific Officer-cryptosporidium/microbiology lab
Technical Co-ordinator Life Sciences (Currently on secondment as Laboratory Operations Manager Life Sciences)
Cryptosporidium Laboratory Team Leader
Technical scientist
Senior Scientist

5. Does your laboratory use PCR for the detection of any micro-organisms in water?
Two respondents said "Yes" to this question; one stated that this was for *Legionella* spp. and that they are currently investigating PCR for Covid-19 analysis, the other that PCR was occasionally used for confirmation of bacterial species, but that this isn't done on their site.

Only respondents answering "Yes" to Question 5 (n=2) answered Question 6
6. For what reasons do you test for these microorganisms using PCR?
These two respondents stated:

- Gives confidence in confirmation of bacterial species recovered. To make comparisons through distribution system or over time.
- Client requirements / rapidity of analysis vs. traditional method

7. On average, how many samples does your laboratory currently test for *Cryptosporidium* each week?
The average across all respondents was 111 tests per week, and the range was 30 - 240 per week.

8. What is the capacity of your laboratory for *Cryptosporidium* testing (samples per week)?
The average across all respondents was 164 tests, with a maximum capacity of 320 tests per week stated by one laboratory. The range was 60 - 320 tests per week.

9. Does your laboratory use PCR to detect or identify *Cryptosporidium* oocysts from routine water monitoring slides?
Two respondents said "Yes" to this question. One stated that they used PCR to identify *Cryptosporidium* species and genotypes, the other stated that they used PCR to both detect and identify *Cryptosporidium* species and genotypes. However, neither of these two labs had responded Yes to question 5.

Only respondents answering "Yes" to Question 9 (n=2) answered Questions 10 – 17
10. Which PCR technology does your laboratory use for detection or identification of *Cryptosporidium* in water (tick all that apply)?

Table 6. Responses to Survey one - question 10

	Detection	Identification
Real time PCR		
Conventional PCR		
Conventional nested PCR	X	X
Restriction Fragment Length Polymorphism (RFLP)		X
Sanger sequencing		X
Next-generation sequencing		

11. Which genes / loci do your PCRs target for detection of *Cryptosporidium* oocysts?
The laboratory that indicated that they used PCR for the detection of oocysts stated that they used nested PCR analysis of the SSU rRNA gene.

12. Which genes / loci do your PCRs target for identification of *Cryptosporidium* species and genotypes?
The two laboratories that indicated that they used PCR for the identification of *Cryptosporidium* species stated that they used nested PCR analysis of the SSU rRNA gene.

13. What PCR equipment and facilities does your laboratory have in-house?
The two laboratories that indicated that they used PCR stated that they had conventional PCR thermocyclers and sequencing capability (SeqStudio Genetic Analyzer, ThermoFisher). One also had real-time PCR instruments, the other had PCR cabinets.

14. What experience / educational level knowledge of PCR does your laboratory have (tick all that apply)?
The two laboratories that indicated that they used PCR each had experienced staff, one also stated that they had PhD and degree level staff as well; the other conceded that their experience was limited to conventional PCR, with less experience of real time PCR.

15. Are you aware of any PCR training opportunities which may be available to your colleagues?
One of the laboratories that indicated that they used PCR said they had received specific / tailored training offered by ThermoFisher Scientific. The other was not aware of any opportunities specific to *Cryptosporidium*.

16. What are the benefits of using PCR for detecting *Cryptosporidium*?
The two laboratories that indicated that they used PCR responded:

- Quicker and less manual labour compared to the current method of detection we use.
- Ability to speciate oocysts

17. What are the drawbacks of using PCR for detecting *Cryptosporidium*?
The two laboratories that indicated that they used PCR responded:

- Limitations with lower counts. Microscopy technique can detect 1 oocyst on a slide whereas PCR can be limited.
- Time constraints

Only respondents answering "No" to Question 9 (n=12) answered Questions 18 – 20
18. Are there any barriers to using PCR for detecting *Cryptosporidium* for routine monitoring in your laboratory (tick all that apply)?
Twelve laboratories responded to this question, one stated that there were no barriers to using PCR. The "other" response was filled in as "N/A". Each of the choices provided was selected by at least one respondent (column labelled with the number of respondents selecting that category):

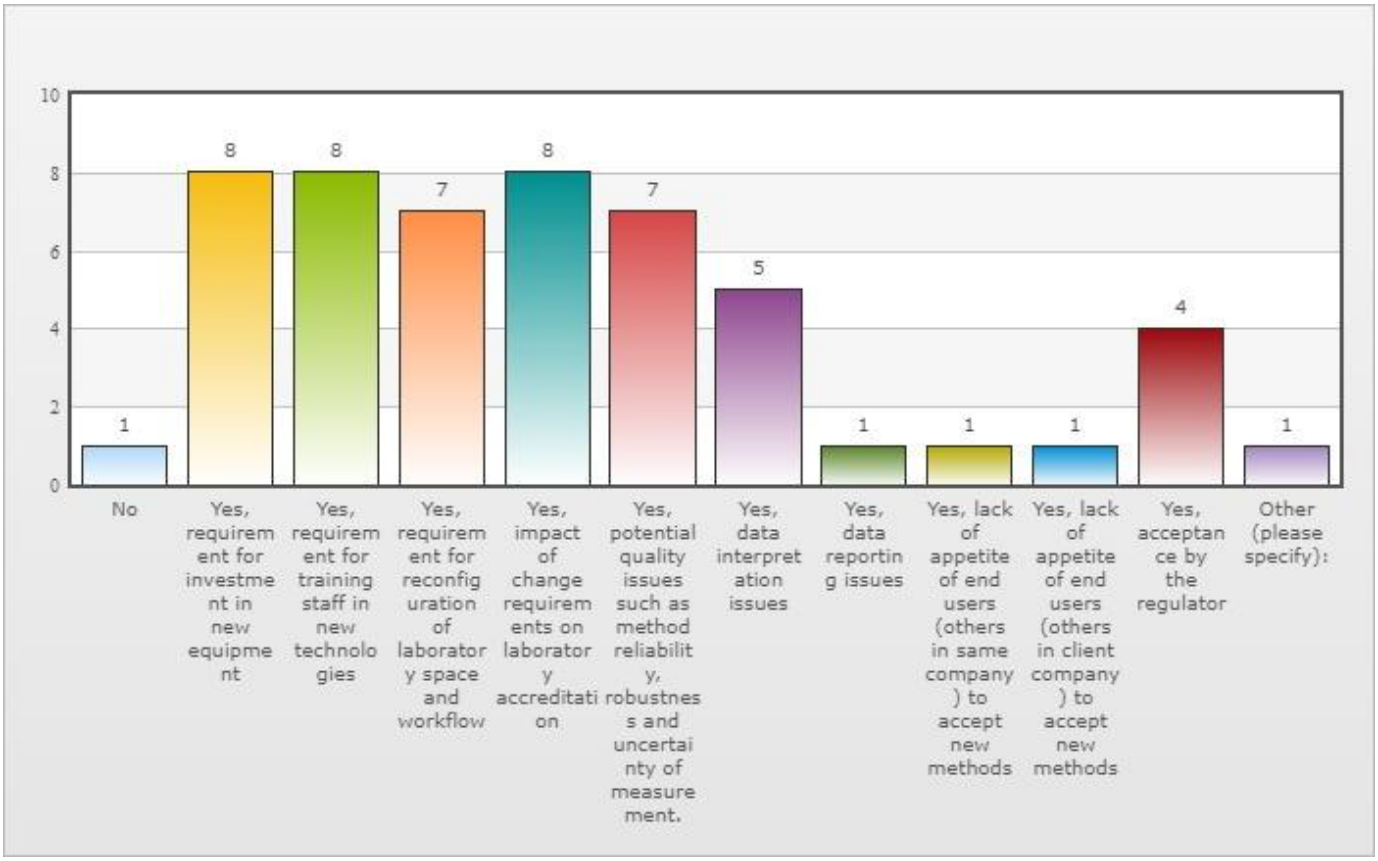

Figure 3. Responses to Survey one - question 18; the y-axis corresponds to the number of respondents.

19. If your laboratory is not using PCR for detecting *Cryptosporidium* oocysts in water is this because (tick all that apply)?

The twelve laboratories who indicated that they are not using PCR responded to this question, each selecting at least one reason. The most popular option was "PCR not being something they have a need for at the moment" (Columns are labelled with the number of respondents selecting that category):

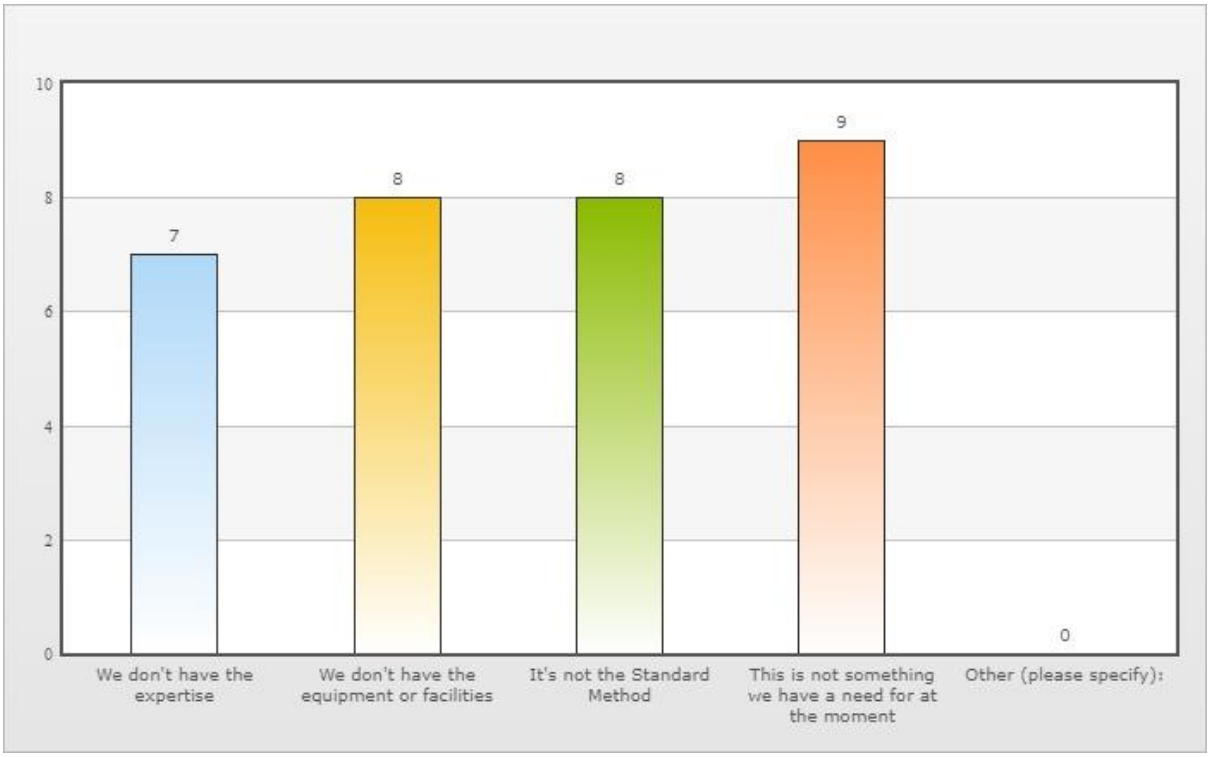

Figure 4. Responses to Survey one - question 19; the y-axis corresponds to the number of respondents.

20. If your laboratory is not using PCR for testing for *Cryptosporidium* oocysts in water, do you have plans to introduce PCR for the detection and/or identification of *Cryptosporidium* in the future?

The twelve laboratories who indicated that they are not using PCR responded to this question:

Table 7. Responses to Survey one - question 20

	No	Yes, in the next 12 months	Yes, in the next 5 years*
Detection	9/12	0/12	3/12
Identification	9/12	0/12	3/12

* Three testing laboratories indicated their intention to introduce PCR for both detection and identification in the next 5 years.

22. Please add any comments or further information which you think we will find helpful:
Five laboratories offered further comments:

Table 8. Responses to Survey one - question 22

ID	Comment
141436303	The Crypto method is something that we have been looking to develop within the laboratory for the last couple of years. The current method needs improving and we have a few potential future projects that will look at DNA methods for detection and identification. We are currently in the process of commencing an MSc project to look at novel methods for detection and identification, one area may be the use of LAMP as this is an area of expertise for the collaborating university.
141569133	- Currently we use nested PCR/RFLP for Cryptosporidium species identification. - We are trialling the Seq Studio and hope to be validated by the end of 2020/start of 2021. - The Rotorgene Real-Time PCR, will then be validated for the confirmation of positive slides previously confirmed as positive by traditional microscopy.
142457779	Current weekly Cryptosporidium sample numbers are typically 240 - 250.
143279421	The survey is a bit unclear as to whether it refers to PCR being used as an alternative to microscopy or to give further information beyond the current methodology.
143803746	We think the major barrier for us is space and equipment but we are open to use PCR technology for Cryptosporidium analysis.

Survey two: *Cryptosporidium* PCR Questionnaire for Water Supply companies

14 / 23 (61 %) Water Supply Companies that were invited to complete this survey, did so.

Survey questions and responses:
1. What is the name of the water supply company you are answering this survey on behalf of? *
2. What is your name? *
3. Please add your contact details (email address preferred) *
4. What is your position? *

The first four questions recorded information about the person completing the survey on behalf of their laboratory. Responses to question 4 were:

Operations team leader
Chief Scientist
Laboratory Manager
Water Quality Monitoring Manager
Water Quality Compliance Manager
Water Quality Regulations Team Manager
Team Leader -- Microbiology
Head of Science
Laboratory and Sampling Manager
Sampling and Analytical Services Manager
Water Quality Manager
Head of Water Quality
Public Health Manager

5. On average, how many *Cryptosporidium* tests does your company commission each week?
The average across all respondents was 91 tests per week, and the range was 5 - 200 per week.

6. Does your company use PCR for the detection of any micro-organisms in water?
Two companies stated that they use PCR; and three comments were offered:

Table 9. Responses to Survey two - question 6

PCR is only used for the genotyping of *Cryptosporidium*, on receipt of a positive slide
Very infrequently. However, PCR have been commissioned from 3rd parties for investigation of specific issues. But for clarification there have been no such investigations in the last 18 months.
Not routinely but we have just commenced some research into the fate of virus in drinking water using PCR. We also send a small number of samples for typing.

7. Does your company use PCR to detect (not genotype) *Cryptosporidium* oocysts in water?

Table 10. Responses to Survey two - question 7

12	No
0	Yes - in-house
2	Yes - by a contract laboratory

Only respondents answering "Yes" to Question 7 (n=2) answered Questions 8 – 12
8. For what reasons does your company use PCR for the detection of *Cryptosporidium* oocysts?
The two companies who responded yes to Q7. Answered as follows:

Table 11. Responses to Survey two - question 8

Operational monitoring	0
During water quality incidents	2
Catchment monitoring	1
Because the regulator expects us to	0
For research	1

9. Has PCR testing for the detection of *Cryptosporidium* ever been used by your company in any of these ways (tick all that apply)?
The two companies that stated that they use PCR both said they had used the technique during water quality incidents, one also added "to determine where the source of contamination comes from".

10. How does your company interpret *Cryptosporidium* PCR data (detection of DNA) compared with results from the Standard Method (microscopy detection of oocysts)?
The two companies that stated that they use PCR agreed that "They both indicate the presence of the organism".

11. What are the benefits of having data from PCR testing for *Cryptosporidium*?
This free text question was answered by the two companies that stated they use PCR for detection of *Cryptosporidium*:

Table 12. Responses to Survey two - question 11

To identify the source of contamination. To determine public health risk.
May help identify source

12. Are any of the following concerns around the use of PCR results for *Cryptosporidium* detection (tick all that apply)?

One of the two companies using PCR gave a response to this question stating that "Method reliability - uncertainty of measurement" was a concern and further, they commented that "Sensitivity of the analytical method, turnaround time" were also important.

Only respondents answering "No" to Question 7 (n=12) answered Questions 13 and 14

13. Are the following likely barriers to the future adoption of PCR results for *Cryptosporidium* detection (tick all that apply)?

The twelve companies and a Water Quality regulator that answered this questions responded as follows (Column labelled with the number of respondents selecting that category):

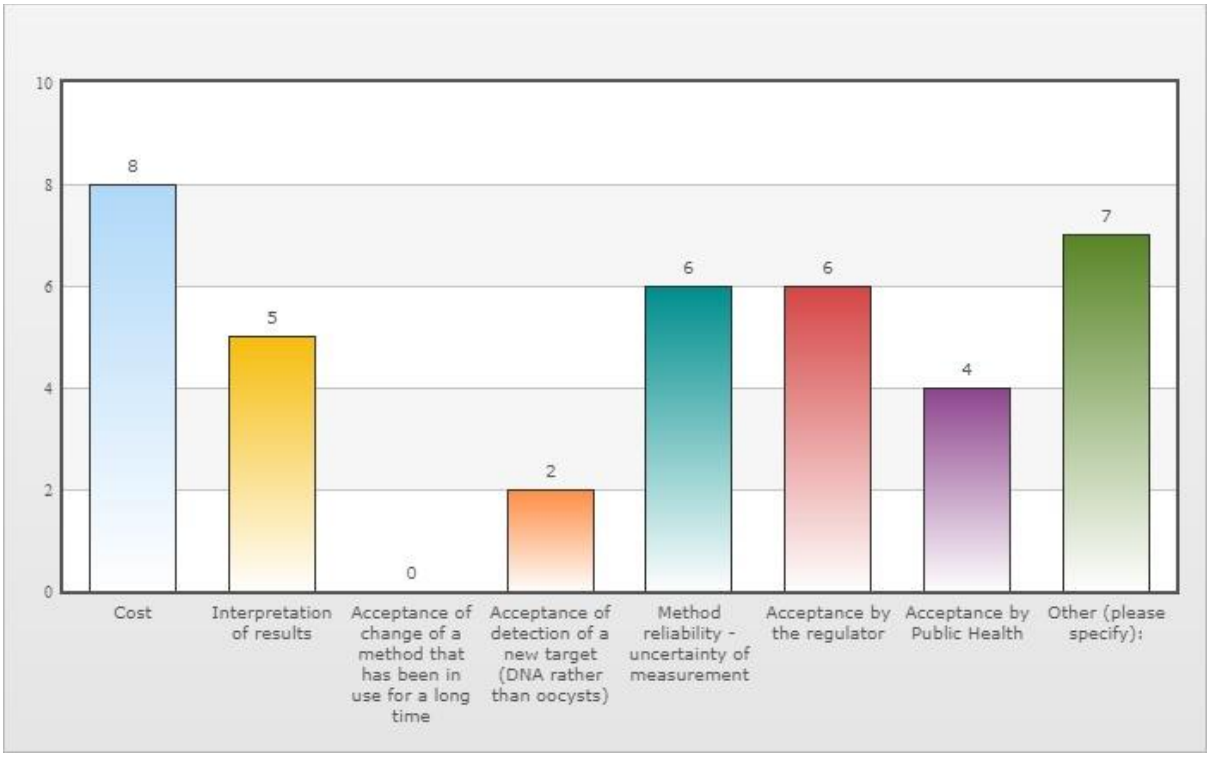

Figure 5. Responses to Survey two - question 13; the y-axis corresponds to the number of respondents.

The other barriers cited were:

- We subcontract our crypto testing to another DWTS accredited lab, who carries out the testing in accordance with the current Regulatory testing protocol (by staining/microscopy/counting).
- Availability by the analytical provider.
- There are no absolute barriers but a good business case for the benefits of new methods is required accounting for all aspects of WQ monitoring (e.g. information/insight, timeliness, cost to analyse etc.)
- One of the main reasons for sampling final water for oocysts is to verify the performance of the coagulation/filtration stages at our WTWs for oocyst (and giardia cyst) removal as other parameters such as turbidity are not entirely reliable as surrogates. If we detect crypto DNA in final water not sure what this tells us about the integrity of our filters as well as the obvious - what does it mean in terms of public health risk? It is difficult enough to interpret what the detection of an intact oocyst means in terms of public health risk.

14. If your company is not using PCR for testing for *Cryptosporidium* oocysts in water, do you have plans to commission PCR for the detection of *Cryptosporidium* in the future?
The twelve companies not currently using PCR for detection of *Cryptosporidium* responded to this question as follows:

Table 13. Responses to Survey two - question 14

Yes, in the next 12 months	0
Yes, in the next five years	3
No	9

16. Please add any comments or further information which you think we will find helpful:
Five companies offered further comments:

Table 14. Responses to Survey two - question 16

ID	Comment
141443099	As regulator we would strongly support the development of a quantitative genetic method for Crypto to replace the current one using IMS and microscopy, provided the necessary equivalence testing is done. A lot of crypto testing is undertaken, and I suspect any new method would represent a significant cost and time saving. Careful liaison with health professionals would need to be undertaken to ensure they understand the change and its implications.
141512267	The real game changer is if we can give an indication of viability. We will continue to use PCR for genotyping of any oocysts that are detected in samples.
142130243	Our company does not operate its own in-house laboratory. We have a long-standing contractual relationship with analytical partner. This allows us flexibility in how we approach gaining analytical data.
142996511	"PCR analysis of cryptosporidium is an interesting area and one that we as a company are interested to explore further. At present we do not employ any PCR methods for routine microbiology so would need to set up a PCR facility - if this would offer a more reliable, cheaper or quicker means to test for cryptosporidium, this would be an area of interest. Also of key interest, following the installation of UV treatment at our highest risk sites, would be the ability to determine viability of any oocysts detected we would also welcome a m" [text incomplete]
143403874	I think a broader discussion around the merits of PCR for a range of water quality determinations and the company would happily facilitate such an event/would be happy to collaborate with others on this.

Discussion and narrative summary of each survey

Further information about the survey outputs as a whole when taking other aspects of the research project into account can be found in Objective 6, including a summary of a follow-up discussion with participants of the UK Water Quality Network meeting in July 2021.

Survey One: drinking water testing laboratories

Two of the fourteen laboratories surveyed used PCR for the detection and/or identification of *Cryptosporidium*. These laboratories had a range of suitable equipment and experienced staff. The stated benefits of using PCR were: "quicker and less manual labour compared to the current method of detection we use", and "the ability to speciate oocysts". The main barriers to adopting the technology were identified by other laboratories as the need for new equipment, staff training and changes in their accreditation requirements. The principal reason for not using the technology was that it is not something the laboratories need at the moment, however three out of the 12 laboratories not currently using PCR, said they might adopt the technology in the next five years.

Survey two: *Cryptosporidium* PCR Questionnaire for Water Supply companies

Two of the fourteen supply companies that responded said that they use PCR for the detection of oocysts. Their main use of PCR was during water quality incidents, and the main benefit was deemed to be the ability to identify the (*Cryptosporidium*) contamination source. However, it was apparent from the responses that there may have been be some mis-interpretation of the terms used in the survey; **Genotyping** is the identification of the species or type of *Cryptosporidium* present and contributes to source identification and risk assessment and management. **Detection** is used to confirm the presence or absence of the parasite, but cannot inform the user about the parasite identity.

The main barriers to adopting PCR (a question also answered by a Drinking Water Quality Regulator) were the cost, that this work is already subcontracted elsewhere, the availability of such tests, the need for a good business case (this from a company who are accepting of the technology) and the interpretation of data in-hand with other water quality parameters. Future adoption (within the next 5 years) of PCR for the detection of *Cryptosporidium* was indicated by three out of the 12 companies not currently using the technology. Other comments from the respondent Water Supply Companies are provided in the section above.

UK Water Quality Network meeting: July 2021

The network reported that although the water industry's main input in wastewater monitoring was in sampling rather than testing, which was done by the Environment Agency and in university laboratories, there was certainly raised awareness and interest in PCR-based technologies. For *Cryptosporidium* testing, one participant at the meeting reported their laboratory was focussing on the challenges of normal testing regimes during the pandemic with which others concurred, but indicated it would be useful to see how PCR might be run alongside or as an alternative method to microscopy in the same laboratory. They welcomed seeing the illustration of the method embedded in the Blue Book. Another participant reported that adopting PCR for slide genotyping had been straight forward although establishing the infrastructure was costly – but noted that this included a sequencer which would not be required for qPCR. The issue of cost was of interest to participants.

Objective 3 – Method development

Development of a PCR-based method based on findings of the literature review and practical investigations.

Objectives

- Select a PCR-based method for development as a quantitative PCR (qPCR)
- Evaluate the effect of the addition of an internal amplification control (IAC) to monitor for PCR inhibition
- Evaluate the PCR according to Minimum Information for Publication of Quantitative Real-time PCR Experiments (MIQE) guidelines
    - the accuracy and precision of the qPCR
    - the ability of the qPCR to discriminate between numbers of oocysts
    - evaluate repeatability and reproducibility of the qPCR
    - Express the analytical sensitivity of the qPCR as limit of detection (LOD)
- Investigate whether testing samples prepared by immunomagnetic separation (IMS) is detrimental to the qPCR.

The qPCR-based method formatted as required by the Standing Committee of Analysts (Anon, 2017), based on both the results of the above and further refined following the Phase 1 trials described in Objective 4, has been presented as example of how such an assay may fit within the Blue Book industry standard (Appendix 6).

Method and Results

Selection of a PCR-based method for development as a qPCR

In Objective 1, a literature review was undertaken which summarised current published research into the use of PCR methods that would be suitable for the analysis and enumeration of *Cryptosporidium* in drinking water. Two existing PCR assays were identified (UKWIR, 2020 and Agulló-Barceló *et al*., 2014) which had potential for development as a qPCR. The UKWIR 2020 assay was chosen for further development having the advantages of prior work up as a pan-genus detection and genotyping assay in the Cryptosporidium Reference Unit laboratory with known ability to detect four *Cryptosporidium* species (*C. parvum, C. hominis, C. andersoni* and *C. ubiquitum*) established experimentally using plasmid DNA and 29 species or genotypes in real-life testing. Analytical specificity was also known to be good. The main disadvantage of the Agulló-Barceló assay was that the 830 bp PCR product was considered too long (830 bp) to allow efficient and linear amplification ideally required for qPCR.

The inclusivity and exclusivity of the primers and probe were evaluated in the UKWIR (2020) project and demonstrated specific amplification from a range of 29 different *Cryptosporidium* species and genotypes and no non-specific amplification from DNA representing 8 other parasites and 15 different species of hosts (UKWIR, 2020).

The UKWIR 2020 assay was adapted for use as a 50 µl volume reaction, using 20 µl DNA template, run on RotorGeneQ (Qiagen, UK) real-time PCR machines. See Appendix 7 for the final qPCR protocol.

For qPCR, each assay run needs a calibration curve, against which the quantitation cycle (Cq) values of the test samples are converted into genome copies or numbers of organisms (in this case oocysts). The calibration curve also provides:

- amplification efficiency (E), determined from the slope of the log-linear portion (M), theoretic maximum 1.00 (which indicates that the amount of product doubles with each cycle). The acceptable range of E = 0.90 – 1.10 (90-110%) (corresponding to values of M between -3.58 and -3.10). E can be affected by amplicon length and sequence, and impurities or inhibitors (especially in highly concentrated samples) which can increase the E value in the sample.

- linearity (R^2) which is the fitness of the data to the calibration curve. Linearity will indicate variability between replicates of the calibration curve; the value of R2 should be >0.98.

These values are usually provided by the real-time PCR instrument for each assay run in which the calibration curve is included / amplified.

There are choices to be made when deciding what to use for the calibration curve reference material. Genomic DNA extracted from target organisms might be the obvious choice, but for *Cryptosporidium* obtaining sufficient, enumerated oocyst suspensions may not be practical going forward (should qPCR be adopted by the industry, for example). Additionally, background material and DNA extraction processes may reduce the robustness of this choice. Plasmid DNA provides perhaps a more consistent reference material but cloning to produce plasmids and handling very high copy number material in a molecular biology laboratory requires strict biocontainment protocols to avoid the risk of cross contamination and false positive results in test samples. Again, this may not be practical going forward as a reference material as there are currently no commercial suppliers. A third option is synthetic DNA that can be manufactured more readily to order and to specification.

Preliminary investigations were undertaken during this project of all three options. Genomic DNA was investigated in an initial Phase 1 trial but did not enable reliable quantification of oocyst numbers by qPCR. This was most likely due to the effect of variable losses during DNA extraction. Plasmid DNA was used to compare the dynamic data of the qPCR and demonstrated comparable results, but due to a lack of commercial options for purchasing *Cryptosporidium* and potential issues around biocontainment of high concentrations of pDNA, the use of synthetic DNA provides a safer and more practical approach. Therefore, synthetic DNA was explored further as reference material for generation of the calibration curve as described here.

A synthetic piece of DNA (oligonucleotide) was newly designed, based on a reference *C. parvum* SSU rDNA sequence (GenBank accession number L16996), the amplicon being the same size (435 bp) but differed in CG content (genomic DNA = 30.3 %, synthetic DNA = 47.8 %) from the reference sequence. This difference was engineered on the manufacturer's instructions into the synthetic strand, in regions which did not contain either primer or probe sequences, to optimise its synthesis. The sequence difference also served to allow discrimination between it and the genomic, reference sequence, should this be necessary (Appendix 8). The synthetic "*C. parvum*" DNA was supplied as a gBlock Gene Fragment, Integrated Gene Technologies, Belgium and is referred to in this report as "gBlock DNA". This calibration curve material was supplied, lyophilised, at very high concentration (7.6 $x10^{11}$ copies per tube). Thus it was suitable for creating a range of dilution series, as oocyst equivalents per PCR, according to the desired application. For example, for some experiments a five-point calibration curve was required, with concentrations representing the mid-range of expected Cq values for the qPCR; used when it was important that all points would be detected by the assay, and return a numerical Cq value. Other applications, such as determining key reaction dynamic data, or limit of detection, required an eight-point calibration curve, to include the vanishing point at which detection was

not successful. The number of points in each calibration curve is detailed for each of the experiments described here.

Preliminary experiments with the gBlock DNA established that it must be stored at no less than 10 ng / µl at -20 °C, and that the prepared calibration curve material should be ideally used on the day it is made up.

Evaluation of the effect of the addition of an internal amplification control to monitor PCR inhibition.

If qPCR is to be used for detection and enumeration of *Cryptosporidium*, surety needs to be provided that the amplification process has worked and was not inhibited by anything in the sample. An IAC can be added to the PCR mix to assess this; an IAC is usually provided as synthetic DNA (an oligonucleotide) with its own primers and probe that can be added to the qPCR; if the IAC is amplified to the expected Cq value then there is no inhibition. If the Cq value of the IAC in a test sample is >3 degrees higher than that in the non-template control tube is then there is inhibition and a negative result for the assay is not reliable. However, first it was desirable to check that incorporating the IAC DNA, its primers and probes was not detrimental to the UKWIR 2020 PCR.

A synthetic 200 bp oligonucleotide (Ultramer™, Integrated Gene Technologies, Belgium) was used as an IAC; this was incorporated into the UKWIR 2020 PCR, amplified and detected using specific primers and a 6-FAM labelled probe (Deer *et al*., 2010).

When investigated over a five-point, ten-fold dilution series of gBlock DNA standard, the reaction dynamics of the qPCR were similar with and without the IAC (Table 15).

Table 15. Results of testing the qPCR with and without the IAC

Reaction dynamics of the qPCR	with IAC	without IAC
PCR amplification efficiency (E)	0.85	0.81
Linearity (R^2)	1.00	0.99
Slope of the calibration curve (M)	-3.74	-3.89

Additionally, the effect of the concentration of the gBlock DNA standard template on the amplification of the IAC was investigated. A 10-fold dilution series of the gBlock DNA standard providing the equivalent of between 1 and 10000 oocysts per PCR was tested using 3 replicates at each concentration with the IAC incorporated in the qPCR. The Cq values of the IAC barely changed (range 20.06-20.55).

It was therefore concluded that the IAC did not affect the reaction dynamics of the qPCR and that the concentration of the target gBlock DNA standard template did not affect the amplification of the IAC.

The IAC was included in the qPCR, and the final qPCR set up is provided in Appendix 7.

Evaluation according to MIQE guidelines for qPCR.

The qPCR assay was evaluated according to MIQE guidelines, which state the essential and desirable attributes of qPCR assays that should be established for assays intended for use in laboratory settings (Bustin *et al*., 2009).

To establish the efficiency (E) and linearity (R^2) of the qPCR assay, an 8 point, 10-fold dilution series of gBlock DNA created a calibration curve that was run in triplicate. All three parameters were acceptable. Figure 6 shows the output from the Rotorgene, the creation of the linear calibration curve and report of PCR reaction dynamic data (E, R^2 and M), with interpretive notes.

qPCR quantification was expressed as oocyst numbers. This was calculated by the PCR platform (Rotorgene) and was determined by comparison of the quantification cycle number (Cq) obtained for each test DNA sample against the calibration curve. The input standard curve material was enumerated and assigned to the PCR platform as "oocysts per PCR" and therefore the outcome measurement for each DNA sample was oocysts per PCR. The calculation of this outcome measurement was influenced by the efficiency of the PCR assay which varies slightly from run to run, in this biological system (PCR), and hence a calibration curve was used with every run to normalise for the run to run variation. There was no cut-off applied to this assay and all samples with a Cq value were considered positive.

In a 100 % efficient assay, the mean difference in Cq values between 10-fold concentrations of DNA is theoretically 3.2 (Bustin *et al*., 2009). A mean difference of 2.29-3.77 Cq values was observed between each 10-fold dilution down to 10 oocyst equivalents per PCR, at which point the Cq values were less reliable with overlap between 1 and 0.1 (Table 16).

In qPCR the lower limits of quantification may be poorly defined and Cq values more variable (Bustin *et al*., 2009), and here the lowest concentration of DNA that appeared to be within the linear interval was somewhere between 1 and 10 oocysts per PCR (Table 16). The limit of quantification would likely lie between these amounts.

Table 16. Cq values for 8pt gBlock calibration curve, and dynamic data

No. "oocysts" equivalent of gBlock DNA / PCR	Mean Cq (n=3)	Cq range	Δ mean Cq
1000000	16.51	16.42-16.58	
100000	19.67	19.61-19.78	+3.16
10000	21.96	21.88-22.02	+2.29
1000	25.15	25.07-25.30	+3.29
100	28.92	28.79-29.03	+3.77
10	32.11	31.92-32.03	+3.19
1	36.32	35.01-37.10	+4.21
0.1	38.17	36.81-39.61	+1.85

R^2	0.99
E	1.05
M	-3.203

Further, at the lower limits of detection, not all replicates would be expected to be consistently positive. This was evidenced from multiple runs (n=21) each using an 8-point calibration curve where only 10/21 (48 %) positive results were observed at the lowest level of 0.1 oocyst equivalents per PCR, whereas 1 oocyst equivalent consistently provided positive results.

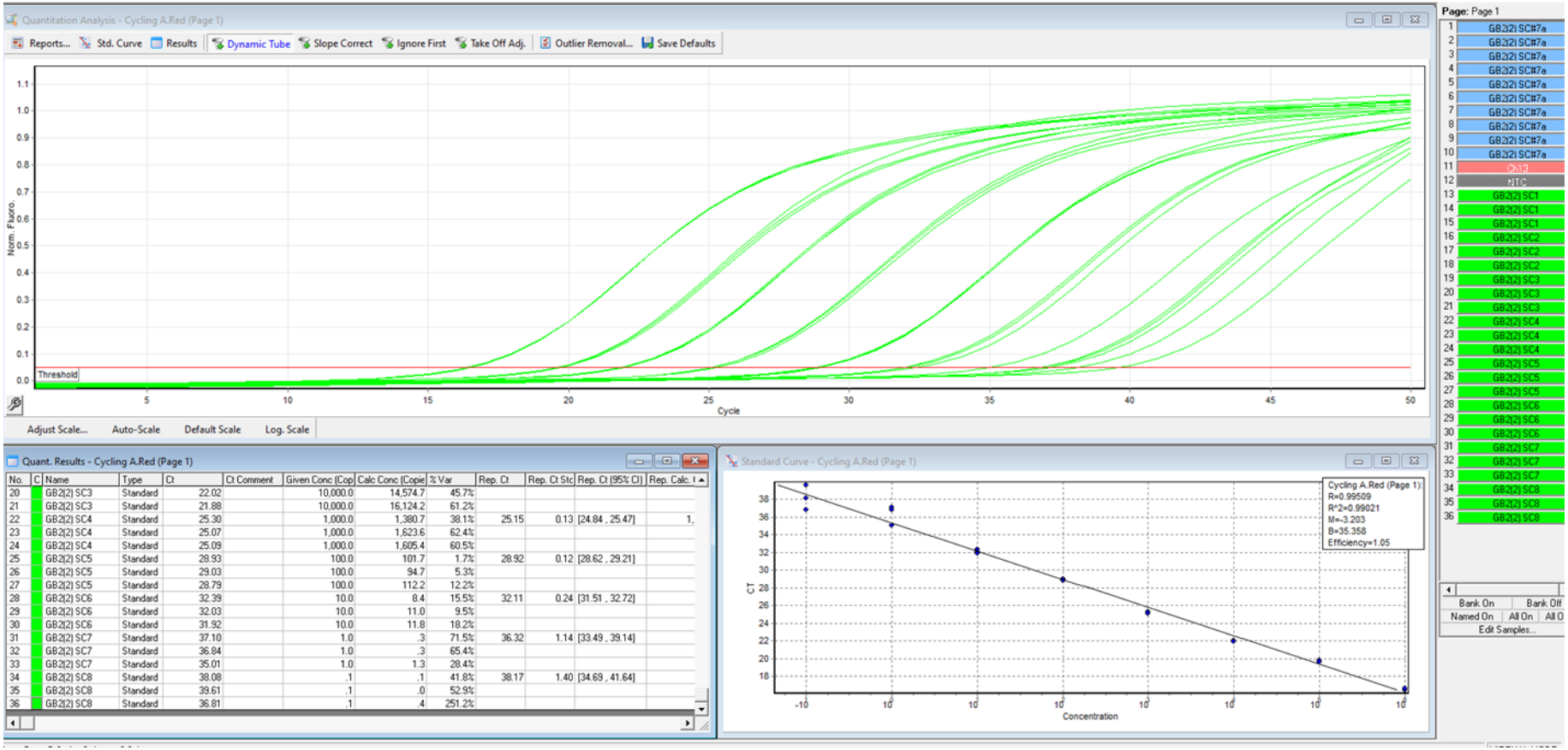

Figure 6. Output from the Rotorgene

The stacked green lines are amplification curves from triplicate samples from the calibration curve. It can be observed here that the triplicate curves to the right of the image are less tight than those to the left. This is visual confirmation that amplification at low concentrations of template is not as consistent as those at higher concentrations. The linear graph at the lower right hand side of the image is the calibration curve and the fit to the straight line (the linearity) of the calibration curve Cq values can be seen and this is confirmed in the data box within the linear graph which reports the R^2 value as 0.99.

LOD is usually defined as the lowest concentration at which 95% of the positive samples are detected, which requires multiple tests. In addition, each assay will have a theoretical LOD, as well as a demonstrable LOD. The theoretical LOD can be estimated as follows:

> If a single copy is the lowest genomic unit that can be detected, then this would need to be present in 20 µl of DNA (the PCR template input volume). If the DNA is a homogenous solution then the full 100 µl extracted from a sample would contain 5 copies of the gene. The SSU rRNA gene is present in 5 copies per sporozoite and there are up to 4 sporozoites in each oocyst (the organismal unit of interest here); thus an oocyst contains 20 gene copies. Therefore the theoretical PCR LOD is 1 copy and the theoretical sample LOD is 5 copies or 0.25 oocyst.

The demonstrable LOD of the assay was evaluated in two ways. A single experiment using the calibration curve gBlock DNA dilutions described in the efficiency and linearity experiment above, tested DNA in triplicate. All three replicates were positive when as few as 0.1 oocysts per PCR (2 copies of the gene) were tested. However, when data from multiple runs were analysed as mentioned previously, only 10/21 (48 %) positive results were observed at the lowest level (0.1 oocyst equivalents per PCR), contrasting with all replicates positive at 1 oocyst equivalent per PCR. Therefore, the demonstrable LOD was 1 oocyst per PCR (20 copies of the gene).

The measurement of uncertainty, based on the accuracy (difference between the actual number of oocysts and measured number reported by the assay) and precision (closeness of values indicated by low standard deviation), of the qPCR was investigated using Cq values from ten replicates of gBlock DNA equivalent to high (100) and low (10) numbers of oocyst equivalents (Table 17).

Table 17. Accuracy and precision assessment of the qPCR

No. "oocysts" equivalent of gBlock DNA / PCR	Mean (range) n=10	Standard deviation	R^2	E	M
100	114.40 (97-133)	9.24	1.00	0.96	-3.411
10	9.30 (6-12)	1.85	1.00	1.01	-3.306

The gBlock DNA from target equivalent numbers of oocysts put into the PCR was accurately enumerated by the assay. The standard deviations indicated that precision was also acceptable.

The robustness of the assay was evaluated by investigating the repeatability and reproducibility.

Repeatability was investigated by testing a single set of gBlock DNA dilutions by the same scientist on three separate occasions. Although there were too few replicates for statistical analysis, graphical representation indicated the closeness of the data points for each occasion (Figure 7).

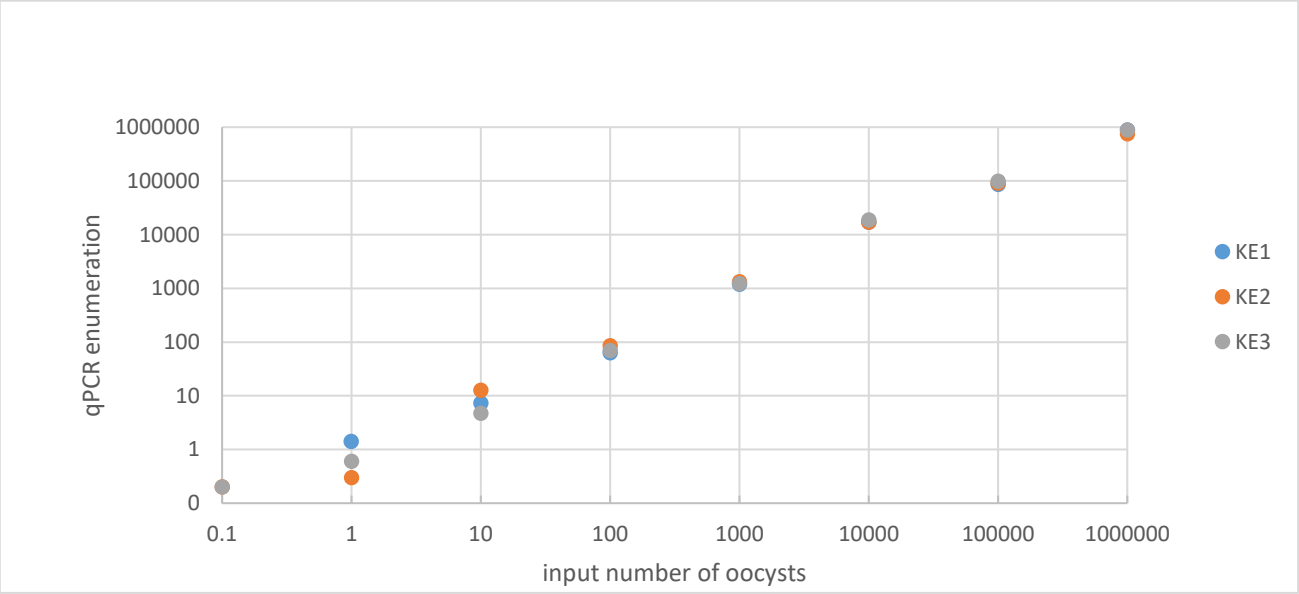

Figure 7. Repeatability of the qPCR

Reproducibility was investigated by a single set of gBlock DNA dilutions being tested three times each by two different scientists. Although there were too few replicates for statistical analysis, graphical representation indicates closeness of data points for each scientist (Figure 8).

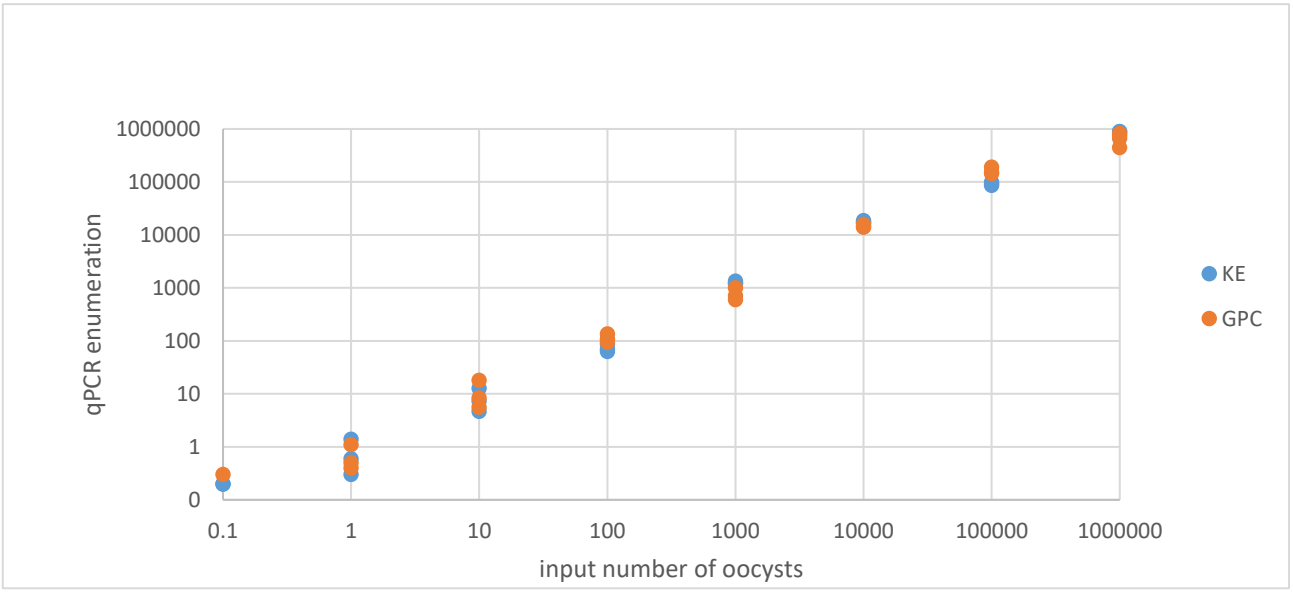

Figure 8. Reproducibility of the qPCR

Investigate whether testing samples prepared by immunomagnetic separation (IMS) was detrimental to the qPCR.

The standard methods for water testing for *Cryptosporidium* rely on high volume sampling, concentration of oocysts by centrifugation and retrieval by immunomagnetic separation (IMS) prior to detection by immunofluorescence microscopy (IFM). For microscopy, IMS provides improved detection as the oocysts are easier to see, having been removed from background debris. To allow for comparison between detection and enumeration by qPCR and IFM, samples processed by IMS would be required in a Phase 1 trial (see Objective 4). It was not known what effect the IMS process and the presence of IMS beads would have on the qPCR.

Additional investigations were therefore made ahead of Phase 1 verification (Objective 4) to investigate whether testing samples prepared by IMS, with the paramagnetic beads still in the sample when DNA was extracted, was detrimental to the qPCR detection and enumeration. Figure 9 shows potential points of losses during both processes.

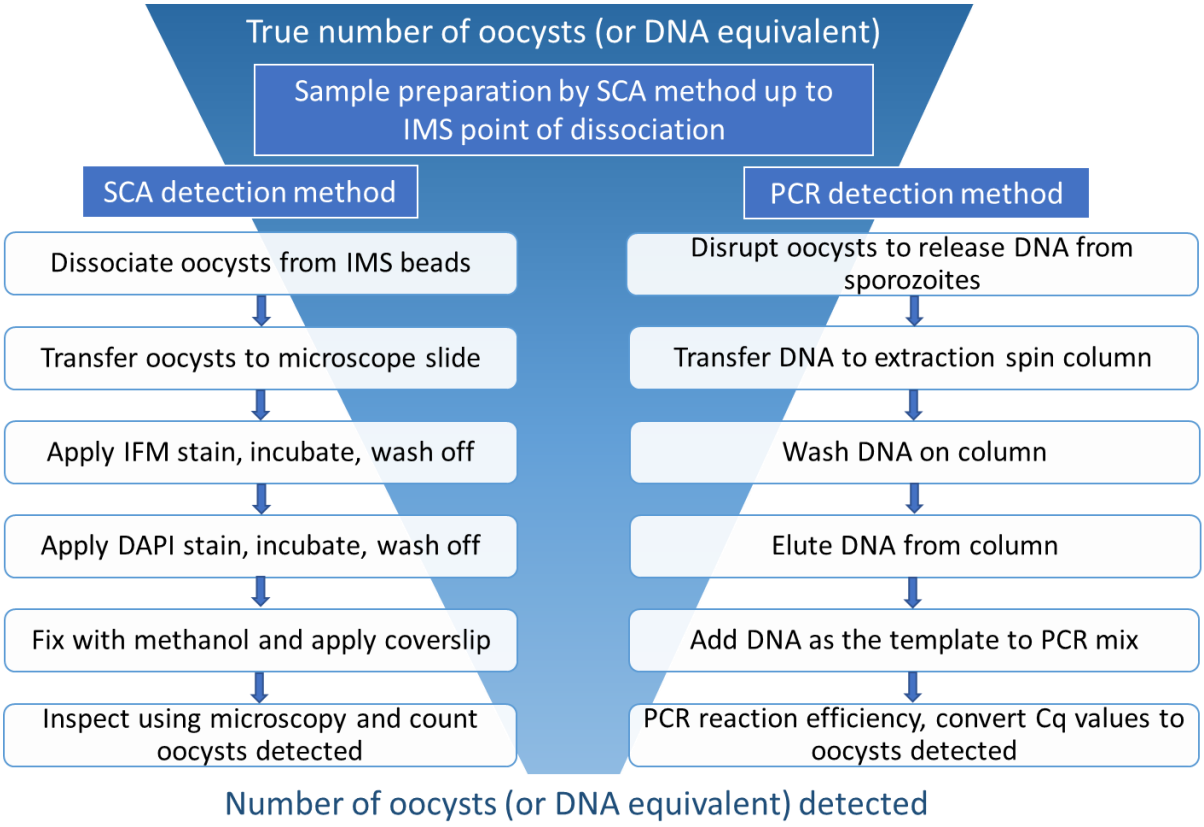

Figure 9. Potential points of losses when samples are processed by the current SCA standard method to the point of dissociation from the IMS beads and then either prepared for IFM or qPCR detection and enumeration.

Ten samples of 10 ml RO water were prepared and spiked with 100, and ten samples spiked with 10, flow cytometry sorted *C. parvum* oocysts (Wisconsin State Laboratory of Hygiene, Wisconsin) in duplicate by IMS (Isolate™, TCS Biosciences) for testing by qPCR and for verification of counts by IFM (CryptoCel, Cellabs). At these spiking levels, 100 oocysts would theoretically generate 20 oocysts per PCR and 10 oocysts 2 oocysts per PCR.

For IMS-qPCR, DNA was extracted directly from the IMS bead-oocyst complexes using oocyst disruption by eight liquid nitrogen freeze-thaw cycles and a standardised membrane spin column process (QIAamp DNA mini-kit, Qiagen). The optimal final elution volume was determined to be 100 µl (details in Appendix 9).

For qPCR alone (no IMS) the DNA from six samples of 100 or 10 flow-cytometry sorted oocysts in 200 µl of RO water with 0.01% Tween 20 was extracted directly without any IMS processing.

Four 20 µl aliquots of DNA were tested per sample using the qPCR protocol and a 5-point calibration curve (Appendix 7). The total number of oocysts detected in the sample by qPCR was calculated from the four aliquots of DNA tested.

The positivity rates for qPCR, IMS-qPCR and IMS-IFM were recorded. The mean number of oocysts detected by qPCR alone were compared with the mean number detected by IMS-qPCR using the t-test.

The verification of IMS by IFM showed that when spiked with 100 oocysts, all ten samples were positive with a mean of 52.40 (SD ± 20.33) oocysts detected (range 12-72). When spiked with 10 oocysts, all ten samples were positive with a mean of 4.50 (SD ± 2.76) oocysts detected (range 2-9).

At each spiking level, by qPCR 6/6 samples were positive and by IMS-qPCR 9/10 samples were positive (Table 18). The mean number of 100 spiked oocysts detected by qPCR was 24.52 which was not significantly different from the mean number of 25.43 detected by IMS-qPCR (p=0.90) (Table 18). The mean number of 10 spiked oocysts detected by qPCR was 5.5 which was not significantly different from the mean number of 3.6 detected by IMS-qPCR (p=0.20) (Table 18), indicating that the qPCR was not adversely affected by the IMS process or presence of the IMS beads.

Table 18. Comparison of oocyst enumeration with qPCR, IMS-qPCR with IMS-IFM

Oocysts spiked / sample	Oocysts detected	qPCR	IMS-qPCR	T-test
100	Mean (SD)	24.5 (± 12.8)	25.4 (± 13.7)	df=1
	Range	0.5-36.4	9.5 – 51.5	p=0.90
	Sample positivity:	6 / 6	9 / 10	
10	Mean (SD)	5.5 (± 2.8)	3.6 (± 2.7)	df=1
	Range	1.1-7.9	0.5 – 9.6	p=0.20
	Sample positivity:	6 / 6	9 /10	

Discussion

In this objective a qPCR method for incorporation in a detection and enumeration assay for *Cryptosporidium* was evaluated. Experimental data showed that synthetic DNA provided a more robust reference material for the calibration curve than genomic DNA due to the losses incurred during DNA extraction from oocysts. The use of plasmid DNA is an alternative approach that removes the DNA extraction losses, but is costly and few laboratories are able to produce plasmids. There are currently no commercial suppliers of *Cryptosporidium* plasmid DNA as verified reference material. The use of synthetic oligonucleotides for calibration curves has been shown in clinical virology to give equivalent qPCR results compared to using either pDNA or quantified DNA from infected cells

(Tourinho *et al*, 2015; Bandeira *et al*., 2020). When using synthetic DNA it was important to ensure the GC content was sufficiently high for the preparation to pass the quality control procedures of the supplier. Increasing the GC content also served another function as the edits to the DNA sequence could then be used to ensure no cross contamination occurred between the calibration curve material, which is often in high concentration, and the test samples. Thus, the calibration curve DNA would be detected if amplicons were confirmed by sequencing for the purpose of genotyping. The storage conditions of the gBlock material were defined (-20°C, >=10 ng/µl, for up to one year) and need to be adhered to prevent degradation of the synthetic DNA.

A calibration curve needs to be used in each and every run as the qPCR efficiency (E) varied slightly in every run, which had a significant effect on quantification. By taking this approach the effect on quantification can be reduced by accounting for run-to-run variation.

The addition of the IAC to the PCR provided an indication if PCR inhibitors were present, and assurance that PCR detection was working and, if used for drinking water monitoring, that non-detections are not false negative results (Hoorfar *et al*., 2003).

The qPCR was evaluated using MIQE guidelines demonstrating that the accuracy and precision were acceptable, and when DNA equivalent to 10 or more oocysts per PCR were present differentiation between 10-fold differences could be detected. Below this level the variation in oocyst enumeration provided less confidence in the results. As the aim was to translate the quantified result from oocysts per PCR into the number of oocysts present in a sample by using the counts from each replicate and adjusting for the whole sample, inaccuracy here will introduce further errors in the per sample count. However, this will be explored further in Objective 4 and 5.

When calculating the LOD of the qPCR it was important to have sufficient data points to accurately establish if 95% of the samples were positive. Where a single experiment used the calibration curve in triplicate and all three were positive at the lowest level (0.1 oocysts per PCR) it would be tempting to suggest this as the limit of detection. Not only is this misleading due to the paucity of data but also more is gained by gathering the data from separate runs so that inter-run variability is also accounted for. Therefore, the LOD of the qPCR was determined to be 1 oocyst per PCR from 21 separate runs, which translates into 5 oocysts per sample. However, this is for the PCR only, and does not take into account any losses during the pre-PCR sample processing. Objectives 4 and 5 provides more information on this, where 10 L water samples were spiked and processed, and the results compared with the current reference method.

The LOQ is difficult to establish for qPCR due to the exponential nature of the reaction (Bustin *et al*., 2009), and particularly when quantification may be greatly affected by upstream processing of samples such as tap water. Although LOQ might be best explored when the qPCR is incorporated into the final method to provide the LOQ of the assay as a whole, inherently variable recovery rates from water samples would contribute to the uncertainty of measurement.

The oocyst enumeration using 10 ml water samples with *C. parvum* oocysts was a first toe in the water to see how the qPCR performed as an actual water testing assay with and without IMS. There was no significant difference in this purified sample matrix, confirming that extraction of DNA from the post-IMS pellet would not be detrimental to the PCR.

Conclusions

It has been demonstrated that the qPCR described here is suitable for exploration of enumeration of *Cryptosporidium* DNA extracted from oocysts. The qPCR is:

- Linear
- Reproducible
- Sensitive to 1 oocyst per PCR
- Specific
- Credible

In addition, the method:

- Requires a calibration curve each time, to reduce run-to-run variability in quantification
- Is subject to sample preparation, including the recovery of oocysts from the sample by IMS (if used) and extraction efficiency of the genomic DNA from oocysts in the sample, which are both variable and unpredictable.

While the qPCR was developed and evaluated in Objective 3, its performance when used as an assay for testing drinking water samples was explored in Objectives 4 and 5 and compared with the current standard method.

Objectives 4 and 5 – Method evaluation

Duplicate analysis of water samples spiked with *Cryptosporidium* oocysts, tested using the current Blue Book method for *Cryptosporidium* analysis alongside qPCR, and collation and review of the results of the spiked sample analysis, to determine the benefits of the use of qPCR against the current Blue Book method.

Objectives

- A Phase 1 study, generating data from both the trial qPCR method and the reference IFM method for the recovery and enumeration of *Cryptosporidium* oocysts spiked into tap water in a single laboratory trial.
- Quantitative and qualitative analysis of the trial data
- Evaluation of the qPCR method for ease of use.

Method

The model provided by Appendix 4 of the Blue Book (Anon, 2010) was used to establish the number of samples and statistical analyses for comparison of qPCR (the test method) with IFM (the reference method), with the null hypothesis that there was no difference in the detection or enumeration between the two methods.

Tap water was used for this Phase 1 study, sampled at one of the Dŵr Cymru Welsh Water (DCWW) treatment works, and 10 L samples were spiked with 0, 10 and 100 *C. parvum* oocysts, sorted and enumerated by flow cytometry (Wisconsin State Laboratory of Hygiene). Quality control data for the oocyst suspensions from the provider, calculated from a minimum of 12 calibration verification samples per set of 10 standards, reported the mean as 100, SD 1.3 for the 100 oocyst suspensions and a mean of 10, SD 0.0 for the 10 oocyst suspensions. Viability was 99.6% and oocysts were used within three months of preparation.

The protocol used for the Phase 1 trial is provided in Appendix 10. Spiking was done in pairs of water samples prior to processing by IMS; processing was according to the Blue Book method at the DCWW laboratory in Glaslyn, Newport. One sample of each pair was then tested by IFM according to the Blue Book method at Glaslyn, and one sample (the concentrate from IMS) was frozen for further testing by the developed qPCR method at the CRU in Swansea as described in Appendix 7.

The set-up of the water sample batches for spiking and processing is shown in Figure 10 below. Each batch comprised three pairs of samples; zero spike control, spiked 10 and spiked 100 oocysts sample for each of the two detection methods.

The Phase 1 trial was undertaken twice to allow for investigation of two different sources of calibration curve material (gBlock DNA and genomic DNA) that would enable quantification of oocyst numbers by qPCR. The gBlock DNA provided the more suitable and sustainable reference material for calibration curve generation and was used to generate the data presented here.

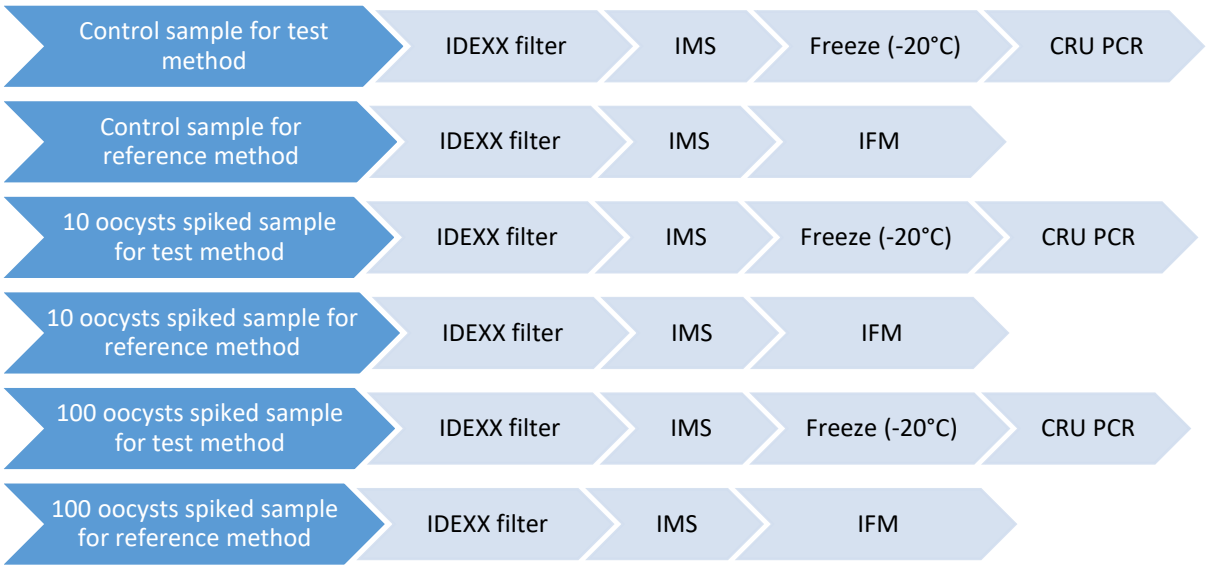

Figure 10. Illustration of the batch setup of samples for the Phase 1 study, to compare the qPCR test method and the IFM reference method.

For qPCR, all of the sample concentrate after IFM was used to extract DNA and then 4 aliquots of 20 µl DNA was tested by qPCR from each sample. The gBlock calibration curve material was run in all qPCR runs as a 5 data point, 10-fold dilution series representing DNA from 1000 to 0.1 oocysts per PCR. To provide equivalence with IFM where the entire final pellet is spotted on to a microscope slide, the final qPCR "count" was the sum of the number of oocysts detected in each aliquot adjusted for the whole DNA extract.

The range, mean, median and standard deviation (SD) of the oocyst counts by IFM and qPCR, the differences between them, and the recovery rates were calculated for each level of spiking and the counts plotted on a graph to illustrate qPCR counts vs IFM counts. The variances in the results were explored to decide which statistical test (parametric or non-parametric) to use to test the null hypothesis that there was no difference in the average counts between the methods. Statistical comparison of counts was undertaken using EpiInfo v6 (CDC) and confidence intervals for the differences using CIA software.

To assess the potential advantages or disadvantages of using qPCR for *Cryptosporidium* enumeration, multiple attributes were assessed through this practical application of the method, including:

- Risk / mitigation of cross contamination
- Mitigation against PCR inhibition
- Sample throughput
- Potential for automation
- Hands on time
- Time to result
- Ease of interpretation of results.

Results

The results for the Phase 1 study are shown below. Ideally, at least 15 samples would be tested by both qPCR and 15 by IFM spiked at each level of 0, 10 and 100 oocysts, and compared. However, due to an unforeseen failure of a Rotorgene qPCR machine used to test four of the batches, only 11 data points were available from qPCR at each spiked level. Therefore, the only the data from the paired spiked sample tested by IFM were used in the comparison of results.

The number of samples positive for *C. parvum* was the same whether tested by qPCR or IFM when spiked with 10 or 100 oocysts, with all 11 samples positive at each level (Table 19). No false positives were detected in the zero spike samples by either method.

Table 19. Summary of counts from the comparison of the trial qPCR method with the reference IFM method

Batch sample	Number of spiked oocysts	Count qPCR	Count IFM	Difference (qPCR-IFM)	Recovery (%) qPCR	IFM
1	10	0.25	6	-5.75	3	60
2	10	0.13	3	-2.88	1	30
3	10	0.75	3	-2.25	8	30
4	10	0.13	5	-4.88	1	50
7	10	3.63	5	-1.38	36	50
8	10	1.88	1	0.88	19	10
11	10	1.50	2	-0.50	15	20
12	10	11.75	2	9.75	118	20
13	10	2.01	5	-2.99	20	50
14	10	1.08	3	-1.93	11	30
15	10	1.38	6	-4.63	14	60
Positivity	10	11/11	11/11	Mean	22.36	37.27
				Median	14	30
1	100	0.50	55	-54.50	1	55
2	100	1.00	45	-44.00	1	45
3	100	29.38	55	-25.63	29	55
4	100	12.75	56	-43.25	13	56
7	100	78.25	29	49.25	78	29
8	100	8.38	38	-29.63	8	38
11	100	102.38	12	90.38	102	12
12	100	14.75	23	-8.25	15	23
13	100	30.41	32	-1.59	30	32
14	100	45.73	51	-5.28	46	51
15	100	39.00	50	-11.00	39	50
Positivity	100	11/11	11/11	Mean	32.91	40.54
				Median	29	45

Although the Blue Book does not state acceptable recovery rates, typical recoveries shown in Appendix 3 of the Blue Book range from 5-97 % in tap or final water. USEPA Method 1623 (USEPA, 2012) which is similar to that in the Blue Book states mean rates of 32-100 % are acceptable from matrix spiked samples for method modifications. The mean recovery rates for IFM detection fell within these ranges at both spiking levels, but the qPCR detection was only within the acceptance criteria at the 100 spiking level (Table 19).

The data were also plotted in Figure 11. This shows the plots of the oocyst counts at 10 and 100 spiked oocysts tested by the two methods and includes the line of equality. At both 10 and 100 oocysts, two points lay above the line of equality where the trial qPCR method gave higher counts and eight points lay below the line where the reference IFM method gave higher counts.

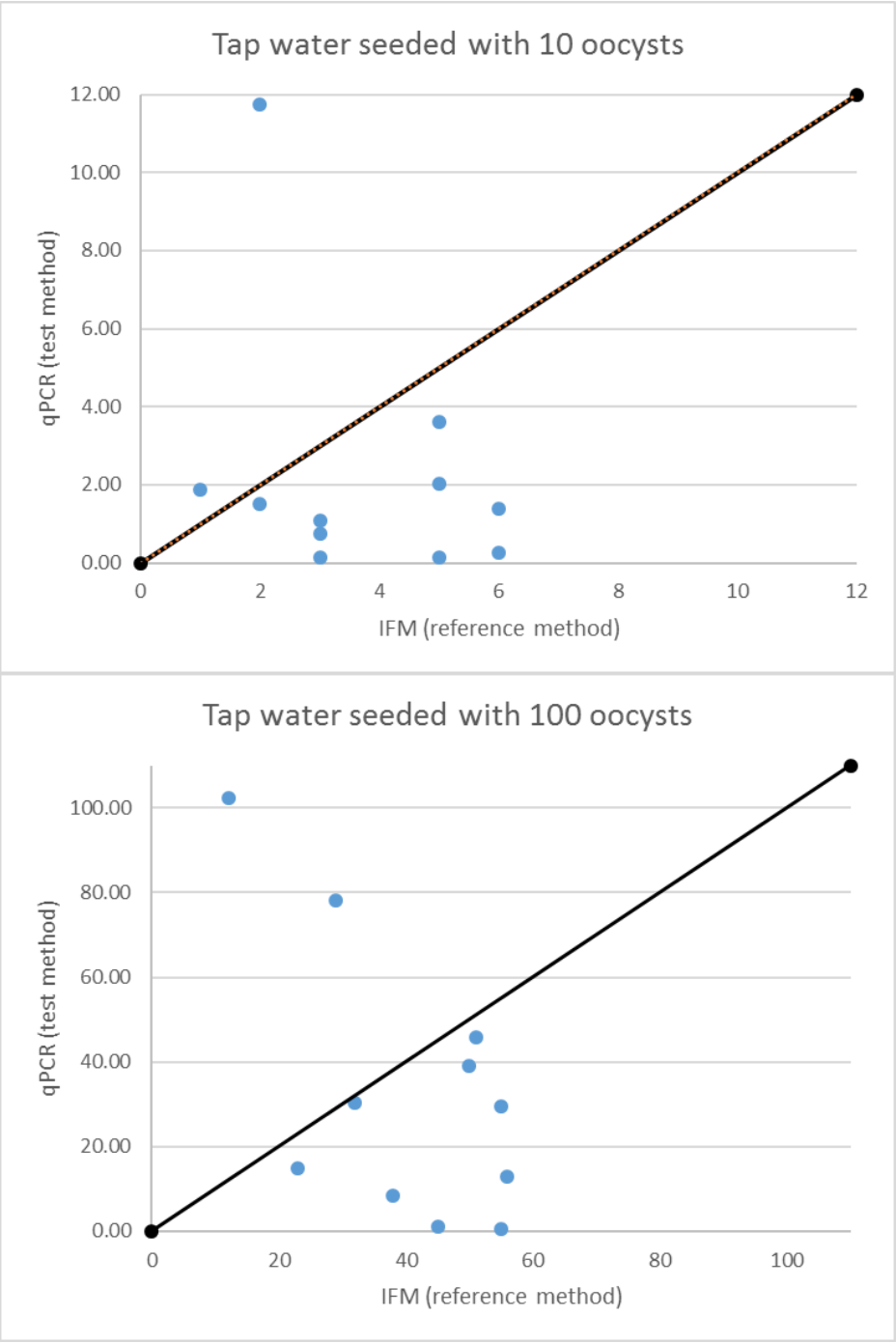

Figure 11. Oocysts detected by qPCR and IFM during the phase 1 study (line is line of equality). The data plotted here are the individual counts. Plots of the percentage recoveries show a similar picture (not shown).

As the data do not appear to be normally distributed, and the variances in the counts differed, a non-parametric test was used to test the null hypothesis that there was no difference in the median counts between the methods (Table 20). For samples spiked with 10 oocysts, the median number of oocysts

detected by qPCR was significantly fewer (1.4, range 0.18-11.8) than detected by the reference method (3, range 1-6) (Mann-Whitney Two-Sample Test = 5.780, p value = 0.016) (Table 20). For samples spiked with 100 oocysts, the median number detected by qPCR was not significantly different (29.4, range 0.5-102.4) than detected by the reference method (45, range 12-56) (Mann-Whitney Two-Sample Test = 1.641, p value = 0.200) (Table 20). The standard deviation was greater by qPCR than the reference test at both spiking levels (Table 20).

Table 20. Summary of the statistical analysis of all the data from the Phase 1 study; comparison of the trial qPCR method with the reference IFM method.

Number of spiked oocysts	Tap water 10 L samples 11 duplicates	Oocysts per sample				
		Trial method qPCR*	Reference method IFM			
0 (Control)	Range	0-0	0-0			
	Mean	0	0			
	Median	0	0			
				Difference in counts	Recovery % qPCR	Recovery % IFM
10 (Spiked)	Range	0.1 to 11.8	1 to 6	-5.8 to 9.8	1 to 118	10 to 60
	Mean	2.2	4	-1.5	22	37
	Median	1.4	3	-2.3	14	30
	SD	3.3	1.74	4.0	32.0	17.37
100 (Spiked)	Range	0.5-102.4	12-56	-54.5 – 90.4	0.5-102	12-56
	Mean	33.0	41	-7.6	33	41
	Median	29.4	45	-11.0	29	45
	SD	32.4	14.87	41.0	31.0	14.89

*Calculated numbers have not been rounded to whole oocysts as qPCR can detect DNA from individual sporozoites or free DNA in the sample

When spiked with 10 oocysts, the Wilcoxon 95 % confidence interval for the difference between medians of the two tests was -3.87 to 2.00. Where the reference method gave a median of 3 oocysts, this suggests an estimated worse-case scenario of the trial qPCR method, on average, finding 29 % of those detected by the reference IFM method (*i.e.* 71 % fewer) and the best-case scenario of 167 % (*i.e.* 67 % more), calculated by (3-3.87)/3 and (3+2)/3 respectively, expressed as percentages.

When spiked with 100 oocysts, the Wilcoxon 95 % confidence interval for the difference between medians of the two tests was -34.81 to 23.56. Where the reference method gave a median of 45 oocysts, this suggests an estimated worse-case scenario of the trial qPCR method, on average, finding 23 % as many oocysts as the reference IFM method (*i.e.* 77 % fewer) and the best-case scenario of 152 % as many oocysts (i.e. 52 % more), calculated by (45-34.81)/45 and (45+23.56)/45, expressed as percentages.

Discussion

The statistical comparisons showed that the oocyst counts by qPCR were significantly lower than by IFM when the spike was 10 oocysts, but there was no significant difference when the spike was 100 oocysts. However, the lower value of the 95 % confidence interval for the difference between the medians of the two tests implied that the qPCR could be very much worse than the reference method.

More samples would need to be examined to increase the power of the study and therefore reduce the confidence interval and clarify the worst-case scenario.

At a spike of 10 oocysts per sample, the theoretical amount of DNA per individual PCR (2 oocysts per PCR) approached the limit of detection of 1 oocyst per PCR (see Objective 3), and was in the region of the calibration curve where there was less linearity and certainty of enumeration. Additional variation in enumeration in the qPCR contributed to the difference between the qPCR and the reference test (IFM).

There are few reports of others truly investigating the quantification of low numbers of oocysts in water samples by qPCR. Staggs et al. (2013) described the evaluation of ten different qPCR methods and spiked water concentrates with various numbers of oocysts. Spiking the concentrate ensured they did not get the losses seen with IMS, but despite this they still showed variability and concluded that a qPCR cannot accurately measure the low numbers of oocysts that are typically found (Staggs et al., 2013). It may be that different and/or optimised DNA extraction processes would provide improvement. Jiang *et al*. (2005) showed how variable different DNA extraction processes can be on the outcome of PCR detection by testing wastewater and storm water samples, as well as filtered storm water pellets spiked with low numbers of flow-cytometry counted oocysts. The best amplification rates were obtained by the same IMS and QIAamp DNA mini kit extraction kit used in this project.

IMS is used in the standard method to reduce the debris on the slide to make observation of oocysts by microscopy easier. It is possible that IMS is an unnecessary step for PCR detection if inhibition can be overcome, as it may incur losses to the detriment of enumeration by qPCR. There have been publications describing IMS-free PCR methods for water samples, some with promising results (Jiang *et al*., 2005; Hill *et al*., 2015). DNA extraction based on bead-beating seems to be most appropriate (Jiang et al., 2005). There is also emerging evidence from testing food samples (mostly leafy greens) that IMS-free preparations are suitable for PCR testing (Marco Lalle, ISS, Rome, personal communication).

Using qPCR for *Cryptosporidium* enumeration provided mostly practical advantages over IFM. Users can be reassured that the potential for cross contamination was mitigated by the closed platform format of the qPCR. The potential for PCR inhibition was mitigated by the components of the DNA extraction process and PCR mix, and monitored by inclusion of an internal amplification control. In our laboratory, sample throughput for DNA extraction was 30 per day and has the potential for automation; for PCR up to 16 samples (with 4 aliquots per sample) can be run simultaneously per PCR instrument and is an already automated process, providing potential for greater throughput than IFM which is highly labour intensive and limited by microscopy time. This would be beneficial in water testing laboratories who reported processing on average 111 tests per week (range 30-240) (Objective 2). Hands on time is mostly for DNA extraction (six hours for 30 extractions) and PCR set up (approximately one hour per batch). Time to result from IMS pellet for 16 samples was 7 hours. Ease of interpretation of results is an advantage of the PCR approach, since the PCR amplification method and interpretation can both be easily written into a standard operating procedure to be followed by technician level staff. An example is provided in Appendix 6 showing how the method could be presented in the Blue Book that describes the current standard method for detection of *Cryptosporidium* from water samples in the UK.

However, it should be noted that just as IFM detects both live and dead oocysts, qPCR will detect DNA from viable and non-viable organisms. Additional steps are required where the current standard for detection or enumeration is of viable organisms. Culture-independent options used in academic studies include the use of PMA-PCR but this has not been included in any standard methods using PCR.

For *Cryptosporidium* the current standard is oocyst detection and enumeration; viability or infectivity are not currently measured so this is not an issue for equivalence with current methods.

Conclusion from the Phase 1 study

The statistical comparisons showed significantly lower counts were obtained by qPCR than IFM when the spike was 10 oocysts, but not when it was 100 oocysts.

Modification of the sample preparation processes are recommended to try and reduce variability; different oocyst disruption and DNA extraction processes could be explored. IMS-free preparations should be explored for testing water samples by PCR. Other PCR-based methods may provide more reliable enumeration of low numbers of oocysts than qPCR.

Further method development and Phase 1 trials, including different water types, and investigation of the performance of the whole assay with other *Cryptosporidium* species, are required before proceeding to Phase 2 trials.

The qPCR-based method has been formatted as required by the Standing Committee of Analysts (Anon, 2017). To aid understanding qPCR in the context of current *Cryptosporidium* testing, this has been done using the Blue Book template for illustrative purposes (Appendix 6).

Objective 6 – Recommendations and future work

Detail recommendations for the industry for the use of PCR in routine analysis of *Cryptosporidium*, and recommendations for any future areas of research that may be required from the results of this study.

Objectives

- Use Objectives 1-5 to determine the evidence for the general use of PCR in routine analysis of *Cryptosporidium* in the water industry.
- Provide the data from all Objectives for external expert consultation by public health professionals, the *Cryptosporidium* community and the water industry to review the evidence presented in the report.

Methods

The findings of Objectives 1-5 were assessed to provide recommendations for industry and identify future areas of research. Expert view was also sought from three leading scientists with different backgrounds (Public Health, *Cryptosporidium* in the water industry and a molecular biologist), who were approached to provide their opinion and review the project report. One of these experts provided a review, one declined to review the project and the other did not respond.

Results

***Summary of findings from Objectives 1-5 in relation to key questions with regard to using PCR for the analysis and enumeration of* Cryptosporidium oocysts in drinking water**

What PCR-based technologies and methods are available?

In Objective 1 the authors undertook two literature reviews, the first of which explored the general use of PCR in the water industry. It was clear that PCR within the water industry is still an emerging technology, and predominantly used for bacteria and viruses. Few reports involved the participation of water utilities or laboratories. Standard Methods were only found for *Legionella* in the UK and *Enterococci*, Enterovirus and Norovirus as well as characterisation of human faecal pollution in the US. As the key requirement for the PCR-based method in this project was enumeration, not all PCR technologies are appropriate with only quantitative (qPCR) and digital PCR (dPCR) providing this potential. The use of qPCR was much more common than dPCR, including all relevant standard methods, likely due to the wider availability and much lower cost of the required equipment.

What are the PCR-based options for unambiguous, reliable and robust detection and enumeration of Cryptosporidium?

The second literature review in Objective 1 was to identify PCR methods that have been or could be applied for the quantification of *Cryptosporidium* oocysts in water. Unlike detecting *Cryptosporidium*

from host animals where host-specificity can limit the expected species, water samples have the potential to be contaminated with any *Cryptosporidium* species or genotype and therefore it is essential that any PCR-based method must be pan-genus. Additionally, while enumeration was the focus of this project, the subsequent characterisation of any detected *Cryptosporidium* would be required, meaning that any method must also be suited to characterisation at least to species level. Of all the papers identified in the literature review only three described assays that met the required criteria, all of which were based on the small subunit (ssu) rRNA gene (Agulló-Barceló *et al*., 2014; Di Giovanni *et al*., 2014; UKWIR, 2020). Currently this gene is considered as the major target for species identification, and has generated a huge amount of historical data from all known species and genotypes as well as many unknown genotypes identified from environmental samples. Of the three papers describing ssu rRNA gene assays, one targeted a region of ~830 bp that was found to have previously struggled on a qPCR platform due to its length (Agulló-Barceló *et al*., 2014). However, as conditions, reagents and platforms can have an impact on the dynamics of PCR it is possible that this assay could be promising if it performs as described by the authors. The other two publications targeted a shorter region of the ssu rRNA gene one using high resolution melt analysis and the other employing a TaqMan probe (Di Giovanni *et al*., 2014; UKWIR, 2020). This target paired with the TaqMan probe has previously been shown to be a sensitive and specific assay for the detection *Cryptosporidium* in water samples in our own laboratory.

What is the current and predicted laboratory capability and capacity for employing PCR-based methods?

Objective 2 included consultation with the water industry through a couple of online questionnaires to gain an understanding of the current and future use and perceptions of PCR-methods. It was clear that currently the use of PCR-based methods in water testing were very low. Four of the drinking water-testing laboratories stated that they use PCR in water testing. One was currently using it for *Legionella* and were also exploring the use of PCR for COVID-19 analysis and another for occasional identification of bacterial species, but not on their site. Two stated that they use PCR to identify *Cryptosporidium* species and genotypes present, but neither used quantitative technology for these assays. Only one water-testing lab indicated that they had real-time PCR instruments that could be used for quantitation. The majority of laboratories that don't use PCR-based methods stated that this was because of a lack of facilities, no standard method and it is something that is not needed at the moment. None of the water-testing laboratories or water supply companies that don't currently use PCR plan to introduce PCR testing within the next 12 months and only three within 5 years. The main barriers were listed as cost, the need for new equipment, staff training and changes in their accreditation requirements.

The DWI have previously funded research on PCR in a project using qPCR to enumerate Adenovirus and Norovirus in water sample concentrates (Merrett *et al*., 2013). They reported that the methods were considered reliable and robust and could be transferred for routine use in water utility laboratories. However, this adoption by the water industry is yet to happen.

Can a PCR-based method be developed to detect and enumerate Cryptosporidium in the IMS preparation?

Objective 3 evaluated the UKWIR18S PCR developed for genotyping as a qPCR to detect and enumerate *Cryptosporidium* oocysts. The qPCR showed acceptable efficiency (1.05) and linearity

(0.99), although this varies from run to run thereby requiring the presence of a calibration curve on every run. The practical limit of detection for the assay was 1 oocysts per PCR, which equated to 5 oocysts per sample. The variability in oocyst quantification was also greater down in the range of 10 oocysts per sample. The variability seen in qPCR means that the enumeration of oocysts within a sample is not absolute and but will provide an approximate value of the quantity of oocysts present. dPCR has been used to provide absolute quantitation of organisms in a sample, but the technology is less accessible, much more expensive and currently it would not be possible to characterise any positives to identify the species or genotypes present by DNA sequencing as the amplicons are not generally available.

This project also tested the effect of the IMS preparation. Losses were seen by both the IMS-PCR and IMS-IFM methods which is to be expected. With the IMS-PCR approach losses can occur at the IMS and DNA extraction stages, whereas with IMS-IFM they can occur during the IMS, staining, rinsing and coverslip-mounting stages. One approach that should be investigated further is whether IMS is actually required when using molecular detection. When examining a sample by microscopy, IMS is vital to reduce the background debris and enable visualisation of oocysts, but this is not necessary with molecular methods as the PCR primers specifically target *Cryptosporidium* DNA in the sample. By leaving out the IMS there is one less stage for losses and the DNA from other organisms in the sample can also act as a carrier and increase the yield of target DNA. Although there are different considerations, such as the inhibitory substances present, emerging evidence suggests that IMS-free processing from complex samples such as food testing leafy greens may work well for PCR testing.

What are the effects on the PCR efficiency of including an Internal Amplification Control (IAC), which shares and therefore competes with the target template for some of the reagents?

Objective 3 also examined the effect of adding an IAC to the assay. This did not affect the dynamics of the qPCR and furthermore the concentration of the gBlock DNA use in the calibration curves did not affect the amplification of the IAC.

What is the surety of a negative result?

In order to assess the surety of a negative result the whole process must be considered, not just the qPCR on highly purified oocysts, but including sample preparation, IMS recovery, DNA extraction and qPCR. Objectives 4 and 5 trialled the qPCR method in spiked tap water against the current reference IFM method. These results demonstrated that all samples were positive by both methods when spiked with 100 and 10 oocysts, indicating at these levels there were no false negatives. However, the qPCR counts and recoveries were highly variable reaching as low as 0.13 oocysts (recovery of 1%) for the 10 oocyst spiking level, so further exploration at numbers below 10 is needed.

Can a PCR-based method be used to reliably detect and enumerate Cryptosporidium in the IMS preparation?

The authors have demonstrated the reliable detection of 10 oocysts from tap water in Objectives 4 and 5. However, enumeration was less reliable and highly variable with qPCR than with IFM.

How do PCR-based methods compare to IFM for the detection and enumeration of low (10) and high (100) numbers of oocysts spiked into tap water samples?

Objective 5 showed that with low numbers of oocysts, the enumeration results were significantly lower and more variable by qPCR than by IFM. While the counts were also lower when high numbers of oocysts were spiked, this difference was not statistically significant.

Is there a difference in detection and enumeration and ease of interpretation when raw waters from different sites are tested?

Following agreement with the funder, this was not done during this project, but will need to be explored in future work before the industry would consider implementing PCR-based methods as a replacement for the current method.

How much automation could be employed to streamline the process?

There are a number of opportunities to streamline the process when using molecular methods including automation. At the front end of the process the possibility of an IMS-free method can be explored, e.g. DNA extraction from the centrifuged pellet, or from micro-fluidic devices. Automated DNA extraction is possible and is frequently used in clinical laboratories. The qPCR developed in this project can readily be set-up using robotics and is something that the Cryptosporidium Reference Unit do with all high throughput PCRs to improve standardisation. Running the qPCR is already an automated process and the analysis of the resulting data can also be automated.

What quality standards are required for PCR and what is their availability?

Calibration curve material is required for qPCR to enumerate oocysts in a sample. Objective 3 explored the use of genomic DNA and also a synthetic piece of DNA (gBlock) that the Cryptosporidium Reference Unit designed based on a reference *C. parvum* ssu rRNA gene sequence. To obtain genomic DNA for standard curves, the use of accurately counted (e.g. flow-cytometry sorted) *Cryptosporidium* oocysts is required, however the yield from DNA extraction, particularly in the absence of other background DNA, was found to be variable and not enable reliable quantification. Additionally, obtaining flow-cytometry sorted material routinely would be costly and not practical. The gBlock synthetic DNA standard has the benefit of being ordered as required at a reasonable price, is provided as a quantified suspension not requiring extraction and can be differentiated from *Cryptosporidium* DNA, due to the engineered sequence differences, allowing contamination events to be detected.

In addition to accurately quantified material for calibration curve production, other quality control materials are also need. For example, flow-cytometry counted oocysts are required for spiking experiments during method validation and performance monitoring of the both the method or analyst. These can be obtained commercially, but are costly and tend to be restricted to *C. parvum* only. To monitor performance of the qPCR itself, know *Cryptosporidium* positive DNA needs to be obtained. This could be self-extracted from purchased oocysts or obtained from reference unit collections, which may have DNA from additional species for testing.

What needs to be done to establish and validate PCR as a standard method?

This proof-of-principle study has shown that it is possible to detect reliably the presence of *Cryptosporidium* oocysts in tap water samples. However, for the method to be established and validated as a standard method the enumeration of oocysts needs to be first improved.

There is potential at a number of stages:

Sample preparation – IMS or no IMS? As described above, the highly purified sample required to visually identify oocysts by microscopy is not required with molecular detection and additional background DNA can improve the carriage of target DNA through extraction.

Oocyst disruption – There are several options for oocyst disruption including freeze-thawing (as in this project), bead-beating and chemical lysis. These methods may also be impacted by the previous sample preparation and the amount of debris present.

DNA extraction - There are also many different DNA extraction approaches and technologies that can be explored further. This includes the addition of Chelex resin to absorb PCR inhibitors and leaving a pure suspension, membrane immobilisation (as in this project) with impurities washes away before eluting in to a pure suspension or binding to magnetic beads to physically pull the DNA out of the sample. Some of these may be better suited to detection and enumeration of *Cryptosporidium* from water and may also be affected by the methods used during sample preparation. Where possible, all parts of the process should make use of options for automation to standardise the processes and reduce the variability introduced by human processing.

The variability in quantification data needs to be reduced, which may prove impossible using qPCR technology. qPCR may be able to provide an indication of the level of oocysts present rather than an absolute number, which may require more specialist platforms such as dPCR that have overcome the challenges with environmental samples and have been successfully used (Kokkoris et al, 2021).

While not currently part of the standard method for the detection and enumeration of *Cryptosporidium*, the ability to genotype from the sample/amplicon is an enormous benefit to the industry. Strategies need to be considered to allow characterisation of species/genotypes and possibly subtypes, whether this is by having sufficient DNA for further tests or involve multiplexing a subtyping assay or changing the detection and enumeration method to one that can potentially subtype.

More Phase 1 (single lab) trials are clearly required using refined methods before Phase 2 (multi lab) trials of a selected method are undertaken. In addition to the investigations mentioned above, it is important to test the final method on different water types (sources, chemistry, etc.) and volumes before proceeding to Phase 2 trials.

Critically, quality control requirements as outlined above and a proficiency testing scheme would need to be developed and provided.

Finally, to establish a PCR-based method as a standard method full documentation of the final procedure (including statistical analysis) is required, similar to the example of how a method would fit in the Blue Book (Appendix 6), with guidance on the correct expression of results.

All of this development into a standard method would need to be underpinned and supported by user and stakeholder acceptability, so continued communication with the industry and public health throughout this development is vital.

What are the limitations of PCR for detection and enumeration?

As with all methods including the current standard method there are limitations with using PCR for detection and enumeration. DNA extraction processes can have a direct impact on the detection and enumeration in the same way as IMS efficiency can influence the enumeration of oocysts in a sample by IFM. Aside from the variability introduced by DNA extraction, with qPCR technologies the accurate and precise enumeration of target organisms at low numbers is challenging (Quan *et al*., 2018), again this is something also seen with the current standard method.

One of the significant differences between PCR and microscopic detection is that PCR doesn't allow for the analyst to see the condition of oocysts or oocyst like bodies (OLBs), which might be informative or indicative of breakthrough in treated water samples. A positive PCR result only confirms that DNA has been detected and cannot confirm that oocysts, whether viable or infective, are actually present in the sample.

While all methods can fall foul of unexpected technical faults, the automated nature of molecular testing means that samples are processed in batches and with the entire sample tested in the PCR a PCR machine failure (as seen with one of our tap water batches) results in the complete loss of multiple samples.

What is the acceptability of PCR if there are public health implications of detection using this method?

As one of the water supply companies questioned during the survey in Objective 2 stated:

"One of the main reasons for sampling final water for oocysts is to verify the performance of the coagulation/filtration stages at our WTWs for oocyst (and giardia cyst) removal as other parameters such as turbidity are not entirely reliable as surrogates. If we detect crypto DNA in final water not sure what this tells us about the integrity of our filters as well as the obvious - what does it mean in terms of public health risk? It is difficult enough to interpret what the detection of an intact oocyst means in terms of public health risk."

A PCR positive result only confirms that DNA has been detected and not that an oocyst was present in the sample so the public health conclusions may have to be carefully considered with Health Professionals. If IMS is used then the chances of detecting "free" DNA in the sample are vastly reduced, but if an IMS-free approach is developed to improve the detection and enumerations then the presence of "free" DNA is more likely. Although free DNA in these samples would also be more prone to damage from various components in the sample, possibly reducing the chance of their detection.

Can a PCR-based method for detection and enumeration also be amenable to downstream characterisation (e.g. by sequence analysis)?

Yes, the PCR used in this study can be analysed to characterise the *Cryptosporidium* species. However, it cannot be used to identify subtypes. Different PCRs and approach would need to be used. This, however, raises a question about the qPCR testing strategy; here the authors used all of the sample concentrate after IMS to extract DNA and then 4 aliquots of 20 µl DNA was tested by qPCR from each sample. The final result was the sum of the number of oocysts detected in each aliquot adjusted for the whole DNA extract volume. This gave equivalence to IFM where the whole IMS concentrate is spotted on to a microscope slide for enumeration. However, it did not leave any DNA for further investigation. While genotyping to establish the *Cryptosporidium* species present could be done by

sequencing the qPCR amplicons, if subtyping (beyond the species level) is needed a different strategy would need to be sought. It is common for analysists to be dealing with single number oocysts, and therefore, will always need to test the entire sample. Previous work has indicated that whole genome amplification is not particularly useful in this respect – maybe even less so if the IMS process is removed. Potential options therefore may include multiplexing the PCR to include subtyping targets, or using alternative technologies such as bait capture and NGS.

Summary of the expert consultation

The expert reviewer found the project report to be a "thorough and interesting piece of work" and had very few comments and suggestions. One comment stated that qPCR was a useful tool in negative screening. This is an important point as using the qPCR to screen out the negative samples could save on the extensive laborious microscopy of negative samples and the positive qPCR samples investigated further. Also suggested was that although easier interpretation of results was among the practical advantages of qPCR over the standard *Cryptosporidium* method, interpretation could be challenging (e.g. unknown viability, or the presence of DNA rather than potentially infective oocysts). Despite the uncertain pathogenicity of some species, the importance of differentiating the species detected in a sample was highlighted as this can assist in incident investigation, suggesting sources and potential risks to Public Health. This justifies our choice of locus and target amplicon, as very few of the other potential assays could sufficiently discriminate between all known *Cryptosporidium* species.

Recommendations for the industry and future areas of research arising from this study.

This proof-of-principle project has demonstrated that a qPCR can be developed for the detection and to a limited extend enumeration of *Cryptosporidium* oocysts in drinking water. However, the results indicate that there is a statistical difference in detection and enumeration of low numbers of oocysts between the qPCR and the current IMS-IFM standard method, with lower counts and greater variability seen with qPCR. There are practical advantages and disadvantages with either method and currently qPCR does not show an improved method for enumeration. Therefore, the qPCR method is not at a point where recommendations can be made to the industry about its adoption, but further discussion with the water industry, and additional developmental work is required.

The industry needs to consider whether to pursue a qPCR-based method, or alternative molecular method, and whether it would be suitable/adoptable considering laboratory requirements and costs. The cost-benefit of molecular detection and enumeration is yet to be seen as it will depend greatly on which technology is used and what additional development work shows.

An additional benefit over the current microscopy method that could be further investigated, is the application of steps to assess the potential viability of *Cryptosporidium* present within a sample. Culture-independent options used in academic studies include the use of PMA-PCR, but this has not yet been included in the Standard Methods for other organisms using PCR.

Alternative PCR methods for quantification such as digital PCR that might provide more robust enumeration, particularly at lower levels should be considered, although the additional cost may be prohibitive and there may be other limitations, such as capability to subsequently genotype.

Modification of sample processing and the qPCR (or use of alternative technology) is recommended to reduce variability. Possibilities of modifications to sample processing include whether IMS is

needed for molecular detection methods, and the effect of different oocyst disruption and DNA extraction processes. An evidence-gathering exercise should be undertaken to explore the suitability of sample concentrates from filters for DNA extraction (e.g. pellet size, nature), potentially eliminating the need for IMS. This would reduce costs and enable multiple pathogen testing, not just parasites but possibly co-sampling viruses (remarkably similar challenges). The increased use of automation is another area that still requires further investigation in order to streamline methods and standardise the processes, reducing the potential for user error and variability.

If the industry feels that the qPCR approach is worth pursuing then further Phase 1 (single laboratory) trials, including different water types, and performance with other *Cryptosporidium* species, must be undertaken before proceeding to Phase 2 (multi-laboratory) trials.

Additional work that should be explored regardless of the technology used to pursue molecular methods for detection and enumeration includes refining the strategy to allow sufficient DNA to be available for subtyping. This will depend on the method of subtyping to be employed and is currently being investigated in other projects.

Interpretation of qPCR detections will require robust validation of methods, encompassing inclusivity and exclusivity of primers and probes, verification of the calibration function of the quantitative PCR phase, verification of the PCR limit of quantification, verification of the PCR limit of detection, robustness, and finally, measurement of uncertainty of the whole method. Some of which has been done as part of this project for this qPCR.

Interpreting results from a public health standpoint must also be considered. Questions need to be addressed around the relationship between quantitative molecular detection and public health risk. What does the detection of *Cryptosporidium* DNA mean for public health? Can high or low Cq values be translated into a relative public health risk?

As with any method occasional false positive and false negatives will occur and the extent of this must be fully evaluated before any method is taken forward as a standard. If potential false positive or negative results are identified, a strategy must be developed on how to resolve and report these.

Criteria for generating reportable results must be decided for any accepted method that has been fully evaluated and validated. With a molecular method that may not give you an absolute number of oocysts detected, should the results be reported in a different manner? The current method that produces an actual oocyst count only reports what is seen on the slide, which is not the true number of oocysts in the sample, due to losses during processing. The actual presence of oocysts and a gauge into the extent of contamination is the important information, and this can probably also be gleamed from molecular results. Other issues around reporting from a molecular assay is that the target for detection is DNA rather than an oocyst and this must be carefully expressed in the way a result is reported.

Before a molecular method can be adopted by the industry there needs to be the establishment of a proficiency scheme as with all of the other parts of the standard methods. This will be particularly important with the introduction of a method and techniques that may be new to analysts.

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Appendix 1 – Structured exclusion and data capture headings used for Question 2 in Objective 1 (Literature Review)

Reference	Full details
Exclusions and reason	Is it a Review (Y/N)
	Using or describing PCR methods for *Cryptosporidium* (Y/N)
	Part of a clinical multi-organism panel kit (Y/N)
	PCR part of method not compatible with quantitation (e.g. not qPCR, dPCR or other format that can be used for quantitation) (Compatible/Not Compatible)
	Pan-genus detection (Y/N) Exclude non-pan genus
	Amplicon also useful for species identification (Y?N)
	Excluded (Y/N)
Data mining and secondary exclusion	Currently set up for quantitation (Y/N)
	Target organisms (all or individual sp.)
	Gene(s)
	Amplicon size
	Sample Types
	DNA extraction
	PCR primers
	PCR probes or dyes
	PCR format (qPCR, dPCR, etc.)
	Calibration requirements
	Reported reaction efficiency (E value from standard curve)
	Sensitivity
	Specificity
	Susceptibility to inhibition
	Quantification accuracy (R2 value)
	Quantification precision
	Potentially suitable for water industry (particularly with low numbers)? And reason why….
	General results and comments

Appendix 2 – List of articles included in synthesis for Question 1 in Objective 1 (Literature Review)

- Aguado, D., Fores, E., Guerrero-Latorre, L., Rusiñol, M., Martínez-Puchol, S., Codony, F., Girones, R., & Bofill-Mas, S. (2019). VirWaTest, A Point-of-Use Method for the Detection of Viruses in Water Samples. Journal of Visualized Experiments, 2019(147). https://doi.org/10.3791/59463
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Appendix 3 – Database of articles and full text assessment included in synthesis for Question 2 in Objective 1 (Literature Review)

Appendix 3 Q2 Refs and Full Text Assessı

Appendix 4 – Industry questionnaires: 1. Water testing laboratories, 2. Water supply companies.

1. *Cryptosporidium* PCR Questionnaire for Water testing laboratories

You are invited to participate in a project conducted on behalf of the Drinking Water Inspectorate (DWI) by the Cryptosporidium Reference Unit (Public Health Wales) by completing this survey. Your participation in this survey is entirely voluntary.

The purpose of this survey is to:
(1) Investigate the current use of the Polymerase Chain Reaction (PCR) by water testing laboratories for the detection of any micro-organisms in water.
(2) Investigate the current use of PCR by water testing laboratories for the detection of *Cryptosporidium* in water.
(3) Enquire about future plans or appetite within the industry to develop / adopt PCR detection of *Cryptosporidium* in water.
(4) Understand the barriers for water testing laboratories to using PCR for the detection of *Cryptosporidium* in water.

The survey forms part of an information gathering exercise that will inform us about the status of PCR as a means of detecting *Cryptosporidium* in water and industry attitudes to further developments in this area. This will assist the DWI in making informed decisions with respect to PCR detection of *Cryptosporidium* in water.

The intention of the survey is to obtain a testing laboratory company response on PCR and *Cryptosporidium* testing. The survey should therefore be completed by an individual who can respond on behalf of their company and not give a personal opinion on the methodology.

The questionnaire has up to 22 questions (depending on your answers) and should take about 20 minutes to complete.
*indicates that this question requires an answer.

PAGE 1

1. What is the name of the water testing laboratory you are answering this survey on behalf of? *

2. What is your name? *

3. Please add your contact details (email address preferred) *

4. What is your position? *

5. Does your laboratory use PCR for the detection of any micro-organisms in water?

- [ ] No *(jumps to PAGE 3 Q7)*
- [ ] Yes, please state which below

Comments:

PAGE 2

6. For what reasons do you test for these microorganisms using PCR?

PAGE 3

7. On average, how many samples does your laboratory currently test for *Cryptosporidium* each week? *(Sliding scale of 0-200)*

8. What is the capacity of your laboratory for *Cryptosporidium* testing (samples per week)? *(Sliding scale of 0-500)*

9. Does your laboratory use PCR to detect or identify *Cryptosporidium* oocysts from routine water monitoring slides?

- ☐ Yes - to detect *Cryptosporidium* oocysts
- ☐ Yes - to identify *Cryptosporidium* species and genotypes
- ☐ Yes - to both detect *Cryptosporidium* oocysts and identify *Cryptosporidium* species and genotypes
- ☐ No *(Jumps to PAGE 5 Q18)*

PAGE 4

10. Which PCR technology does your laboratory use for detection or identification of *Cryptosporidium* in water (tick all that apply)?

	Detection	Identification
Real time PCR	☐	☐
Conventional PCR	☐	☐
Conventional nested PCR	☐	☐
Restriction Fragment Length Polymorphism (RFLP)	☐	☐
Sanger sequencing	☐	☐
Next-generation sequencing	☐	☐

11. Which genes / loci do your PCRs target for detection of *Cryptosporidium* oocysts?

12. Which genes / loci do your PCRs target for identification of *Cryptosporidium* species and genotypes?

13. What PCR equipment and facilities does your laboratory have in-house?

14. What experience / educational level knowledge of PCR does your laboratory have (tick all that apply)?

- [ ] Expertise from years of experience
- [ ] Degree level
- [ ] PhD level
- [ ] Ex-biotechnology industry experience
- [ ] Other (please specify):

15. Are you aware of any PCR training opportunities which may be available to your colleagues?

- [ ] No
- [ ] Yes, please comment below

Comments:

16. What are the benefits of using PCR for detecting *Cryptosporidium*?

17. What are the drawbacks of using PCR for detecting *Cryptosporidium*? *(Jumps to PAGE 6 Q21)*

PAGE 5

18. Are there any barriers to using PCR for detecting *Cryptosporidium* for routine monitoring in your laboratory (tick all that apply)?

- [ ] No
- [ ] Yes, requirement for investment in new equipment
- [ ] Yes, requirement for training staff in new technologies
- [ ] Yes, requirement for reconfiguration of laboratory space and workflow
- [ ] Yes, impact of change requirements on laboratory accreditation
- [ ] Yes, potential quality issues such as method reliability, robustness and uncertainty of measurement.
- [ ] Yes, data interpretation issues
- [ ] Yes, data reporting issues
- [ ] Yes, lack of appetite of end users (others in same company) to accept new methods
- [ ] Yes, lack of appetite of end users (others in client company) to accept new methods
- [ ] Yes, acceptance by the regulator
- [ ] Other (please specify):

19. If your laboratory is not using PCR for detecting *Cryptosporidium* oocysts in water is this because (tick all that apply)?

- [ ] We don't have the expertise
- [ ] We don't have the equipment or facilities
- [ ] It's not the Standard Method
- [ ] This is not something we have a need for at the moment
- [ ] Other (please specify):

20. If your laboratory is not using PCR for testing for *Cryptosporidium* oocysts in water, do you have plans to introduce PCR for the detection and/or identification of *Cryptosporidium* in the future?

	No	Yes, in the next 12 months	Yes, in the next 5 years
Detection	☐	☐	☐
Identification	☐	☐	☐

PAGE 6

21. Please could you indicate if we may contact you about this survey, or indicate a colleague if you prefer?

- [ ] Yes
- [ ] No
- [ ] Please contact my colleague by email:

Colleague email address:

22. Please add any comments or further information which you think we will find helpful:

2. *Cryptosporidium* PCR Questionnaire for Water Supply companies

You are invited to participate in a project conducted on behalf of the Drinking Water Inspectorate (DWI) by the Cryptosporidium Reference Unit (Public Health Wales) by completing this survey. Your participation in this survey is entirely voluntary.

The purpose of this survey is to:
(1) Investigate the current use of the Polymerase Chain Reaction (PCR) results for the detection of any micro-organisms in water.
(2) Investigate the current use of PCR results specifically for the detection of *Cryptosporidium* in water.
(3) Enquire about future plans or appetite within the industry to develop / adopt PCR detection of *Cryptosporidium* in water.
(4) Understand reservations or challenges for water supply companies in using PCR instead of current methods for detecting *Cryptosporidium* in water, and any perceived impact on operational monitoring and interpretation.

The survey forms part of an information gathering exercise that will inform us about the status of PCR as a means of detecting *Cryptosporidium* in water and industry attitudes to further developments in this area. This will assist the DWI in making informed decisions with respect to PCR detection of *Cryptosporidium* in water.

The intention of the survey is to obtain a company response on the use of PCR for *Cryptosporidium* detection. The survey should therefore be completed by an individual who can respond on behalf of their company and not give a personal opinion on the methodology or its application.

The questionnaire has up to 16 questions (depending on your answers) and should take about 20 minutes to complete.
* Indicates that this question requires an answer

PAGE 1

1. What is the name of the water supply company you are answering this survey on behalf of? *

2. What is your name? *

3. Please add your contact details (email address preferred) *

4. What is your position? *

5. On average, how many *Cryptosporidium* tests does your company commission each week?

6. Does your company use PCR for the detection of any micro-organisms in water?

- [ ] No
- [ ] Yes, please state which below

Comments:

7. Does your company use PCR to detect (not genotype) *Cryptosporidium* oocysts in water?

- [ ] No *(Jumps to PAGE 3 Q13)*
- [ ] Yes - in-house
- [ ] Yes - by a contract laboratory

PAGE 2

8. For what reasons does your company use PCR for the detection of *Cryptosporidium* oocysts?

Operational monitoring ☐

During water quality incidents ☐

Catchment monitoring ☐

Because the regulator expects us to ☐

For research ☐

9. Has PCR testing for the detection of *Cryptosporidium* ever been used by your company in any of these ways (tick all that apply)?

☐ To inform change of practice (in a catchment for example)

☐ To alert operators of issues at a works

☐ During water quality incidents

☐ Other (please specify):

10. How does your company interpret *Cryptosporidium* PCR data (detection of DNA) compared with results from the Standard Method (microscopy detection of oocysts)?

☐ They both indicate the presence of the organism

☐ With the Standard Method oocysts are enumerated, this is not always possible with PCR

Comments:

11. What are the benefits of having data from PCR testing for *Cryptosporidium*?

12. Are any of the following concerns around the use of PCR results for *Cryptosporidium* detection (tick all that apply)? *(Jumps to Page 4)*

- [ ] Cost
- [ ] Interpretation of results
- [ ] Acceptance of change of a method that has been in use for a long time
- [ ] Acceptance of detection of a new target (DNA rather than oocysts)
- [ ] Method reliability - uncertainty of measurement
- [ ] Acceptance by the regulator
- [ ] Acceptance by Public Health
- [ ] Other (please specify):

PAGE 3

13. Are the following likely barriers to the future adoption of PCR results for *Cryptosporidium* detection (tick all that apply)?

- [ ] Cost
- [ ] Interpretation of results
- [ ] Acceptance of change of a method that has been in use for a long time
- [ ] Acceptance of detection of a new target (DNA rather than oocysts)
- [ ] Method reliability - uncertainty of measurement
- [ ] Acceptance by the regulator
- [ ] Acceptance by Public Health
- [ ] Other (please specify):

14. If your company is not using PCR for testing for *Cryptosporidium* oocysts in water, do you have plans to commission PCR for the detection of *Cryptosporidium* in the future?

- [ ] Yes, in the next 12 months
- [ ] Yes, in the next five years
- [ ] No

PAGE 4

15. Please could you indicate if we may contact you about this survey, or indicate a colleague if you prefer?

- [ ] Yes
- [ ] No
- [ ] Please contact my colleague by email:

Colleague email address:

16. Please add any comments or further information which you think we will find helpful:

Appendix 5 – Lists of Water testing Laboratories and Water supply companies questionnaires sent to in Objective 2.

Water testing laboratories to which the "*Cryptosporidium* PCR Questionnaire for Water testing laboratories" was sent:

Affinity Water
ALS Coventry
ALS Wakefield
Anglian Water
Dŵr Cymru Welsh Water
Northumbrian Water
Severn Trent laboratories
South East Water
South West Water
Thames Water
United Utilities
Wessex Water
Northern Ireland Water
Scottish Water

Water supply companies to which the "*Cryptosporidium* PCR Questionnaire for Water Supply companies" was sent:

Affinity Water
Albion
Albion Eco
Anglian Water
Bristol Water
Cambridge Water
Dŵr Cymru Welsh Water
Essex and Suffolk Water
Hafren Dyfrdwy (was Dee Valley)
Icosa Water
Independent Water Networks Limited
Leep Water Networks Limited
Northumbrian Water Limited
Portsmouth Water
Severn Trent Water
South East Water
South Staffordshire Water
South West Water
Southern Water
Sutton and East Surrey Water
Thames Water
United Utilities
Veolia Water
Wessex Water
Yorkshire Water

Appendix 6 – Example of how a qPCR method may be presented in the Standing Committee of Analysts' Blue Book edited in green to illustrate how quantitative PCR might be included

The Microbiology of Drinking Water (2010) - Part 14 - Methods for the isolation,identification and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts

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Whilst specific commercial products may be referred to in this document, this does not constitute an endorsement of these products but serves only as illustrative examples of thetype of products available. Equivalent products may be available and it should be understood that the performance of the method might differ when other materials are used and all should be confirmed by validation of the method.

Contents

Chapters without edits have been removed from this document, but remain here for reference purposes

Disclaimer

This adapted version is for illustrative purposes only and is not a recommended method as further developmental work needs to be undertaken. It is designed to show how qPCR might be included in laboratory testing for the detection and enumeration of *Cryptosporidium* from water samples. The methods included were developed as part of a DWI-funded project and criteria required to verify and validate modifications to the method have also been added to the appendices.

Warning to users

The analytical procedures described in this booklet should only be carried out under the proper supervision of competent, trained analysts in properly equipped laboratories.

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc Act 1974 and all regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 2002 (SI 2002/2677). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted.

Numerous publications are available giving practical details on first aid and laboratory safety. These should be consulted and be readily accessible to all analysts. Amongst such publications are; “Safe Practices in Chemical Laboratories” and “Hazards in the Chemical Laboratory”, 1992, produced by the Royal Society of Chemistry; “Guidelines for Microbiological Safety”, 1986, Portland Press, Colchester, produced by Member Societies of the Microbiological Consultative Committee; and “Safety Precautions, Notes for Guidance” produced by the Public Health Laboratory Service. Another useful publication is “Good Laboratory Practice” produced by the Department of Health.

Glossary

ACDP	Advisory Committee on Dangerous Pathogens
ANOVA	analysis of variance
bar	unit of pressure equal to 1 atmosphere (15 lb in^{-2}, 101.325 kPa, 760 mm of mercury.
break-through	penetration of a filter medium, for example a membrane filter, by particulate material that would otherwise be trapped by the filter medium.
by-pass	passage of sample water through a filter apparatus such that the water does not pass through the filtration module itself but leaks to waste, past for example an O-ring, allowing (oo)cysts to by-pass the unit.
calibration curve	The result of a set of serially diluted DNA samples of known concentration. The calibration curve is used to calculate the efficiency, linear range and reproducibility of a PCR and allows for the quantification of an unknown amount of the target in a sample.
cpm	cycles per minute
Cq value	Cycle number of the real-time PCR at which the fluorescence crosses a theoretically applied threshold
Cryptosporidium	a protozoan parasite
Cryptosporidium oocyst	the environmentally resistant transmissive life cycle stage in which *Cryptosporidium* occurs in the environment. The oocyst (which is shed in the faeces of an infected person or animal) contains 4 sporozoites capable of causing infection.
DAPI	4',6-diamidino-2-phenylindole
DIC	differential interference contrast (microscopy)
DNA	deoxyribonucleic acid
DNA Extraction	The process by which DNA is released from cells, purified and isolated for use in molecular assays.
excystation	process by which the infective bodies contained in cysts or oocysts are released
FITC	fluorescein isothiocyanate
Giardia	a protozoan parasite
Giardia cyst	the resting life cycle stage in which *Giardia* occurs in the environment, and which is capable of causing infection
hydrophobic / hydrophobicity	the tendency of a surface to repel wetting by water
IMS	immuno-magnetic separation
Inhibition	The lack of PCR amplification caused by the presence of inhibitors that can directly interact with the DNA or components of a PCR reaction to reduce or stop the PCR reaction
Internal amplification control	A non-target sequence of DNA added to the sample and amplified simultaneously in the PCR to identify false negatives due to PCR inhibition.
MAb	monoclonal antibody
NTC	No template control

(oo)cyst	this term signifies either *Cryptosporidium* oocyst or *Giardia* cyst or both, as appropriate, depending on the context
OLBs	(oo)cyst-like bodies, i.e. organisms that resemble (oo)cysts but are not.
PCR	polymerase chain reaction
qPCR	quantitative real-time PCR
protoplasmic “residual body”	protoplasmic material remaining within an oocyst after sporozoites have excysted.
rcf	relative centrifugal force, equivalent to force of gravity (g)
RFLP	restriction fragment length polymorphism
V_{PV}	pellet volume
V_{SV}	final mixture volume

Methods for the isolation, identification and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts

Introduction

Cryptosporidium is a genus of coccidian protozoan parasites, found worldwide in a variety of vertebrate hosts, including humans. Some species of this parasite, notably *Cryptosporidium hominis* and *Cryptosporidium parvum*, cause disease (cryptosporidiosis) usually manifested as diarrhoea in humans (*Cryptosporidium hominis* and *Cryptosporidium parvum*) and young livestock (*Cryptosporidium parvum*).

Giardia is a genus of flagellated protozoan parasites. *Giardia duodenalis* (sometimes referred to as *Giardia intestinalis* and *Giardia lamblia*) is recognised as one of the most common worldwide protozoan parasites causing diarrhoea (giardiasis), infesting the small intestine of humans, and other vertebrates.

Transmission of infection can occur by any route where infective *Cryptosporidium* oocysts or *Giardia* cysts are ingested. Both oocysts and cysts can survive for prolonged periods of time in cool, moist environments, with *Cryptosporidium* oocysts being more resistant to chlorination than *Giardia* cysts.

Information on the biology, transmission and public health significance of *Cryptosporidium* and *Giardia* is given in Appendix 1.

A qPCR has been developed and is described here to provide a feel for how this technology might sit alongside or even replace microscopy for enumeration, with the example provided for *Cryptosporidium*.

Increasingly, molecular typing of environmental and outbreak isolates is being conducted to better understand the transmission and epidemiology of *Cryptosporidium*. The techniques employed are outside the scope of this document, but an overview of the rationale and principles of such investigations is presented in Appendix 2.

Definitions

Cryptosporidium oocysts are the environmentally resistant transmissive life cycle stages in which *Cryptosporidium* occurs in the environment. The oocyst (which is shed in the faeces of an infected person or animal) contains 4 sporozoites capable of causing infection. When ingested, disease is transmitted and a new cycle of infection set up. The oocysts are resistant to adverse conditions in the environment and can remain dormant but viable for months in waters, sediments and soils.

Limitations

Environmental monitoring is problematical owing to the low numbers and uneven distribution of *Cryptosporidium* oocysts and *Giardia* cysts normally found in many waters, and sand filter materials. The lack of *in-vitro* culture methods for increasing (oo)cyst numbers also causes difficulties in detection, which currently relies on examination by microscopy. Consequently, it can be difficult to accurately identify (oo)cysts from other particulate material and debris found in concentrated suspensions obtained from waters.

The procedures used for the separation, recovery and identification of (oo)cysts are labour

intensive and time consuming. The presence of particulate material in a sample may interfere with every stage of the sample collection and analytical processes, including the microscopic examination. The presence of inhibitory substances may interfere with qPCR.

The failure to detect (oo)cysts or their DNA in a sample does not ensure or guarantee that the sample is indeed *Cryptosporidium*- or *Giardia*-free. No *Cryptosporidium* or *Giardia* method is capableof achieving 100 % recovery, and indeed actual recoveries are much lower. Reported recovery data for waters obtained using procedures described in this booklet are presented in Appendix 3.

The methods presented here only detect the presence of *Cryptosporidium* and give no indication of viability.

Health and safety

The analytical procedures described in this booklet should only be carried out by competent, trained persons with adequate supervision where necessary.

Reagents and organisms used in this method are covered by the Control of Substances Hazardous to Health Regulations 2002[1] and appropriate risk assessments should be carried out before using this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[2].

Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted. Field operations should be conducted with due regard for possible local hazards, and appropriate safety equipment should be used when required. *Cryptosporidium* and *Giardia* are classified as ACDP Hazard Group 2[3] organisms, i.e. the organisms can cause human disease and may be hazardous to laboratory staff. Laboratory procedures should only be performed in properly equipped laboratories within at least a category 2 containment facility[3]. Filters that have been used to separate (oo)cysts from water samples, may contain significant numbers of (oo)cysts and other potentially pathogenic micro-organisms, and the ova and cysts of other parasites. Filters should therefore only be processed in an appropriate laboratory.

If used in a viable state, *Cryptosporidium* oocysts and *Giardia* cysts used for seeding or quality assurance purposes may pose a risk of infection to humans. A non-viable form of (oo)cysts may be produced by the irradiation or heat treatment of viable (oo)cysts. If non-viable (oo)cysts are used, consideration should be given to the possibility that the inactivation treatment used may affect, for example the surface characteristics of the (oo)cyst and potentially affect recoveries. Comparison work may therefore be advisable depending on the type of inactivation used and the information being sought. Consistent use of one type of treatment should be used within a laboratory.

In addition, equipment (for example, mercury bulbs) should be treated cautiously and handled appropriately if damaged. Mercury vapour lamps have a limited safe working life of 100 - 200 hours depending on their specification. Beyond this period the fluorescence output may fade and bulbs may explode, damaging the lamp housing and posing a risk of exposure to mercury vapour. Bulbs should therefore be changed at regular intervals and their fluorescence output calibrated using a fluorescence calibration control slide.

References

1. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No 2677. See also Approved Code of Practice and Guidance L5 (Fourth edition) HSE Books 2002 ISBN 0 7176 2534 6.

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3. Advisory Committee on Dangerous Pathogens, The Approved List of Biological Agents. Health and Safety Executive 2004.

4. Drinking Water Inspectorate. Guidance on assessing risk from *Cryptosporidium* oocysts in treated water supplies to satisfy the Water Supply (Water Quality) regulations 1999. (Available from: http://www.dwi.gov.uk). These regulations have now been revoked.

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9. UKWIR (2020). *Cryptosporidium*: Enhancing the water industry's capability to respond. UKWIR Project 20/DW/06/22.

10. Deer, D.M., Lampel, K.A. & González-Escalona, N. (2010). A versatile internal control for use as DNA in real-time PCR and as RNA in real-time reverse transcription PCR assays. Letters in Applied Microbiology, 50, 366–372.

E Enumeration of *Cryptosporidium* by quantitative PCR

E1 Introduction

Using PCR as an alternative to microscopic examination can be beneficial: including streamlining the analytical process, as DNA extraction and PCR are amenable to automation; providing a shorter time to result; improved specificity of detection; and generation of amplicons that could be further processed for species identification (Chalmers *et al.*, 2010). Additionally, the application of a calibration curve can enable enumeration as a quantitative PCR (qPCR). To allow for molecular detection and enumeration of *Cryptosporidium* DNA must be extracted from the sample. This may be from the concentrated pellet (see sections A-C) or from the oocyst bead complexes formed during the IMS process (see Section D up to the point of dissociation in D5.4). Following DNA extraction, the entire sample must be tested by PCR so as to detect low numbers if present. This is done by running four replicates of the PCR and the results combined.

E2 Scope

This document describes a molecular based method (qPCR) for the detection and quantification of *Cryptosporidium* spp. in water samples, as collected and concentrated using procedures described in Sections A-D up to the point of dissociation in D5.4.

Technical details specified in this document are given for information only. Any other technical solutions using commercially available kits or in-house developed methods complying with the performance requirements are suitable.

This methodology can be used to identify and quantify *Cryptosporidium* to the genus level, however the resulting amplicon can be sequenced to identify the species or genotype of *Cryptosporidium* present but this procedure is not described here.

The information in this document is intended to be applied in the microbiological investigation of all types of water. However, some additives, e.g. chemicals or processes used for water treatment, the nature and/or content of suspended matter and/or accompanying microbiota can interfere with the method and affect its sensitivity.

Users wishing to employ these methods should verify its performance under their own laboratory conditions as described in Appendix 5.

E3 Principle

Using PCR allows the DNA from a specifically targeted organism to be detected in a sample. The addition of a calibration curve produced from known concentrations of DNA standards allows the target DNA to be quantified. With knowledge of the number of gene copies within an oocyst (i.e. 20 copies of small subunit rRNA gene per oocyst) this quantified DNA can be converted to give an estimation of the oocyst number in the sample.

As with all methods there are advantages and disadvantages. The use of PCR does not rely on the visualization of the oocyst itself, which requires highly skilled analysts and can be problematic particularly in samples with high levels of background debris. Molecular

methods such as PCR are more amenable to streamlining through automation, improving workflow and standardizing procedures. However, the detection of DNA in a sample by PCR does not necessarily reflect the presence of intact oocysts (unlike microscopy) and need to be considered if public health or operational decisions are required.

The detection and enumeration of *Cryptosporidium* by either microscopy or qPCR face some of the same limitations. Neither currently provides any information on the viability or infectivity, and the enumeration of *Cryptosporidium* does not provide true numbers of organisms within a sample as both methods are heavily reliant on the pre-detection sample processing which results in variable losses.

E4 Apparatus

Some of the following procedures are based on proprietary products, and if used, manufacturer's instructions should be followed. Inclusion in this document does not constitute an endorsement of these products but serves only as illustrative examples of the type of products that are available. Users should decide which product or procedure is appropriate for their own requirements and should verify its performance under their own laboratory conditions with appropriate samples.

E4.1 DNA extraction

- 1.5 ml screw-capped tubes
- 1.5 ml flip-topped tubes
- Heating Block (95°C and 56°C)
- Hand held digital thermometer
- Liquid nitrogen dewar
- Rack for freezing 1.5 ml tubes in liquid nitrogen dewar
- Variable volume pipettes (capable of pipetting volumes 20 µl – 1000 µl)
- Filter-protected pipette tips
- Vortex
- Microcentrifuge

E4.2 Quantitative PCR

- Vortex
- Microcentrifuge
- Variable volume pipettes (capable of pipetting volumes 2 µl – 1000 µl)
- Filter-protected pipette tips
- 1.5ml flip-topped tubes
- PCR tubes
- Rotor-Gene qPCR instrument
- Suitable PCR suite with uni-directional workflow; dedicated laminar flow cabinets for reagent set up and preparation, and DNA handling

E5 Reagents

Some of the following procedures are based on proprietary products, and if used, manufacturer's instructions should be followed. Inclusion in this document does not constitute an endorsement of these products but serves only as illustrative examples of

the type of products that are available. Users should decide which product or procedure is appropriate for their own requirements and should verify its performance under their own laboratory conditions with appropriate samples.

Alternative commercial reagents are available, but may possess minor variations to their formulation. The performance of all reagents should be verified prior to their use in the method. Further guidance on assessing the performance of methods or parts of methods used for drinking water analysis is given in Appendices 4 and 5.

Variations in the preparation and storage of reagents should also be verified.

Commercially available reagents should be used and stored according to manufacturer's instructions. Where reagents are stored in a refrigerator they should be allowed to reach room temperature before use.

E5.1 DNA extraction

- Liquid Nitrogen
- Qiagen QIAamp DNA mini extraction kit (containing Proteinase K, Buffer AL, Buffer AW1, Buffer AW2, Buffer AE)
- Ethanol (98-100%)
- Reverse osmosis (RO) water

E5.2 Quantitative PCR

- Bioline SensiMix™ II probe kit
- Forward primer [50 µM] JF1/2 (1:1 mix of each JF1 and JF2) (UKWIR, 2020)
  - JF1 AAGCTCGTAGTTggatTTCTG (601-621 L16996 *C. parvum*)
  - JF2 AAGCTCGTAGTTaatcTTCTG (601-621 L16996 *C. parvum*)
- Reverse primer [50 µM] JR (UKWIR, 2020)
  - JR CCTTACTCCTTCAGCACCTTA (1015-1035 L16996 *C. parvum*)
- TaqMan Probe [10 µM] JT2 (UKWIR, 2020)
  - JT2 Cy5-TCAGATACCGTCGTAGTCT-MGB-EQ (958-976 L16996 *C. parvum*)
- Internal amplification control (IAC) reagents (Deer *et al*., 2010)
- gBlock synthetic oligonucleotide (sequence adapted from L16996 *C. parvum* to increase the GC content and produce a unique sequence to detect in the event of cross-contamination)
  - AAGCTCGTAGTTGGATTTCTGTTAgTgATcTgTgTgAgAcAcTcTGATGAgTg TcTgcATgAcAcTggCATAgTcCATATcACTAcAcATTccAGTgcgcGAggTTTTA CTTTGAGAgAgTcAGAGTGCTTAgAGCAGGCATATGCCTTGAATACTCCA GCATGGAgcggTgTcAggGATTTTTATCTTTCTTATTGGTTCTAAGATgAGA gcggcGATTggcAGGGACAGTTGGGGGTACTTGTgccTAACAGTCAGAGGT GAAATTCTTAGATTTGcTAgAGACAAgCcgATGCGgAAGCAccTGCCAAGG AcGTcTTCgTcAgTCggGAACGggAGccAGGGGATCGAAGACGATCAGATA CCGTCGTAGTCTTAACCATAAACTATGCCAACTAGAGATTGGAGGTTGT TCCTTACTCCTTCAGCACCTTAT
- Nuclease free water

E6 Analytical procedure

This procedure starts with the samples processed to the point in section D5.4 just prior to

dissociation and the IMS bead/oocyst complexes have been transferred from the Leighton tubes to 1.5 ml screw-capped tubes. Screw-capped tubes are required to prevent the lids popping off during the freeze-thaw process and causing sample losses and / or cross-contamination. If the samples are not to be processed immediately the screw-capped tubes can be frozen until DNA extraction.

E6.1 DNA extraction

Prepare a positive and negative DNA extraction control by adding 200 µl RO water to two 1.5 ml screw-cap tubes and spiking one with 50-100 oocysts. Next prepare a set of extraction tubes by labelling a QIAamp column and a 1.5 ml flip-topped tube, and setting out 2 x Qiagen collection tubes for each sample (including the controls). Prepare the QIAamp DNA mini kit reagents as per the manufacturer's instructions, by adding ethanol to Buffers AW1 and AW2.

Set a heating block to 100 °C and pour enough liquid nitrogen into the dewar to last for eight cycles of freeze-thawing. Snap freeze all samples (including the controls) in the liquid nitrogen for 1 minute and then transfer to the 100 °C block to thaw. Gently flick the tubes to resuspend any settled oocysts and repeat the freeze-thaw process 7 more times. Gently flick every tube after each thaw – it is important not to vortex the samples to resuspend at this point as vortexing the samples while hot can damage the DNA in the sample so that it can longer amplify by PCR. Following the final thaw allow the samples to cool to room temperature and centrifuge briefly to spin contents and any condensation in the lid to the bottom of the tube.

Extract the DNA from the sample using the Qiagen QIAamp DNA mini extraction kit following the manufacturer's instructions. Add 20 µl of Proteinase K and 200 µl of Buffer AL to each tube, vortex for 15s and centrifuge briefly before incubating at 56°C for 10 minutes. Let the samples cool on the bench for 5 minutes and centrifuge briefly. Add 200 µl of Ethanol to each tube, vortex for 15s and centrifuge briefly. Transfer each sample to its labelled QIAamp column, centrifuge at 6000 x *g* (rcf not rpm) for 1 minute. Discard the collection tubes and place the QIAamp columns in fresh collection tubes. Add 500 µl of Buffer AW1 to each column, centrifuge at 6000 x *g* for 1 minute, discard the collection tubes and place the columns in fresh collection tubes. Add 500 µl of Buffer AW2 to each column, centrifuge at 20,000 x *g* for 4 minutes and discard the collection tubes. Insert the columns into the labelled 1.5 ml flip-topped tubes and add 100 µl of Buffer AE to all tubes. Incubate all samples at room temperature for 5 minutes and then centrifuge at 6000 x *g* for 1 minute before discarding the spin columns and capping the tubes. Store the tubes containing the DNA extracts at -20°C until ready to test.

E6.2 Quantitative PCR

Extreme care must be taken to avoid contamination of PCR reagents and equipment with genomic and amplified DNA. Work in a low risk to high risk direction: reagent tubes and blocks should be handled before sample tubes and blocks. Gloves should be changed after touching sample tubes/blocks or other potentially contaminated areas such as the discard box.

This section describes manual PCR set up and assumes the use of dedicated cabinets for each of reagent preparation and DNA handling. PCR amplification and analysis are described using the Qiagen RotorgeneQ real time PCR instrument.

Preparation of the gBlock calibration curve template:

The gBlock calibration curve template is supplied lyophilized in a known copy number per mass (e.g. 7.6 x10^{11} copies in 500 ng). The gBlock should be resuspended in an appropriate volume of TE buffer or nuclease free water to ensure that this stock preparation is >=10 ng/µl (e.g: >=50 µl); this should be stored at -20°C for up to 1 year, the preparation degrades over time if stored at a lower concentration. Using this known volume, the number of copies per µl can be calculated from the amount supplied. From this stock solution, create a dilution series of the template, adjusted from copies per µl to oocysts equivalents by dividing by 20, which is suitable for the qPCR. An eight point calibration curve for example would range from 1x10^{6} to 0.1 oocysts per PCR (20 µl template addition).

Preparation of the IAC reagent:

The Ultramer™ oligonucleotide IAC template is supplied and prepared as described in Deer *et al*. (2010). Briefly, the template is stored (at -20°C for up to 1 year) as a stock at 10^{7} copies / µl and combined with forward and reverse primers (100 nM final concentration), and Taqman™ (hydrolysis) probe (6'FAM labelled; 200 nM final concentration) to create a single reagent for addition to the qPCR.

qPCR set up and performance:

In the reagent cabinet prepare a qPCR master mix containing all of the reagents described below. The volume prepared is determined by the number of samples (and their replicates) that require testing*. A small extra volume (the equivalent of 2 extra single samples per 10 reactions) is added to allow for small pipetting errors ensuring sufficient master mix is prepared. Prepare this into a nuclease free plastic tube of appropriate volume.

		No. PCRs
	x1	10*
SENSIMIX II	25	250.0
JF1/2 (50uM)	0.6	6.0
JR (50uM)	0.6	6.0
JT2 (10uM)	0.38	3.8
IAC primer/probe/ template mix	2.5	25
[DNA]	20	200.0
nfH_2O	0.92	9.2
total	50	500.0

In a suitable rack, set out sufficient 0.2 ml PCR tubes for each of the test sample replicates (four per sample), two tubes for the two extraction controls (one positive and one negative, from section E6.1), one for a verified positive control DNA sample (PCR IQC positive) and one tube for the non-template control, NTC (PCR IQC negative). In addition set out a minimum of 5 tubes for the gBlock calibration curve templates. Aliquot 30 µl of the qPCR master mix into each PCR tube.

In the DNA handling cabinet, add 20 µl of the test sample DNA, four per sample, the extraction control samples (2), the PCR controls (2) and the gBlock calibration curve templates (5 or more) to the PCR tubes containing the master mix.

Load the PCR tubes into the real time PCR instrument (RotorgeneQ). Ensure the ring holding the PCR tubes is locked and close the instrument lid. Set the instrument to run the

following qPCR program:

Initial hot start:	95 °C 10 minutes
Thermocycling:	95 °C 15s
	60 °C 60s
	72 °C 30 s
	x50 (total number of cycles)

The *Cryptosporidium* assay data is gathered from the red channel (Cy5 fluorophore) and the IAC from the green channel (FAM); both acquiring fluorescence data at the end of the 60 °C annealing phase. The program includes autogain calibration to set the correct fluorescence detection level.

E6.3 Reading of results

When the qPCR run has completed, amplicons may be either discarded or kept for further analysis by Sanger sequencing to establish the species or genotype present.

Quantitative analysis of the qPCR is carried out as follows:
Open the RotorgeneQ run file, Select “Analysis” and in the pop-up box double-click on “Cycling A. Green (Page 1)” and “Cycling A. Red (Page 1). Nominate the test samples and their replicates, extraction and PCR controls and the calibration curve templates appropriately in the “samples” area of the software. Ensure that the calibration curve templates are nominated as standards and the oocyst equivalents are entered correctly under “given concentration”. Set the threshold for both channels to 0.05. Consult the standard curve graph in the analysis window to record the reaction dynamics R^2, E and M to check they fall within the correct ranges as described previously. If this is the case then the quantification data for the test samples can be accepted from the “Quant. Results. Cycling red” window. Data from this window (Cq and “calc. conc”) can be copied and exported into a spreadsheet or other document for ease of reporting. Data from the IAC, reported in the “Quant. Results. Cycling green” window (Cq only) can be similarly exported. If the Cq value for the IAC in any test sample replicate is more than 3 Cq higher than the IAC in the NTC, and the test sample replicate is negative, then consider the test sample PCR to have been inhibited and the result to be falsely negative.

E6.4 Confirmation tests

Any samples positive by qPCR as described here can have their amplicons sequenced, using the same primers as in the qPCR, to identify the species or genotype of *Cryptosporidium* present in the sample.

E7 Calculations

The number of oocysts per sample is calculated by combining the oocyst counts for each of the four PCR replicates and adjusting for the entire sample. E.g.: 4+2+5+5=16, 16x5/4=20. This represents the number of oocysts detected per volume of water originally sampled.

E8 Expression of results

As the calibration curve is set up to establish a dilution series of oocyst DNA, the results are expressed from the qPCR instrument as the number of *Cryptosporidium* oocysts per PCR (oo / PCR). By using the calculation described above in section E7 which gives consideration to the starting volume of DNA extracted from the sample, this can be converted into oocysts per sample or per volume of water.

E9 Quality assurance

The quality assurance of the enumeration by qPCR relies upon the inclusion of suitable controls and performance monitoring.

The inclusion of the calibration curve enables performance monitoring of the qPCR assay as concentrations beyond the limit of detection are included. Any change in performance should be apparent from the results of the calibration curve.

The inclusion of an internal amplification control monitors for inhibition within each sample that may be caused by components of the sample that have been extracted alongside the DNA and have an inhibitory effect on the PCR, providing a potential false negative result.

The inclusion of positive and negative controls at the DNA extraction and PCR stages ensure that each of these procedures has worked properly (protecting against false negatives) and that there is no cross-contamination between samples (protecting against false positives).

There is currently no proficiency scheme available for enumeration by qPCR and laboratories should monitor their own performance using the controls described above and where possible spiking samples with known number of oocysts (preferably flow-counted and suitable for qPCR, e.g. not gamma irradiated which can affect molecular amplification).

Appendix 5 Validation procedures – enumeration by quantitative PCR

5.1 General

All technical criteria described here should be applied if any part of the method, so described, is altered (e.g. change in PCR master mix, change in qPCR platform) as this constitutes primary validation. For method verification (secondary validation) simplified requirements can be used in order to implement this validated method in the laboratory of a third party. The specificity of the assay (inclusivity) for newly described species or genotypes must be established and not assumed.

5.2 Inclusivity and exclusivity of probes and primers

Primers and probes used must give the expected results for all accepted *Cryptosporidium* species (i.e. human and non-human infectious species and genotypes). This should be established *in vitro* as far as possible, but it is acknowledged that due to the availability of some DNA *in silico* analyses may be required.

- Suggested inclusivity list – detection by PCR of genomic DNA from all available species and genotypes of *Cryptosporidium*, or homology *in silico* with primers and probes.
- Suggested exclusivity – tests on non-*Cryptosporidium* genomic DNA from organisms phylogenetically related to *Cryptosporidium*, or which have plausible presence in water samples. Examples of related organisms: *Neospora caninum, Toxoplasma gondii, Cyclospora cayatenensis, Eimeria tenella, Eimeria acervulina, Sarcocystis tenella.* Examples of those plausibly present: *Giardia duodenalis,* aquatic organisms, wildlife hosts, livestock and humans.

5.3 Verification of the calibration function of the quantitative PCR phase

5.3.1 Principle:

A calibration curve which is derived from Cq measurements from amplification of varying quantities of DNA can be constructed and the parameters of the line established. It is possible to use the equation of this line to calculate the number of oocysts present in an unknown sample corresponding to a particular Cq value.

The calibration curve may be constructed using plasmid DNA, genomic DNA or a synthetic oligonucleotide provided it is close in sequence and amplified product size to the template of interest. The calibration curve template must share the same primer and (if relevant) probe sequences.

qPCR instruments (e.g. RotorgeneQ) calculate these parameters for the user, and quantitative data can be read directly from the instrument output (after the establishment and application of an appropriate standard curve). The instrument calculates the quantitation based on the following equation where M = slope and B = y intercept:

$Cq = M \log_{10}[\text{concentration}] + B$

And is rearranged to:

$\log_{10}[\text{concentration}] = Cq - B / M$

For example:

Cq=26.92 (automatic RotorGene output = 1440 oocysts = 28,800 gene copies)

B= 38.777, M= -3.753

log_{10}[concentration] = (26.920-38.777) / -3.753

log_{10}[concentration] = 3.159

Manual Concentration = $10^{3.159}$ = 1442 = 28,840 gene copies

5.3.2 Calibration curve evaluation

Calibration should be evaluated under conditions suitable for determining reproducibility, carried out on at least different days, and/or with different operators. Prepare a range of DNA concentrations nominally defined as oocysts per PCR (oo / PCR) from 10^5 to 10^{-1}. From this range of concentrations a calibration curve can be produced by the qPCR instrument with the following acceptable parameters:

- amplification efficiency (E), determined from the slope of the log-linear portion (M), theoretic maximum 1.00 (which indicates that the amount of product doubles with each cycle). The acceptable range of E = 0.90 – 1.10 (90-110%)(corresponding to values of M between -3.58 and -3.10). E can be affected by amplicon length and sequence, and impurities or inhibitors (especially in highly concentrated samples) which can increase the E value in the sample.

- linearity (R2) which is the fitness of the data to the calibration curve. Linearity will indicate variability between replicates of the calibration curve; the value of R2 should be >0.98.

5.3.3 Use of the calibration curve

Use the formula in A5.3.1 above to calculate manually from the sample Cq value, or use the enumeration value returned by the qPCR machine.

5.4 Verification of the limit of quantification (LOQ)

The limit of quantification corresponds to the lowest concentration that can be quantified accurately. This needs to be established for the whole method as the quantification should be a reflection of the true number of organisms present in a sample, and is therefore affected by the upstream processing as well as the qPCR.

5.5 Verification of the limit of detection (LOD)

The limit of detection corresponds to the smallest number of genome units (oocysts or copies) that provides a PCR positive results in 95 % of tested samples (Bustin *et al*., 2009). This is determined by running replicate calibration curves that include samples at low-concentrations and identifying the level where there is positive amplification in at least

95% of the samples at a specific concentration. Additionally, this should be confirmed for the whole method and not just the qPCR by testing multiple spiked samples processed through all stages of the procedure.

5.6 Robustness

The repeatability (same user and same lab) and reproducibility (different user, different lab, and different day) of the method should be evaluated as a measure of the robustness of the assay.

Additionally, robustness could be determined through small changes in the protocol which may reasonably occur from time to time, these changes could include:

- qPCR instruments
- PCR master mixes or different lots of the same mix
- staff
- template origin (e.g. Flow-counted oocysts, in-house oocysts, spiked Tap Water, pDNA, gDNA and synthetic oligonucleotides)

5.7 Measurement of uncertainty

The measurement of uncertainty (MoU) considers the accuracy and precision of the method and when using qPCR can be determined for the PCR itself or the method as a whole including the processing, concentration and extraction of DNA from the oocysts. The accuracy describes how close the result is to the true value and is represented by the mean, whereas the precision describes the variation between the results and is indicated by the standard deviation.

To evaluate the MoU of the just the PCR, ten replicates of DNA containing known copy numbers / oocysts equivalents must be tested at low and higher levels (e.g. 10 and 100 oocysts). The DNA can be from measured pDNA or synthetically produced oligonucleotides.

To evaluate the MoU of the method as a whole, at least ten replicates of accurately enumerated oocysts must be spiked into water samples and processed according to the standard method. Ideally the oocysts should be flow-cytometry counted to obtain the level of accuracy required in the spiking suspensions.

5.8 Recovery method

The recovery of *Cryptosporidium* when detection and enumeration by qPCR does not only include the recovery of oocysts, but also the extraction of DNA from the sample. The recovery efficiency of the method as a whole should be determined using a known low (10) and high (100) number of oocysts spiked into at least 15 replicate samples as described in Appendix 4 Validation Procedures – enumeration by microscopy. The results should be compared with those obtained from paired samples processed by the reference method (IMS-IFM).

5.9 References

Bustin, S., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry, 55, 611-622.

Appendix 5A – Example data

5A.1 Verification of qPCR

5A.1.1 Inclusivity and exclusivity of probes and primers

The inclusivity and exclusivity of the primers and probe demonstrated specific amplification from a range of 29 different *Cryptosporidium* species/genotypes and no non-specific amplification from DNA representing 8 other parasites and 15 different species of hosts (UKWIR, 2020) (Table 5A1).

Table 5A1. Analytical specificity of the UKWIR18S assay (reproduced from UKWIR, 2020)

Cryptosporidium species and genotypes	Amplification	Non-*Cryptosporidium*	Amplification
Cryptosporidium hominis	Yes	Other parasites:	
Cryptosporidium parvum	Yes	*Giardia duodenalis* assemblage A	No
Cryptosporidium cuniculus	Yes	*Giardia duodenalis* assemblage B	No
Cryptosporidium ubiquitum	Yes	*Neospora caninum*	No
Cryptosporidium felis	Yes	*Toxoplasma gondii*	No
Cryptosporidium canis	Yes	*Cyclospora cayatenensis*	No
Cryptosporidium andersoni	Yes	*Eimeria tenella*	No
Cryptosporidium viatorum	Yes	*Eimeria acervulina*	No
Cryptosporidium meleagridis	Yes	*Sarcocystis tenella*	No
Cryptosporidium xiaoi	Yes		
Cryptosporidium occultus	Yes	Mammalian species:	
Cryptosporidium bovis	Yes	Human DNA	No
Cryptosporidium ditrichi	Yes	Field Vole	No
Cryptosporidium suis	Yes	Wood mouse	No
Cryptosporidium varanii	Yes	Ovine	No
Cryptosporidium ryanae	Yes	Bovine	No
Cryptosporidium baileyi	Yes	Grey seal	No
Cryptosporidium muris	Yes	Fox	No
Cryptosporidium erinacei	Yes	Polecat	No
Cryptosporidium proliferans	Yes	Red Deer	No
Cryptosporidium galli	Yes	Badger	No
Cryptosporidium serpentis	Yes	Horse	No
Cryptosporidium ducismarci	Yes	Serval	No
Cryptosporidium testudinis	Yes	Lion	No
Cryptosporidium tyzzeri	Yes	Goat	No
Cryptosporidium horse *gt*	Yes	Cat	No
Cryptosporidium skunk *gt*	Yes		
Cryptosporidium chipmunk gt 1	Yes		
Cryptosporidium ferret *gt*	Yes		

5A.1.2 Calibration curve

To establish the efficiency (E) and linearity (R2) of the qPCR assay, an 8 point, 10-fold dilution series of gBlock DNA created a calibration curve that was run in triplicate. All three parameters were acceptable (Table 5A2).

Table 5A2. Cq values for 8pt gBlock calibration curve, and dynamic data

No. "oocysts" equivalent of gBlock DNA / PCR	Mean Cq (n=3)	Cq range	Δ mean Cq
1000000	16.51	16.42-16.58	
100000	19.67	19.61-19.78	+3.16
10000	21.96	21.88-22.02	+2.29
1000	25.15	25.07-25.30	+3.29
100	28.92	28.79-29.03	+3.77
10	32.11	31.92-32.03	+3.19
1	36.32	35.01-37.10	+4.21
0.1	38.17	36.81-39.61	+1.85

R2	0.99
E	1.05
M	-3.203

5A.1.3 Limit of quantification

The limits of quantification of this PCR and the method as a whole are not yet understood due to the variation seen and the impact that this has on enumeration, particularly at low numbers of oocysts. There are several factors influencing this including losses in sample preparation and DNA extraction as well as within the qPCR itself. Additional work is required to further investigate this.

5A.1.4 Limit of detection

The limit of detection was determined to be 1 oocyst per PCR with all samples positive, and the next concentration (0.1 oocysts/PCR) only 10/21 positive (Table 5A3).

Table 5A3. Data from calibration curves to determine the qPCR limit of detection

PCR run	Cq Value at each concentration in the calibration curve							
	1,000,000 oo/PCR	100,000 oo/PCR	10,000 oo/PCR	1,000 oo/PCR	100 oo/PCR	10 oo/PCR	1 oo/PCR	0.1 oo/PCR
1	16.53	19.61	21.97	25.30	28.93	32.39	37.10	38.08
2	18.73	21.88	24.06	27.64	31.61	34.52	36.76	Neg
3	19.02	22.03	24.42	28.06	31.98	34.70	40.26	40.46
4	19.34	22.27	24.48	28.10	31.92	35.52	38.15	39.48
5	17.67	20.77	22.99	26.51	30.47	33.78	38.67	Neg
6	18.00	20.78	23.30	26.70	30.47	33.62	39.89	Neg
7	18.77	21.17	23.55	27.23	31.16	34.37	37.54	Neg
8	16.12	18.67	22.23	25.64	28.80	32.70	36.79	Neg
9	17.48	18.66	22.62	25.53	29.15	33.41	36.39	40.52
10	15.99	18.33	21.35	24.85	28.15	32.14	35.66	39.21
11	15.67	16.56	21.19	24.77	28.74	33.68	36.65	39.40
12	16.15	19.50	23.05	26.65	30.55	33.92	38.62	39.87
13	17.00	19.80	23.37	27.73	30.79	34.68	38.13	43.66
14	16.72	19.22	22.73	27.66	31.37	34.84	38.00	Neg
15	17.48	19.90	23.42	27.56	31.18	34.76	36.73	Neg
16	18.46	20.45	24.15	28.23	31.57	34.83	37.79	39.02
17	18.90	21.03	24.50	28.83	31.57	35.28	38.26	Neg
18	18.99	21.01	24.59	28.97	31.51	35.42	38.88	39.38
19	19.27	21.07	24.59	28.99	32.17	35.39	41.43	Neg
20	20.10	21.37	24.98	29.16	32.59	35.11	36.57	Neg
21	20.22	21.56	25.26	29.05	32.83	35.39	38.17	Neg
Positivity	21/21	21/21	21/21	21/21	21/21	21/21	21/21	10/21

5A.1.5 Robustness

Repeatability was investigated by testing a single set of gBlock DNA dilutions by the same scientist on three separate occasions. Although there were too few replicates for statistical analysis, graphical representation indicated the closeness of the data points for each occasion (Figure 5A1).

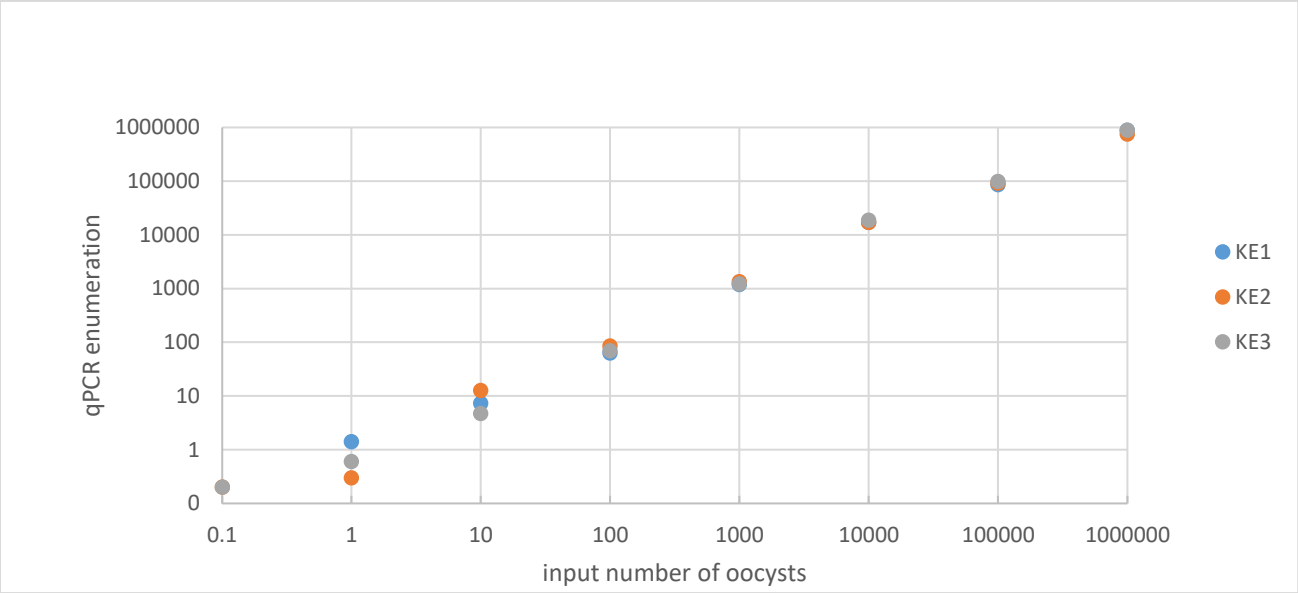


Figure 5A1. Repeatability of the qPCR

Reproducibility was investigated by a single set of gBlock DNA dilutions being tested three times each by two different scientists. Although there were too few replicates for statistical analysis, graphical representation indicates closeness of data points for each scientist (Figure 5A2).

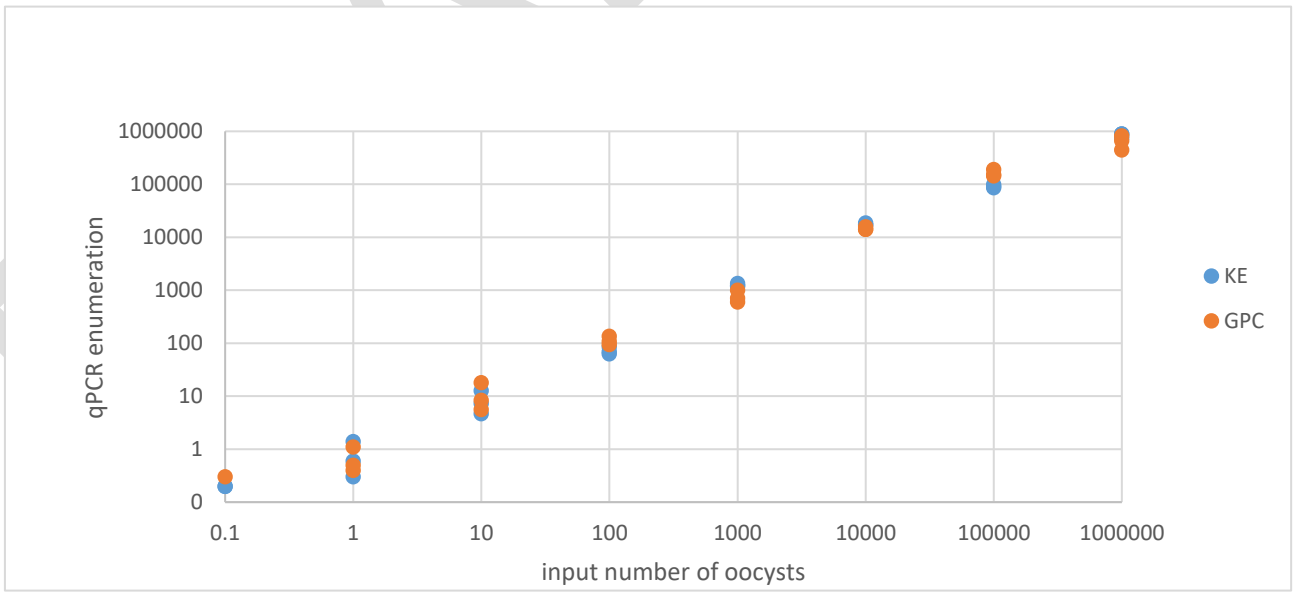


Figure 5A2. Reproducibility of the qPCR

5A.1.6 Measurement of uncertainty

The measurement of uncertainty, based on the accuracy (difference between the actual number of oocysts and measured number reported by the assay) and precision (closeness of values indicated by low standard deviation), of the qPCR was investigated using Cq values from ten replicates of gBlock DNA equivalent to high (100) and low (10) numbers of oocyst equivalents (Table 5A4).

Table 5A4. Accuracy and precision assessment of the qPCR

No. "oocysts" equivalent of gBlock DNA / PCR	Mean (range) n=10	Standard deviation	R^2	E	M
100	114.40 (97-133)	9.24	1.00	0.96	-3.411
10	9.30 (6-12)	1.85	1.00	1.01	-3.306

The gBlock DNA from target equivalent numbers of oocysts put into the PCR was accurately enumerated by the assay. The standard deviations indicated that precision was also acceptable.

5A.2 Phase 1 Validation of detection and enumeration by IMS-qPCR

Tap water was used for this Phase 1 study, sampled at one of the Dŵr Cymru Welsh Water (DCWW) treatment works, and 10 L samples were spiked with 0, 10 and 100 *C. parvum* oocysts, sorted and enumerated by flow cytometry (Wisconsin State Laboratory of Hygiene). Quality control data for the oocyst suspensions from the provider, calculated from a minimum of 12 calibration verification samples per set of 10 standards, reported the mean as 100, SD 1.3 for the 100 oocyst suspensions and a mean of 10, SD 0.0 for the 10 oocyst suspensions. Viability was 99.6% and oocysts were used within three months of preparation.

Spiking was done in pairs of water samples prior to processing by IMS; processing was according to the Blue Book method at the DCWW laboratory in Glaslyn, Newport. One sample of each pair was then tested by IFM according to the Blue Book method at Glaslyn, and one sample (the concentrate from IMS) was tested by qPCR method at the CRU in Swansea.

The set-up of the water sample batches for spiking and processing is shown in Figure 5A3 below. Each batch comprised three pairs of samples; zero spike control, spiked 10 and spiked 100 oocysts sample for each of the two detection methods.

For qPCR, all of the sample concentrate after IFM was used to extract DNA and then 4 aliquots of 20 µl DNA was tested by qPCR from each sample. The gBlock calibration curve material was run in all qPCR runs as a 5 data point, 10-fold dilution series representing DNA from 1000 to 0.1 oocysts per PCR. To provide equivalence with IFM where the entire final pellet is spotted on to a microscope slide, the final qPCR "count" was the sum of the number of oocysts detected in each aliquot adjusted for the whole DNA extract.

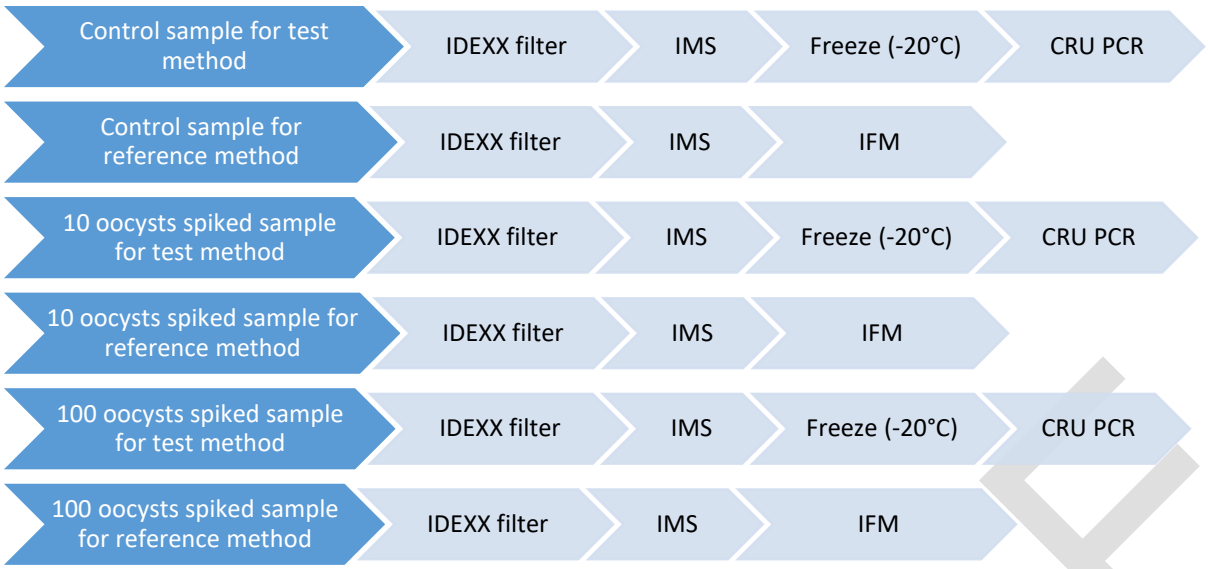


Figure 5A3. Illustration of the batch setup of samples for the Phase 1 study, to compare the qPCR test method and the IFM reference method.

Ideally, at least 15 samples would be tested by both qPCR and 15 by IFM spiked at each level of 0, 10 and 100 oocysts, and compared. However, due to an unforeseen failure of a Rotorgene qPCR machine used to test four of the batches, only 11 data points were available from qPCR at each spiked level. Therefore, the only the data from the paired spiked sample tested by IFM were used in the comparison of results.

Table 5A5. Summary of counts from trial qPCR method and reference IFM method

Batch sample	Number of spiked oocysts	Count qPCR	Count IFM	Difference (qPCR-IFM)	Recovery (%) qPCR	IFM
1	10	0.25	6	-5.75	3	60
2	10	0.13	3	-2.88	1	30
3	10	0.75	3	-2.25	8	30
4	10	0.13	5	-4.88	1	50
7	10	3.63	5	-1.38	36	50
8	10	1.88	1	0.88	19	10
11	10	1.50	2	-0.50	15	20
12	10	11.75	2	9.75	118	20
13	10	2.01	5	-2.99	20	50
14	10	1.08	3	-1.93	11	30
15	10	1.38	6	-4.63	14	60
Positivity	10	11/11	11/11	Mean	22.36	37.27
				Median	14	30
1	100	0.50	55	-54.50	1	55
2	100	1.00	45	-44.00	1	45
3	100	29.38	55	-25.63	29	55
4	100	12.75	56	-43.25	13	56
7	100	78.25	29	49.25	78	29
8	100	8.38	38	-29.63	8	38
11	100	102.38	12	90.38	102	12
12	100	14.75	23	-8.25	15	23
13	100	30.41	32	-1.59	30	32
14	100	45.73	51	-5.28	46	51
15	100	39.00	50	-11.00	39	50
Positivity	100	11/11	11/11	Mean	32.91	40.54
				Median	29	45

The number of samples positive for *C. parvum* was the same whether tested by qPCR or IFM when spiked with 10 or 100 oocysts, with all 11 samples positive at each level (Table 5A5). No false positives were detected in the zero spike samples by either method.

USEPA Method 1623 (USEPA, 2012) states mean recovery rates of 32-100 % are acceptable from matrix spiked samples for method modifications. The mean recovery rates for IFM detection fell within acceptable ranges at both spiking levels, but the qPCR detection was only within the acceptance criteria at the 100 spiking level (Table 5A5).

The data were also plotted in Figure 8. This shows the plots of the oocyst counts at 10 and 100 spiked oocysts tested by the two methods and includes the line of equality. At both 10 and 100 oocysts, two points lay above the line of equality where the trial qPCR method gave higher counts and eight points lay below the line where the reference IFM method gave higher counts.

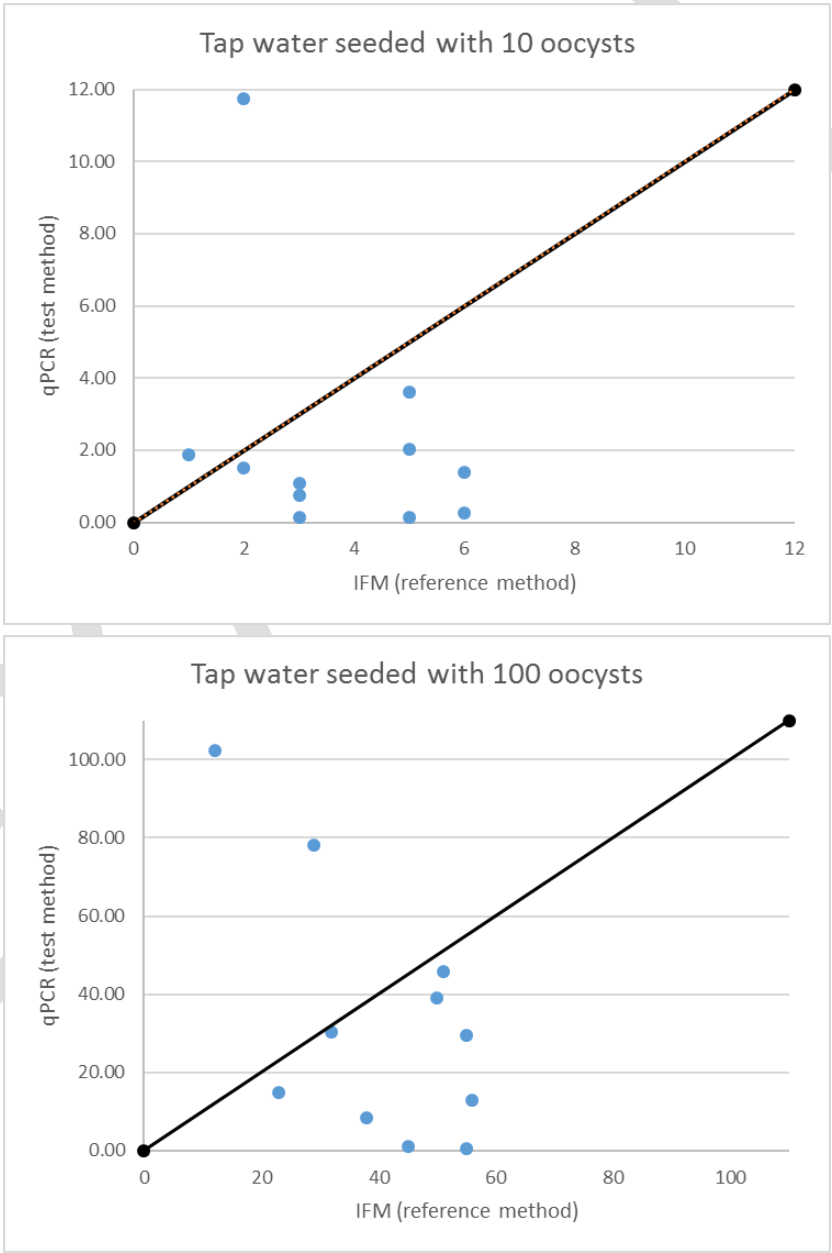


Figure 5A4. Oocysts detected by qPCR and IFM during the phase 1 study (line is line of equality)

As the data do not appear to be normally distributed, and the variances in the counts differed, a non-parametric test was used to test the null hypothesis that there was no difference in the median counts between the methods (Table 5A6). For samples spiked with 10 oocysts, the median number of oocysts detected by qPCR was significantly fewer (1.4, range 0.18-11.8) than detected by the reference method (3, range 1-6) (Mann-Whitney Two-Sample Test = 5.780, p value = 0.016) (Table 5A6). For samples spiked with 100 oocysts, the median number detected by qPCR was not significantly different (29.4, range 0.5-102.4) than detected by the reference method (45, range 12-56) (Mann-Whitney Two-Sample Test = 1.641, p value = 0.200) (Table 5A6). The standard deviation was greater by qPCR than the reference test at both spiking levels (Table 5A6).

Table 5A6. Summary of the statistical analysis of all the data from the Phase 1 study; comparison of the trial qPCR method with the reference IFM method.

Number of spiked oocysts	Tap water 10 L samples 11 duplicates	Oocysts per sample				
		Trial method qPCR*	Reference method IFM			
0 (Control)	Range	0-0	0-0			
	Mean	0	0			
	Median	0	0			
				Difference in counts	Recovery % qPCR	Recovery % IFM
10 (Spiked)	Range	0.1 to 11.8	1 to 6	-5.8 to 9.8	1 to 118	10 to 60
	Mean	2.2	4	-1.5	22	37
	Median	1.4	3	-2.3	14	30
	SD	3.3	1.74	4.0	32.0	17.37
100 (Spiked)	Range	0.5-102.4	12-56	-54.5 – 90.4	0.5-102	12-56
	Mean	33.0	41	-7.6	33	41
	Median	29.4	45	-11.0	29	45
	SD	32.4	14.87	41.0	31.0	14.89

*Calculated numbers have not been rounded to whole oocysts as qPCR can detect DNA from individual sporozoites or free DNA in the sample

When spiked with 10 oocysts, the Wilcoxon 95 % confidence interval for the difference between medians of the two tests was -3.87 to 2.00. Where the reference method gave a median of 3 oocysts, this suggests an estimated worse-case scenario of the trial qPCR method, on average, finding 29 % of those detected by the reference IFM method (i.e. 71 % fewer) and the best-case scenario of 167 % (i.e. 67 % more), calculated by (3-3.87)/3 and (3+2)/3 respectively, expressed as percentages.

When spiked with 100 oocysts, the Wilcoxon 95 % confidence interval for the difference between medians of the two tests was -34.81 to 23.56. Where the reference method gave a median of 45 oocysts, this suggests an estimated worse-case scenario of the trial qPCR method, on average, finding 23 % as many oocysts as the reference IFM method (i.e. 77 % fewer) and the best-case scenario of 152 % as many oocysts (i.e. 52 % more), calculated by (45-34.81)/45 and (45+23.56)/45, expressed as percentages.

The statistical comparisons showed there was a significant difference in the oocyst counts by qPCR and IFM when the spike was 10 oocysts, but no significant difference when the spike was 100 oocysts. However, the lower value of the 95 % confidence interval for the difference between the medians of the two tests implied that the qPCR could be very much worse than the reference method. More samples would need to be examined to

increase the power of the study and therefore reduce the confidence interval and clarify the worst-case scenario.

Appendix 7 – UKWIR 2020 qPCR Protocol

Assay name: UKWIR18S
Test target: 18S gene of All *Cryptosporidium* species and genotypes
Description: a Taqman probe-based qPCR for the detection and characterisation of *Cryptosporidium* spp.
This assay has been validated as described; any modification of these conditions may alter its performance.

Assay design:

Element	Name	Sequence 5'-3' (nt location in specified reference sequence)
Forward primer	JF1/2	F1: AAGCTCGTAGTTggatTTCTG (601-621 L16996 *C. parvum*) F2: AAGCTCGTAGTTaatcTTCTG (601-621 L16996 *C. parvum*)
Reverse primer	JR	R: CCTTACTCCTTCAGCACCTTA (1015-1035 L16996 *C. parvum*)
Probe [Cy5-XXXX-MGB-EQ]	JT2	TCAGATACCGTCGTAGTCT (958-976 L16996 *C. parvum*)

Assay components:

Component	Specific reagent	Volume (µl)
PCR master mix	Bioline SensiMix™ II probe kit	25
Forward primer [50µM]	JF1/2 (1:1 mix of each JF1 and JF2)	0.6
Reverse primer [50µM]	JR	0.6
Probe [10µM]	JT2	0.38
Internal amplification control (IAC) reagents (Deer *et al*., 2010)	IAC primers and probe	2.5
Water	Any nuclease free / molecular grade	0.9
Template DNA		20
Total volume:		50

Reaction conditions:

Stage	Thermocycler: RotorgeneQ 6000, or 6-plex		
	Temperature	Duration (sec)	Comments
Hold (Hot start)	95 °C	600	
Cycling (50 cycles)	95 °C	15	
	60 °C	60	Acquiring on appropriate channel, with calibration = autogain optimisation on first acquisition.
	72 °C	30	

Assay format:

Transparent (polypropylene) tube type Gene-disc 72 0.2 ml (36-well rotor) 0.1 ml (72-well rotor)	**For quantification of oocyst numbers in spiked or field samples, test 4 aliquots of DNA and normalise the count for the total sample processed** **Include single replicate calibration curve standards; at least 5 dilution points from 10000 oocysts or equivalents per PCR.**

Interpretation:

Analyte	Comments
Fluorophore	Analyse data from the [Cy5 (red)] fluorophore for *Cryptosporidium* target, and [FAM (green)] for IAC
Slope correct	Apply the (noise) slope correction to normalise against background fluorescence.
Apply threshold	Apply a fluorescence threshold of 0.05.
Ct values	Check Ct values obtained for samples/controls and that the NTC has no value. Apply personal upper limits if required.
Amplification curve	All *Cryptosporidium* curves should be sigmoidal and reach high fluorescence.
Expected detection range	Lower limit 1 oocyst per PCR [from gBlock calibration curve]
Expected specificity	All *Cryptosporidium* species and genotypes
Quantification	For the calibration curve use a minimum 5-point 10-fold serial dilution of quantified gBlock DNA gBlock synthetic oligonucleotide (sequence adapted from L16996 *C. parvum* to increase the GC content and produce a unique sequence to detect in the event of cross-contamination) AAGCTCGTAGTTGGATTTCTGTTAgTgATcTgTgTgAgAcAcTcTGATG AgTgTcTgcATgAcAcTggCATAgTcCATATcACTAcAcATTccAGTgcgcGA ggTTTTACTTTGAGAgAgTcAGAGTGCTTAgAGCAGGCATATGCCTTG AATACTCCAGCATGGAgcggTgTcAggGATTTTTATCTTTCTTATTGGTT CTAAGATgAGAgcggcGATTggcAGGGACAGTTGGGGGTACTTGTgccTA ACAGTCAGAGGTGAAATTCTTAGATTTGcTAgAGACAAgCcgATGCGg AAGCAccTGCCAAGGAcGTcTTCgTcAgTCggGAACGggAGccAGGGGAT CGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCA ACTAGAGATTGGAGGTTGTTCCTTACTCCTTCAGCACCTTAT For test samples, normalise 4 results from the oocysts per PCR to oocysts per sample.

Example PCR processing batch record sheet – see next page.

CRU DWI UKWIR18S qPCR run sheet

A. Sample & PCR preparation:

CRU PCR batch No.		

	Date	Initials	
EpMotion UV'd			

CRU Extraction date:		
DNA mini kit lot#:		
56 / 100C block checked / date		
PCR date		
Operator		
Rotorgene		

	Sample Ring position	CRU ref:	Red channel Ct	Green channel ct
1	A1	*sample1-1*		
2	A2	*sample1-2*		
3	A3	*sample1-3*		
4	A4	*sample1-4*		
6	A6	*sample 2-2*		
7	A7	*sample 2-3*		
8	A8	*sample 2-4*		
9	B1	*sample 3-1*		
10	B2	*sample 3-2*		
11	B3	*sample 3-3*		
12	B4	*sample 3-4*		
13	B5	*sample 4-1*		
14	B6	*sample 4-2*		
15	B7	*sample 4-3*		
16	B8	*sample 4-4*		
17	C1	*sample 5-1*		
18	C2	*sample 5-2*		
19	C3	*sample 5-3*		
20	C4	*sample 5-4*		
21	C5	*sample 6-1*		
22	C6	*sample 6-2*		
23	C7	*sample 6-3*		
24	C8	*sample 6-4*		
25	D1	*sample 7-1*		
26	D2	*sample 7-2*		
27	D3	*sample 7-3*		
28	D4	*sample 7-4*		
29	D5	*NTC*		
30	D6	*CC 10000*		
31	D7	*CC 1000*		
32	D8	*CC 100*		
33	E1	*CC 10*		
34	E2	*CC 1*		
35	E3			
36	E4			

	Total Vol.	Lot number	Expiry date
SENSIMIX	270.0		
JF1/2 (50uM)	26.0		-
JR (50uM)	26.0		-
JT2 (10uM)	23.8		-
nfH_2O	29.2		-
IAC / P+P	220.0		
Calibration curve	-		

		No. PCRs
	x1	10
SENSIMIX	25	250.0
JF1/2 (50uM)	0.6	6.0
JR (50uM)	0.6	6.0
JT2 (10uM)	0.38	3.8
IAC / P+P [FAM]	2.5	25.0
[DNA]	20	200.0
nfH_2O	0.92	9.2
	50	500.0

Notes:	
PCR programme	DWI UKWIR
Taqman probe	JT2 - Cy5, red channel
IAC probe	FAM - green channel
Template volume	20 µl x 4

Appendix 8 – Sequence of the synthetic gBlock DNA [L16996 gBl], compared with the native, reference *C. parvum* sequence [L16996 C.p]

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                       410        420        430        440        450
F PRIMER        ---------A AGCTCGTAGT TGGATTTCTG ---------- ----------
R PRIMER        ---------- ---------- ---------- ---------- ----------
TAQMAN MGB      ---------- ---------- ---------- ---------- ----------
L16996 C.p      ~~~~~~~~~A AGCTCGTAGT TGGATTTCTG TTAATAATTT ATATAAAATA
L16996 gBl      ~~~~~~~~~A AGCTCGTAGT TGGATTTCTG TTAgTgATcT gTgTgAgAcA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       460        470        480        490        500
F PRIMER        ---------- ---------- ---------- ---------- ----------
R PRIMER        ---------- ---------- ---------- ---------- ----------
TAQMAN MGB      ---------- ---------- ---------- ---------- ----------
L16996 C.p      TTTTGATGAA TATTTATATA ATATTAACAT AATTCATATT ACTATATATT
L16996 gBl      cTcTGATGAg TgTcTgcATg AcAcTggCAT AgTcCATATc ACTAcAcATT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       510        520        530        540        550
F PRIMER        ---------- ---------- ---------- ---------- ----------
R PRIMER        ---------- ---------- ---------- ---------- ----------
TAQMAN MGB      ---------- ---------- ---------- ---------- ----------
L16996 C.p      TTAGTATATG AAATTTTACT TTGAGAAAAT TAGAGTGCTT AAAGCAGGCA
L16996 gBl      ccAGTgcgcG AggTTTTACT TTGAGAgAgT cAGAGTGCTT AgAGCAGGCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       560        570        580        590        600
F PRIMER        ---------- ---------- ---------- ---------- ----------
R PRIMER        ---------- ---------- ---------- ---------- ----------
TAQMAN MGB      ---------- ---------- ---------- ---------- ----------
L16996 C.p      TATGCCTTGA ATACTCCAGC ATGGAATAAT ATTAAAGATT TTTATCTTTC
L16996 gBl      TATGCCTTGA ATACTCCAGC ATGGAgcggT gTcAggGATT TTTATCTTTC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       610        620        630        640        650
F PRIMER        ---------- ---------- ---------- ---------- ----------
R PRIMER        ---------- ---------- ---------- ---------- ----------
TAQMAN MGB      ---------- ---------- ---------- ---------- ----------
L16996 C.p      TTATTGGTTC TAAGATAAGA ATAATGATTA ATAGGGACAG TTGGGGGTAC
L16996 gBl      TTATTGGTTC TAAGATgAGA gcggcGATTg gcAGGGACAG TTGGGGGTAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       660        670        680        690        700
F PRIMER        ---------- ---------- ---------- ---------- ----------
R PRIMER        ---------- ---------- ---------- ---------- ----------
TAQMAN MGB      ---------- ---------- ---------- ---------- ----------
L16996 C.p      TTGTATTTAA CAGTCAGAGG TGAAATTCTT AGATTTGTTA AAGACAAACT
L16996 gBl      TTGTgccTAA CAGTCAGAGG TGAAATTCTT AGATTTGcTA gAGACAAgCc
```

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                       710        720        730        740        750
F PRIMER        ---------- ---------- ---------- ---------- ----------
R PRIMER        ---------- ---------- ---------- ---------- ----------
TAQMAN MGB      ---------- ---------- ---------- ---------- ----------
L16996 C.p      AATGCGAAAG CATTTGCCAA GGATGTTTTC ATTAATCAAG AACGAAAGTT
L16996 gBl      gATGCGgAAG CAccTGCCAA GGAcGTcTTC gTcAgTCggG AACGggAGcc


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       760        770        780        790        800
F PRIMER        ---------- ---------- ---------- ---------- ----------
R PRIMER        ---------- ---------- ----------~ ~~~~~~~~~~ ~~~~-~~~~~
TAQMAN MGB      ---------- ------TCAG ATACCGTCGT AGTCT----- ----------
L16996 C.p      AGGGGATCGA AGACGATCAG ATACCGTCGT AGTCTTAACC ATAAACTATG
L16996 gBl      AGGGGATCGA AGACGATCAG ATACCGTCGT AGTCTTAACC ATAAACTATG


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       810        820        830        840        850
F PRIMER        ---------- ---------- ---------- ---------- ----------
R PRIMER        ~~~~~~~~~~ ~~~------- ---CCTTACT CCTTCAGCAC CTTA------
TAQMAN MGB      ---------- ---------- ---------- ---------- ----------
L16996 C.p      CCAACTAGAG ATTGGAGGTT GTTCCTTACT CCTTCAGCAC CTTAT.....
L16996 gBl      CCAACTAGAG ATTGGAGGTT GTTCCTTACT CCTTCAGCAC CTTAT.....
```

Appendix 9 – Test of optimal DNA extraction elution volume

To determine the effect of elution buffer volume on PCR outcome, DNA from flow cytometry-counted oocysts was extracted and tested using the UKWIR18S qPCR at the CRU. The DNA extraction method included 8 cycles of freeze-thawing followed by purification using QIAamp DNA mini kit.

As the testing is for the detection and enumeration of oocysts in water it is important that the entire DNA sample is tested. Additionally, as the number of oocysts in an operational monitoring sample would be unknown, the 20 µl DNA template format UKWIR18S PCR must be used in case the number of oocysts, if present, is <5.

Experimental Plan

This experiment will use 10 tubes of flow cytometry-counted oocysts (from the batch received in March 2020), each containing 10 oocysts. The DNA extraction will start by freeze-thawing the oocysts in the original tubes. Five extracts will be eluted from the spin columns in 50 µl of elution buffer and the other five eluted in 100 µl of elution buffer. Multiple replicates of UKWIR18S PCR (20 µl template) will be used to test the DNA (2 for 50 µl elution and 4 for 100 µl elution – there is always less eluted than the volume loaded onto the column).

The aim is to identify the optimal volume for elution in the DNA extraction by comparing the difference between 50 µl elution with 100 µl elution (rather than the variability between individual PCRs as this has been addressed previously during validation of the PCR). Therefore the Ct values generated from all 50 µl PCRs will be investigated as a group and compared with those generated from 100 µl using EpiInfo.

Results and Discussion

All of the PCRs were positive regardless of elution volume. The 50 µl elution samples (n=10) produced a range of Cqs from 34.05 – 41.76, with a median of 35.10 and a mean of 36.30 (+/- 2.59 SD). The 100 µl elution samples (n=20) generated a range of 34.89 – 37.31, with a median of 35.70 and a mean of 35.83 (+/- 0.56 SD). The results and statistical analyses are shown below.

Oocyst tube	Elution volume µl	PCR1 Ct	PCR2 Ct	PCR3 Ct	PCR4 CT
1	50	34.05	34.20	N/A	N/A
2	50	37.48	37.39	N/A	N/A
3	50	34.41	34.35	N/A	N/A
4	50	34.87	35.33	N/A	N/A
5	50	41.76	39.17	N/A	N/A
6	100	35.63	36.33	36.32	35.05
7	100	36.40	35.35	34.89	35.55
8	100	35.37	36.09	35.60	35.53
9	100	35.50	35.77	35.55	35.78
10	100	36.02	36.13	36.38	37.31
-50 (Neg control)	50	Neg	Neg	N/A	N/A
-100 (Neg control)	100	Neg	Neg	Neg	Neg

ELUTION	Obs	Total	Mean	Variance	Std Dev
50	10	363	36.301	6.718	2.592
100	20	717	35.828	0.310	0.557
Difference			0.474		

ELUTION	Minimum	25%ile	Median	75%ile	Maximum	Mode
50	34.050	34.350	35.100	37.480	41.760	34.050
100	34.890	35.515	35.700	36.225	37.310	35.550

ANOVA
(For normally distributed data only)

Variation	SS	df	MS	F statistic	p-value	t-value
Between	1.495	1	1.495	0.631	0.433763	0.794193
Within	66.352	28	2.370			
Total	67.847	29				

Bartlett's test for homogeneity of variance
Bartlett's chi square = 28.069 deg freedom = 1 p-value = 0.000000

Bartlett's Test shows the variances in the samples to differ.
Use non-parametric results below rather than ANOVA.

Mann-Whitney or Wilcoxon Two-Sample Test (Kruskal-Wallis test for two groups)

Kruskal-Wallis H (equivalent to Chi square) =	0.627
Degrees of freedom =	1
p value =	0.428371

Although the mean Cqs are similar (36.30 for 50 µl and 35.83 for 100 µl), the Ct values for 100 µl elutions are a lot more consistent than those from the 50 µl elution. This is particularly visible in tubes 2 and 5 where the Cqs are higher than the other 50 µl tubes and is also supported by the variance and standard deviations around the means for the two groups (6.7 and 2.6 for 50 µl compared to 0.3 and 0.6 for 100 µl). Although the difference between the two elution volumes is not statistically significant ($p = 0.4$) the variation between samples eluted in 50 µl could have an impact on the quantitation using the standard curve in the qPCR assay.

Also, while the PCR has shown during validation to give good reproducible results, at very low amounts of template variation between PCR assays can increase as the DNA is not completely homogenous. This is often seen with DNA from single figure oocysts when genotyping and it looks like the same may have happened in tube 5 where very high Cqs were observed with more variation between the two replicates than in the other samples, suggesting a low amount of target DNA have been extracted from this tube.

This is very important with the aim of producing a qPCR as variation with very low numbers of oocysts will result in inaccurate quantification results and false counts of oocyst numbers.

Conclusion

Elution in 100 µl provided the most consistent results which although increases the cost of testing suggests that quantitation should be more precise and with the addition of additional replicates has the added benefit of increasing the potential of detecting multiple species if present in the sample.

Appendix 10 – Spiking trial protocol for Objective 4 at Dwr Cymru Welsh Water (DCWW)

The initial stages of the *Cryptosporidium* sample preparation protocol will follow those routinely used by DCWW either through to microscopic screening (half of the samples in each batch), or to the indicated point where the testing will halt and the concentrated samples (with oocysts still attached to the beads) will be frozen prior to shipping to the Cryptosporidium Reference unit (CRU) in Swansea for DNA extraction and PCR testing (the other half of the samples).

Overview (from the contract)

Objective 4. Duplicate analysis of spiked *Cryptosporidium* samples, using the current blue book method for *Cryptosporidium* analysis, alongside the method of PCR deemed suitable.

Samples will be spiked and prepared using industry standard equipment (IDEXX Filta-Max Xpress™ and Dynabeads™ anti-*Cryptosporidium* IMS Kit) according to the Blue Book method in subcontractor Dŵr Cymru Welsh Water's Glaslyn laboratory, which is accredited by UKAS in accordance with the International Standard ISO17025 and Lab37 and Drinking Water Testing Specifications.

Fifteen samples per assay will be prepared, using high numbers of oocysts (100) and low numbers of oocysts (10) spiked, with each sample having a negative control (Blue Book Appendix 4) (Figure 1 below). As the detection target is different for each assay (PCR detects DNA, IFM detects oocyst wall antigens) the negative controls will also need to be tested by each assay. It is proposed that the spiked and control samples are set up in parallel batches for true comparison, and to facilitate working within the laboratory's capacity (Figure 2 below).

There will be 15 batches of 6 samples to process to meet the requirements of Figure 1; if these are done on a weekly basis the experiments will be run over 15 weeks. This timescale is in consideration of the current workload of the DCWW laboratory, and well within capacity and will therefore mitigate against delay even if the laboratory is required to test extra samples for other commitments (such as investigation of an incident) during the course of the project.

The samples for PCR will be frozen to -20 ^{o}C post-IMS and stored until transported frozen to the CRU in Swansea for DNA extraction and PCR. Keeping the samples frozen until preparation for testing by a PCR-based method mitigates against the risk of samples needing to be tested should the workload of the laboratory at the time be unexpectedly increased for example during the investigation of a large-scale outbreak.

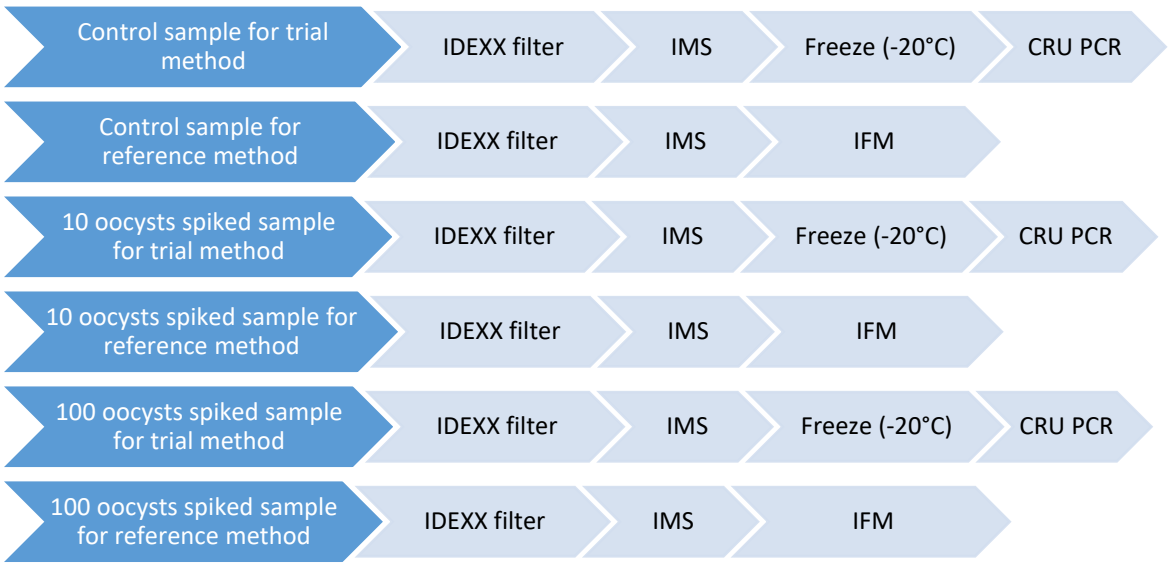
The *Cryptosporidium* oocysts for the spiking experiments will be purchased as commercially available flow cytometry sorted oocysts in suspension supplied with manufacturer's certification of numbers. The oocysts will be transferred according to the manufacturer's instructions.

The methods will be compared in terms of oocyst "counts" and recovery, as well as other attributes (such as ease of use, interpretation, turnaround time and so on). We therefore propose that, should the findings of the spiked tap water experiment prove promising, the comparison should progress to further in-house validation, should the funders wish this. We therefore include an option to investigate whether detection and enumeration, including ease of interpretation, is significantly affected by specific types of water. To do this,

Figure 1: Proposed design of initial comparison (Phase 1) of a PCR-based method with reference method (IFM).

Number of spiked oocysts	Tap water 10 L samples	Trial method counts	Reference method counts			
0 (Control)	1-15 (duplicate samples)					
	Range Mean Median					
				Difference in counts	**Recovery % Trail method**	**Recovery % reference method**
10 (Spiked)	1-15 (duplicate samples)					
	Range Mean Median Standard deviation					
100 (Spiked)	1-15 (duplicate samples)					
	Range Mean Median Standard deviation					

Figure 2. Illustration of how a batch of 6 samples (4 x spiked and 2 x negative controls) will be set up for analysis by one PCR-based trial method and the IFM reference method.



three sites will be selected to include a variety of raw waters of different characteristics (pH, conductivity, turbidity, colour and suspended solids will all be recorded). The test will be repeated on five separate (not necessarily consecutive) days (Figure 3). Paired samples plus controls will be used on each occasion. At one site using half (i.e. 0.5x) the target number and twice (i.e. 2x) the target number of oocysts will be used. Again, the timescale scheduled for the spiking work (15 weeks) is in consideration of the current workload of the laboratory, and well within capacity and will therefore mitigate against delay even if the laboratory is required to test extra samples for other commitments (such as investigation of an incident) during the course of the project.

Figure 3: Proposed design and analysis for the optional comparison (Phase 2) of developed PCR-based method with the reference method, IFM, at raw water three sites.

Number of spiked oocysts	**Site; days (duplicate 10 L samples)**	**Trial method counts**	**Reference method counts**			
0	1; 1-5					
	2; 1-5					
	3; 1-5					
	Range Mean Median					
				Difference in counts	**Recovery % trail method**	**Recovery % reference method**
100	1; 1-5					
	2; 1-5					
	3; 1-5					
	Range Mean Median SD/95% CI*					
50	3; 1-3					
200	3; 1-3					

*SD = standard deviation, CI = confidence interval

Methods

The methods used for this project will be based on the routine methods used by DCWW according to their standard operating procedures (LPM071 The preparation, recovery and identification of *Cryptosporidium* oocysts in treated and raw water samples) when spiking and testing 10 L grab samples for quality control. There are a few important changes:

- The samples will be spiked with the oocyst suspensions supplied specially for the project, but the spiking will be done according to Step 10.6 in DCWW's protocol LPS108 (*Cryptosporidium* Daily Quality Control Sample). This protocol is focused on the spiking of AQC rigs with EasySeed, but the process is the same for 10 L samples. There is one exception: as the tubes containing the spiking oocysts are smaller than EasySeed, the volumes for spiking have been adjusted.

- Half the samples in each batch will not proceed to dissociation and microscopy, but will be frozen instead
- There are special batch record sheets for the project.

More details are provided below.

The 10 L samples will be tap water from one of the DCWW treatment works in the first phase and raw water (source to be confirmed) in the optional second phase.

The oocysts to be spiked will be provided by the CRU from Wisconsin State Laboratory of Hygiene as flow cytometry-counted suspensions containing 10 or 100 oocysts for the tap water phase and 50, 100 or 200 oocysts for the raw water phase, in 200 µl reverse osmosis (RO) water with 0.01% Tween20. They will be transferred from the CRU to DCWW chilled, and DCWW will email the CRU to record receipt on arrival. Spiking suspensions must be stored immediately in an appropriate refrigerator at 5±3°C.

The 1.5 ml tubes labelled in step 19.10 of LPM071, and used to transfer the beads from the Leighton tubes in step 19.13 of LPM071 must be screw-capped tubes as these will be used directly in DNA extraction of the test method samples at the CRU; these tubes must also be used for the reference method samples to standardise the recovery method for comparison of results. These tubes will be supplied by the CRU.

For the reference method samples, DCWW will supply the microscope slides used routinely.

The nuclease free water for the re-suspension of oocyst-bead complexes will be supplied by the CRU.

The processed tubes and slides must be labelled according to the results sheet for that batch (see sheets below). The sample naming format is based on Batch Number (1-15 for tap [Figure 1.] and 16-30 for raw Figure 3.]), Method Type (Trial Method is TM and Reference Method is RM) and number of oocysts spiked (0, 10, 100 for tap and 0, 50, 100, 200 for raw). For example, the sample from Batch 1 processed by the Trial Method and spiked with 10 oocysts is 1TM10, and the sample from Batch 2 processed by the Reference method and spiked with 100 oocysts is 2RM100.

Spiking 10 L samples
Spike the 10 L samples using the method described in Section 10.6 LPS108 with the following adjustments to the volumes as oocysts supplied are 200 µl of suspension in 2 ml tubes. Add 1 ml of 0.05% of Tween20 to the vial of oocysts, replace the cap and vortex for approximately 20 seconds. Transfer as usual. Add 1.5 ml of DI water to the vial and vortex for approximately 20 seconds. Transfer as usual. Add another 1.5 ml of DI water and vortex for approximately 20 seconds. Transfer as usual.

Tap water samples
For each tap water batch, six 10 L samples will be spiked each with the appropriate number of oocysts (0, 10 and 100 oocysts in duplicate – total of 6 samples), processed through IDEXX Filta-Max Xpress™, then IMS with Dynabeads™ anti-*Cryptosporidium* IMS Kit.

In each batch of six, the three trial method samples (one at each spiking level) will proceed up to completion of point 19.17 in protocol LPM071, at which point 200 µl of nuclease free water (supplied by the CRU) will be added to re-suspend the beads, and the samples frozen upright in the provided storage box at -20°C (Figure 2).

The three reference method samples will proceed all the way through to microscopic examination (Figure 2) and the results recorded on the results sheets provided.

Raw Water Samples

For each raw water batch, 10 L samples will be spiked each with the appropriate number of oocysts (Batches 16-27: 0 and 100 oocysts in duplicate – total of 4 samples, Batches 28-30: 0, 50, 100 and 200 oocysts in duplicate – total 8 samples), processed through IDEXX Filta-Max Xpress™, then IMS with Dynabeads™ anti-*Cryptosporidium* IMS Kit.

In each batch, the two (or four in Batches 28-30) trial method samples (one at each spiking level) will proceed up to completion of point 19.17 in protocol LPM071, at which point 200 µl of nuclease free water (supplied by the CRU) will be added to re-suspend the beads, and the samples frozen upright in the provided storage box at -20°C (Figure 2).

The reference method samples will proceed all the way through to microscopic examination (Figure 2) and the results recorded on the results sheets provided.

Results

The results of the microscopy for the reference method samples, and documentation of re-suspension and freezing for the trial method samples, will be recorded on the prepared result sheets at the end of this document. The results sheets should then be scanned and emailed to the CRU (Swansea.Crypto@wales.nhs.uk), with the subject line "DWI Project".

Storage of samples

Reference Method Samples

The reference method samples will end up as microscopy slides having been screened by an analyst at DCWW. The coverslip must be sealed properly to prevent drying and the slides must be stored in an appropriate slide storage box (opaque to protect from quenching by external light) in the allocated sample fridge in the microscopy dark room. These must be stored until the end of the project, but do not need transferring to the CRU unless requested. Slides must be labelled with the sample number as per the results sheets below.

Trial Method Samples

The trial method samples must remain in the -20°C freezer (the appropriate freezer will be identified by Matthew Jones) in the provided sample box with each 1.5 ml screw-capped tube labelled with the sample number as per the results sheets below, until packaging and transfer to the CRU when requested to do so.

Packaging and transfer of trial method samples to the CRU

The trial method samples, must remain frozen during transfer to CRU. This is vital as once thawed, oocysts could let in components that damage DNA and/or affect the downstream PCR testing. The logistics for the transfer of these samples to the CRU will be decided upon prior to shipping. Options may include shipping with a courier licenced to ship dry ice or someone from the project transferring the samples by car.

Results: Batch 1 - Tap Water (Samples: 1TM0, 1RM0, 1TM10, 1RM10, 1TM100, 1RM100)

	Date	Analyst	Additional Details
6 x 10 L samples collected			Source and location:
Duplicate 10 L samples spiked with 0, 10 & 100 oocysts			
10 L samples filtered			Filter Batch No.:
Filters eluted			
Immunomagnetic separation			IMS Kit Batch No.:
Trial method samples re-suspended in 200 µl and stored at -20°C			
Reference Method samples dissociated and fixed on slides			
Slides stained (inc. +ve stain control)			Staining Kit Batch No.:
Slides examined			FITC Control slide lot #: +ve Stain Control Checked:
Slides stored at 5 ± 3°C			

Sample number	Total number of intact oocysts (with contents inside or just outside the oocyst by DAPI & DIC)	Comments
1RM0		
1RM10		
1RM100		