Report No. 137

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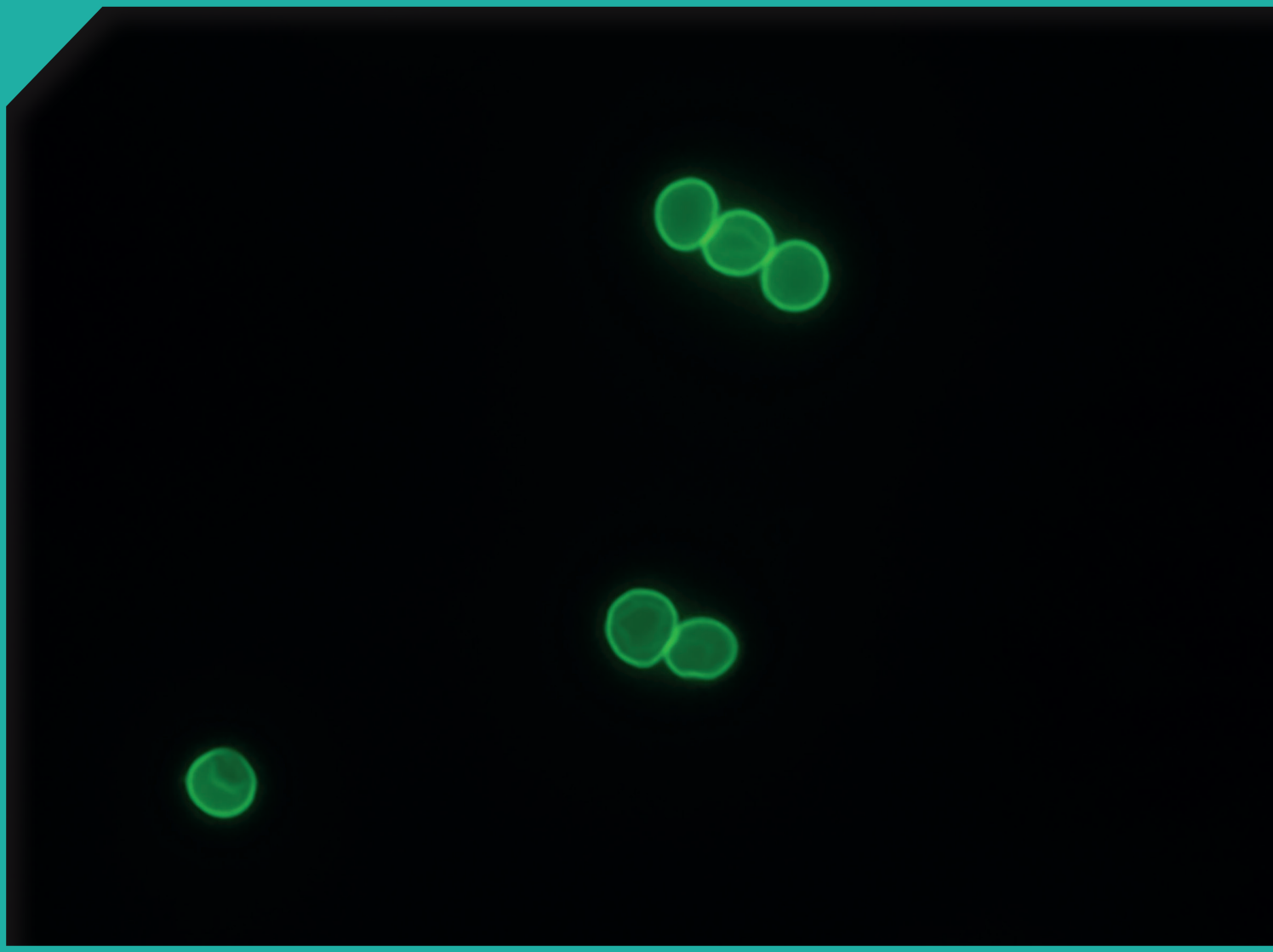
Identifying pressures • Informing policy • Developing solutions

Towards Developing a Cryptosporidium Monitoring Protocol



Comhshaol, Pobal agus Rialtas Áitiúil
Environment, Community and Local Government

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ENVIRONMENTAL PROTECTION AGENCY

The Environmental Protection Agency (EPA) is responsible for protecting and improving the environment as a valuable asset for the people of Ireland. We are committed to protecting people and the environment from the harmful effects of radiation and pollution.

The work of the EPA can be divided into three main areas:

Regulation: *We implement effective regulation and environmental compliance systems to deliver good environmental outcomes and target those who don't comply.*

Knowledge: *We provide high quality, targeted and timely environmental data, information and assessment to inform decision making at all levels.*

Advocacy: *We work with others to advocate for a clean, productive and well protected environment and for sustainable environmental behaviour.*

Our Responsibilities

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We regulate the following activities so that they do not endanger human health or harm the environment:

- waste facilities *(e.g. landfills, incinerators, waste transfer stations)*;
- large scale industrial activities *(e.g. pharmaceutical, cement manufacturing, power plants)*;
- intensive agriculture *(e.g. pigs, poultry)*;
- the contained use and controlled release of Genetically Modified Organisms *(GMOs)*;
- sources of ionising radiation *(e.g. x-ray and radiotherapy equipment, industrial sources)*;
- large petrol storage facilities;
- waste water discharges;
- dumping at sea activities.

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- Conducting an annual programme of audits and inspections of EPA licensed facilities.
- Overseeing local authorities' environmental protection responsibilities.
- Supervising the supply of drinking water by public water suppliers.
- Working with local authorities and other agencies to tackle environmental crime by co-ordinating a national enforcement network, targeting offenders and overseeing remediation.
- Enforcing Regulations such as Waste Electrical and Electronic Equipment (WEEE), Restriction of Hazardous Substances (RoHS) and substances that deplete the ozone layer.
- Prosecuting those who flout environmental law and damage the environment.

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- Monitoring and reporting on the quality of rivers, lakes, transitional and coastal waters of Ireland and groundwaters; measuring water levels and river flows.
- National coordination and oversight of the Water Framework Directive.
- Monitoring and reporting on Bathing Water Quality.

Monitoring, Analysing and Reporting on the Environment

- Monitoring air quality and implementing the EU Clean Air for Europe (CAFÉ) Directive.
- Independent reporting to inform decision making by national and local government *(e.g. periodic reporting on the State of Ireland's Environment and Indicator Reports)*.

Regulating Ireland's Greenhouse Gas Emissions

- Preparing Ireland's greenhouse gas inventories and projections.
- Implementing the Emissions Trading Directive, for over 100 of the largest producers of carbon dioxide in Ireland.

Environmental Research and Development

- Funding environmental research to identify pressures, inform policy and provide solutions in the areas of climate, water and sustainability.

Strategic Environmental Assessment

- Assessing the impact of proposed plans and programmes on the Irish environment *(e.g. major development plans)*.

Radiological Protection

- Monitoring radiation levels, assessing exposure of people in Ireland to ionising radiation.
- Assisting in developing national plans for emergencies arising from nuclear accidents.
- Monitoring developments abroad relating to nuclear installations and radiological safety.
- Providing, or overseeing the provision of, specialist radiation protection services.

Guidance, Accessible Information and Education

- Providing advice and guidance to industry and the public on environmental and radiological protection topics.
- Providing timely and easily accessible environmental information to encourage public participation in environmental decision-making (*e.g. My Local Environment, Radon Maps)*.
- Advising Government on matters relating to radiological safety and emergency response.
- Developing a National Hazardous Waste Management Plan to prevent and manage hazardous waste.

Awareness Raising and Behavioural Change

- Generating greater environmental awareness and influencing positive behavioural change by supporting businesses, communities and householders to become more resource efficient.
- Promoting radon testing in homes and workplaces and encouraging remediation where necessary.

Management and structure of the EPA

The EPA is managed by a full time Board, consisting of a Director General and five Directors. The work is carried out across five Offices:

- Office of Climate, Licensing and Resource Use
- Office of Environmental Enforcement
- Office of Environmental Assessment
- Office of Radiological Protection
- Office of Communications and Corporate Services

The EPA is assisted by an Advisory Committee of twelve members who meet regularly to discuss issues of concern and provide advice to the Board.

EPA Research Programme 2014–2020

Towards Developing a *Cryptosporidium* Monitoring Protocol

EPA Research Report 137

Prepared for the Environmental Protection Agency

by

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DISCLAIMER

The EPA Research Programme addresses the need for research in Ireland to inform policymakers and other stakeholders on a range of questions in relation to environmental protection. These reports are intended as contributions to the necessary debate on the protection of the environment.

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Executive Summary

The enteric protozoan parasite, *Cryptosporidium*, is an important cause of diarrhoeal disease worldwide. *Cryptosporidium* oocysts are hardy and can persist for longer than *Escherichia coli*; they cause infection at low levels (probably <10 oocysts). Of the 26 species and over 40 genotypes described to date, at least eight can infect humans, but only three are considered major human pathogens: *Cryptosporidium hominis*, *C. parvum* and *C. meleagridis*. While *C. hominis* is largely restricted to humans, *C. parvum* has been reported from a large range of mammals and is very prevalent in young ruminants. The third species, *C. meleagridis*, is primarily an avian parasite that occasionally infects humans. Conventional disinfectants, such as free chlorine, chlorine dioxide or chloramine at the concentrations normally used to treat drinking water, do not effectively inactivate *Cryptosporidium* oocysts. The most effective means to inactivate oocysts during drinking water treatment is exposure to ozone or ultraviolet irradiation. In January 2004, cryptosporidiosis became a notifiable disease in Ireland. Since then between approximately 370 and 610 cases (with an average of 472) have been reported each year. These figures correspond to a crude incidence rate of between 8.7 and 14.4 cases per 100,000 of population annually, one of the highest rates in Europe.

The prevalence and persistence of *Cryptosporidium* – particularly with respect to drinking water supplies – is one of the key environment and health issues for the Environmental Protection Agency (EPA). This project was established through the EPA's Environmental Research Centre in conjunction with the Department of Agriculture Food and the Marine's laboratory complex at Backweston, Co. Kildare, to initiate the development of a national *Cryptosporidium* monitoring protocol.

The project was divided into the following tasks:

- Establishment of a reference laboratory for the detecting and identification of *Cryptosporidium* species in drinking water using filtration/ immunomagnetic separation/microscopy and molecular biology techniques through the participation of a number of water supplies on the EPA's Remedial Action List (RAL) (Chapters 2 and 3);
- Survey of *Cryptosporidium* monitoring in Irish drinking water and treatment plants (Chapter 4); and
- Evaluation of emerging contaminants (biological, chemical) in an Irish context via literature review and pilot testing (Chapter 5).

In relation to these objectives the key achievements of the project were:

- Establishment of an Irish National Accreditation Board (INAB) ISO 17025-accredited facility in May 2012 for the detection of *Cryptosporidium* oocysts in water (*Cryptosporidium* Reference Laboratory (CRL)).
- Establishing of methods, based on current international benchmark methods, for the genotyping of *Cryptosporidium* in drinking water and providing genotyping services to commercial primary testing laboratories and the Health Service Executive (HSE) during outbreak investigations.
- Monitoring of a number of water supplies on the EPA RAL for *Cryptosporidium*. Oocysts were identified and enumerated using three confirmative microscopic techniques: fluorescein isothiocyanate (FITC) staining, 4',6-diamidino-2-phenylindole (DAPI) staining and differential interference contrast (DIC) microscopy, followed by genotyping. A total of 560 samples were submitted to the CRL by seven local authorities. The samples were taken from 65 different drinking water supplies spread throughout Ireland. More than 90% of supplies tested had no treatment in place other than chlorination. Twenty supplies were routinely monitored over the course of the project. Overall, 199 (36%) tested

positive for *Cryptosporidium*. The number of oocysts per sample ranged from 0 to 264 (0–2.4 oocysts per 10 l), with 125 (63%) containing ≤5 oocysts. Genotyping resulted in 92 (46%) samples successfully genotyped to species/ genotype level. An additional 135 slides were submitted to the CRL as part of the slide referral service, of which 55 (40%) were successfully genotyped.

- Overall, eight species of *Cryptosporidium* and four genotypes were detected, with *C. andersoni* (39 (6.6%)), *C. ubiquitum* (35 (5.9%)), *C. parvum* (10 (1.5%)) and *C. bovis* (7 (1.1%)) the most frequent species detected from the different drinking water supplies. This study has shown that *Cryptosporidium* contamination of drinking water catchments in Ireland is widespread and probably mainly of animal origin. However, the majority of the species found are considered of low or no known risk to public health.

- The relationship between turbidity monitoring and *Cryptosporidium* oocyst concentration in a catchment system was also investigated and results have shown that there is a positive relationship between events in the catchment and the numbers of oocysts in the raw water supply, with oocyst numbers higher just after a rainfall event when turbidity has spiked. This highlights the fact that monitoring for triggers to events and timing sampling to events provide a more efficient and cost-effective sampling regime and, more importantly, provide more valuable data on the *Cryptosporidium* risk than random sampling.

- An online survey of public and private water schemes was conducted in 2011 to seek information on *Cryptosporidium* monitoring, such as reasons why a supply was or was not tested, the monitoring history, testing frequency and water type tested (raw or treated). A second part of the survey dealt with general *Cryptosporidium* testing issues. According to the survey, 227 public water supplies are currently monitored for *Cryptosporidium*. This represents roughly one quarter of all public water supplies in Ireland. The main reason for monitoring was that the supply was listed on the EPA RAL and lacked an effective barrier against *Cryptosporidium* oocysts. However, monitoring frequencies are generally very low – less than 10 times per year – and a lack of resources was cited as the main reason. In another survey conducted in 2013, Water Services Authorities (WSAs) indicated that approximately 247 drinking water samples could be genotyped for *Cryptosporidium* annually. The reasons provided by 65% of WSAs for not genotyping positive samples included cost, lack of genotyping service, a small number of oocysts on the slide or the fact that supplies were fed by groundwater.

- The occurrence of emerging or newly identified contaminants in Irish water supplies is of continued concern for the health and safety of the consuming public. This is in part due to their physical and chemical properties which make these products difficult to remove by conventional water treatment processes. Following a comprehensive literature review, seven groups of emerging contaminants were chosen to be examined at two drinking water treatment plants (WTPs) in Ireland. All were selected based on their applicability to Ireland's drinking water supplies and the likelihood of their occurrence due to agricultural practices, industrial activities or as a result of water treatment processes. Two WTPs were sampled over a period of 10 months between October 2010 and July 2011. Results from the 10-month survey found that, overall, the majority of the substances were not present. The two contaminants that were detected (galaxolide at WPT1 and oestrone at WTP2) were at low levels. Galaxolide was above the 0.1 µg/l surface water limit in February and April 2012 in WTP1. In June 2012, oestrone was detected above the guide value of 7 ng/l in WTP2. The project was a snapshot of two water treatment facilities and may not completely reflect the real presence of emerging contaminants in drinking water. This will be an area of importance for Ireland over the coming years and will require future extensive research across all WTPs.

- The establishment of the CRL provides services previously only available outside of Ireland to local authorities and primary testing laboratories for the genotyping of *Cryptosporidium* isolates from water. The CRL has nurtured and developed expertise in the area of *Cryptosporidium* detection and genotyping, providing an opportunity to initiate a comprehensive co-ordinated approach to sampling and analysis for this parasite in drinking water in Ireland. The project is continuing with current funding from Irish Water.

1 General Introduction

The enteric protozoan parasite, *Cryptosporidium*, is an important cause of diarrhoeal disease worldwide. In immunocompetent individuals, infections may be mild, moderate or severe, lasting for up to 14 days. In contrast, immunocompromised patients can suffer severe chronic or recurring cryptosporidiosis that may be fatal (Tzipori and Ward, 2002). Of the 26 species and over 40 genotypes described to date, at least eight can infect humans (Chako et al., 2010; Chalmers and Katzer, 2013), but only three are considered major human pathogens: *Cryptosporidium hominis*, *C. parvum* and *C. meleagridis*. While *C. hominis* is largely restricted to humans, *C. parvum* has been reported in a large range of mammals and is very prevalent in young ruminants. The third species, *C. meleagridis*, is primarily an avian parasite that occasionally infects humans.

Cryptosporidiosis is transmitted by microscopic (4–5.5 µm in diameter), thick-walled oocysts. This life-cycle stage is extremely long-lived and highly resistant to environmental stressors (Robertson et al., 1992). Under cool, moist conditions oocysts remain viable for up to 6 months (Robertson et al., 1992) and can even survive slow freezing to between –15 and –20°C (Carey et al., 2004). In fact, an outbreak in a Norwegian hotel was traced to drinks contaminated by infected ice cubes (Hajdu et al., 2008). The infective dose of cryptosporidiosis depends on host age, immune status and the *Cryptosporidium* species and is, therefore, difficult to determine accurately. It appears, however, to be low compared with other enteric pathogens. According to studies with healthy volunteers, the 50% infectious dose of *C. parvum* is approximately 132 and 1,880 oocysts in seronegative and seropositive humans, respectively (Chako et al., 2010). On the other hand, mathematical modelling of data from previous outbreaks suggests that some people become infected following ingestion of a single oocyst (Haas and Rose, 1994).

Infection occurs via the faecal–oral route, chiefly by ingestion of contaminated water (and more rarely food), recreational exposure in swimming pools, close contact with young ruminants in farms or petting zoos and horizontal transmission in childcare and nursing-home settings. Most large-scale outbreaks are due to the presence of *Cryptosporidium* oocysts in the drinking water supply (Davies and Chalmers, 2009). Once oocysts are present in the community, outbreaks are probably exacerbated by cross-infection via contaminated swimming pools and human-to-human contact. Water used for drinking water abstraction, particularly surface water sources, can become contaminated through agricultural run-off, discharge from wastewater treatment plants (WWTPs), and leakage from septic tanks or sewage pipes. As infected people and animals can pass up to 10 billion oocysts per gram of faeces, only a small amount of infected material is sufficient to contaminate large quantities of water (Carey et al., 2004). At the same time, conventional disinfectants, such as free chlorine, chlorine dioxide or chloramine, at concentrations normally used to treat drinking water, do not effectively inactivate *Cryptosporidium* oocysts. The most effective means to inactivate oocysts during drinking water treatment is exposure to ozone or ultraviolet irradiation. In order to ensure optimum penetration, the latter must be preceded by adequate pretreatment such as membrane filtration or coagulation followed by sedimentation or dissolved air flotation and filtration (EPA, 2010a). Sequential use of several disinfectants can enhance inactivation (Carey et al., 2004).

1.1 *Cryptosporidium* in Ireland

In January 2004, cryptosporidiosis became a notifiable disease in Ireland. Since then between approximately 370 and 610 cases (with an average of 472) have been reported each year. These figures correspond to a crude incidence rate of between 8.7 and 14.4 cases per 100,000 of population annually (HPSC, 2005–2010a,b), one of the highest rates in Europe. This high incidence is probably largely due to the predominant use of surface water for drinking water abstraction. Almost 82% of the drinking water in Ireland is derived from surface water sources (EPA, 2011). Moreover, due to the distinctive hydrogeology in many parts of the

island, a lot of springs and groundwater sources are also impacted upon by surface water, especially during heavy rainfall. As already mentioned, surface water sources are at high risk from contamination with *Cryptosporidium* oocysts. This is particularly the case in Ireland, where the high prevalence of livestock farming, an abundance of septic tanks many of which are thought to be inefficient or faulty (EPA, 2005), combined with high annual rainfall and a moist and cool climate, are likely to promote the distribution and survival of oocysts. The situation is exacerbated further by the fact that the many public water supplies in Ireland are small (serving less that 5,000 people) and lack the resources to provide adequate treatment and disinfection to effectively inactivate oocysts (EPA, 2011).

1.2 Water Testing Methods

To determine whether raw water sources or treated drinking water supplies are contaminated with *Cryptosporidium* oocysts, standard water testing methods have been developed by the United States Environmental Protection Agency (US EPA) and the UK Drinking Water Inspectorate (DWI) (US EPA, 2005; UK Environment Agency, 2009). First, water samples are concentrated either by filtration (in the case of large volume samples of clean, usually treated water) or chemical flocculation (in the case of small volumes of raw water) using calcium carbonate, ferric sulfate or aluminium sulfate as flocculants (Carey et al., 2004). Subsequently, the oocysts are recovered from the large amount of environmental debris by immunomagnetic separation. This technique involves capture of the oocysts by antibody-coated magnetic beads while extraneous material is washed away. The concentrated oocysts are then visualised by staining with an immunofluorescent antibody and enumerated using epifluorescent microscopy. Vital dyes such as DAPI (4',6-diamidino-2-phenylindole) and PI (propidium iodide) are used to determine whether the oocysts are intact and contain sporozoites or whether they are empty shells. Unfortunately, these testing methods are expensive, labour intensive and take too long to provide real-time information about the safety of the water supply. In addition, recovery efficiencies are highly variable but generally low (ranging between 20% and 55%) (Lloyd and Drury, 2002; Carey et al., 2004). Due to poor reproducibility, analysis of duplicate samples collected at the same time and location can result in very different oocyst counts, which, because of the small number of oocysts generally present in water samples, can lead to drastic differences in the calculated number of oocysts per sample (Xiao et al., 2006). Finally, many oocysts in the environment belong to species that are not infective to humans (Xiao and Ryan, 2008), while others, although microscopically intact, may no longer be viable.

The standard water testing methods, however, provide no information on species identity or infectivity. While useful, therefore, for evaluating the efficiency of the treatment process (operational control), they are of limited value for assessing the actual risk to public health.

1.3 Irish Guidelines and Legislation for Drinking Water in Relation to *Cryptosporidium*

In Ireland, 34 Water Service Authorities (WSAs), located in city and county councils, are responsible for the production, distribution and monitoring of all public water supplies (currently numbered at a total of 956, serving about 85% of the population) (EPA, 2011). Under the European Communities (Drinking Water) (No. 2) Regulations, 2007 (SI 278/2007), they have the duty to ensure that the water is free from any microorganisms, parasites or substances that constitute a potential danger to public health. However, there are no regulatory requirements to monitor drinking water directly for the presence of *Cryptosporidium* (EPA, 2010a). Instead, supplies must be monitored for the presence of *Clostridium perfringens*, a spore-forming microorganism with high resistance to chlorination. Only if levels exceed 0 organisms per 100 l of water, must the water be tested for *Cryptosporidium.* In addition, the regulations recommend *Cryptosporidium* testing if turbidity levels in the treated water exceed 1 NTU[1] and/or if the supply is at high risk from potential sources for *Cryptosporidium* in the catchment but has insufficient treatment and disinfection barriers in place. In the latter case, monitoring frequencies are to be determined in consultation with the Health Service Executive (HSE).

1. NTU, nephelometric turbidity unit.

Where appropriate treatment and disinfection methods are installed, some operational monitoring is recommended in order to establish whether the barriers are effective. *Cryptosporidium* may also be included as a parameter in the raw water operational programme if it is considered important for the source (EPA, 2010a).

A more comprehensive approach was developed by the Irish EPA Environmental Enforcement Network *Cryptosporidium* Working Group. This group recommends a regular monitoring schedule to be carried out over a period of 2 years. According to this schedule, treatment works serving over 20,000 people are to be tested weekly, while monitoring frequencies in smaller supplies are to be defined according to the *Cryptosporidium* risk and the results of treatment works monitoring. It is advised that, after the initial 2-year period, monitoring results are reviewed and schedules adapted to the specific requirements and characteristics of the supply. Notwithstanding the numerous recommendations on water testing and monitoring, there are currently no national guidelines on the course of action to be taken when oocysts are detected in the water supply. In fact, even a defined standard above which action must be taken is lacking not only in Ireland but also anywhere else in Europe. A previous cut-off of between 10 and 30 oocysts per 100 l of treated water, based on a comparison of oocyst levels in treated water during waterborne outbreaks and when no outbreaks were detected (Haas and Rose, 1995), has since been revoked in acknowledgement of the fact that due to the variations in host susceptibility and species infectivity and virulence, it is not feasible to establish a health-related standard to protect the population as a whole. Unless continuous monitoring is carried out it is also not possible to determine the concentration of oocysts at this level of accuracy. Instead, some jurisdictions have introduced treatment standards that are consistent with the capabilities of a well-designed treatment plant, operated under optimum conditions (Lloyd and Drury, 2002). In addition to *Cryptosporidium* testing, WSAs must assess each public water supply for the potential risk from *Cryptosporidium*. This *Cryptosporidium* risk assessment is part of the overall Drinking Water Safety Plan adopted by the EPA in 2008 in line with World Health Organization (WHO) guidelines (WHO, 2008b).

The plan considers all potential risks that may arise in the catchment and the treatment works, but does not deal with potential hazards in the distribution network (EPA, 2010a). Similar testing guidelines and recommendations apply to public and private group water schemes (GWSs). Currently there are 672 public GWSs in Ireland, serving about 2.7% of the population (EPA, 2011). These public GWSs are supplied by larger public water supplies that are tested according to the Regulations. Private GWSs, on the other hand, source and distribute their own water, under supervision of the local WSA. The 529 private GWSs in the country supply 5.3% of the population (EPA, 2011). While they are not obliged under the Regulations to carry out *Cryptosporidium* risk assessments, they are strongly encouraged to do so as a matter of good operational practice. If risk assessments have been carried out, the WSAs are advised to include the risk scores in their annual report to the EPA (EPA, 2010b). Finally, small private supplies that supply industrial or commercial premises and public buildings, such as schools or nursing homes, are recommended to adopt a highly simplified risk assessment approach and put in place treatment systems that adequately reduce the risk (EPA, 2010b). There are currently 1,245 small private supplies serving 0.6% of the Irish population (EPA, 2011).

1.4 Objectives

The project was established through the EPA's Environmental Research Centre in conjunction with the Department of Agriculture, Food and the Marine's laboratory complex at Backweston, Co. Kildare, to initiate the development of a national *Cryptosporidium* monitoring protocol.

The overall objective of the project was the development of best practice in relation to *Cryptosporidium* monitoring for normal and incident conditions.

The project was divided into the following tasks:

- Establishment of a reference laboratory for the detection and identification of *Cryptosporidium* in drinking water using molecular biology techniques and sequencing through the participation of a number of water supplies on the

EPA's Remedial Action List (RAL) (Chapters 2 and 3);

- Survey of *Cryptosporidium* monitoring in Irish drinking water and treatment plants (Chapter 4); and
- Evaluation of emerging contaminants (biological, chemical) in an Irish context via literature review and pilot testing (Chapter 5).

2 Development of a *Cryptosporidium* Reference Laboratory in Ireland

2.1 Introduction

2.1.1 Primary testing

Both the US EPA and the UK DWI have published comprehensive methods for the primary testing of drinking water for *Cryptosporidium* oocysts. The method established in the *Cryptosporidium* Reference Laboratory (CRL) is based on the US EPA Method 1622 (US EPA, 2005) and the UK method (UK Environment Agency, 2009). The method outlines the identification of *Cryptosporidium* in water to genus level but does not identify species of *Cryptosporidium.* It also does not distinguish between viable and non-viable oocysts, the origin of oocysts or the public health risk associated with the presence of the oocysts in drinking water. This method is suitable for the detection and enumeration of *Cryptosporidium* oocysts in drinking water that have been filtered through IDEXX Filta-Max xpress® filters. Filters are sent to the CRL and oocysts are eluted from the filter using an IDEXX Filta-Max xpress® Pressure Elution Station, separated from other contaminants in the sample using immunomagnetic separation (IMS) and stained using fluorescently labelled anti-*Cryptosporidium* antibodies and DAPI. Identification and enumeration are carried out using three confirmative microscopic techniques: fluorescein isothiocyanate (FITC) staining, DAPI staining and differential interference contrast (DIC) microscopy (Fig. 2.1).

2.1.2 Molecular genotyping

In order to gain a better understanding of the source and public health risk associated with the detection of *Cryptosporidium* in drinking water catchments, molecular genotyping methods must be applied to the oocysts recovered from primary testing. Whilst molecular diagnostics have become standard in some bacteriological laboratories, the molecular identification of *Cryptosporidium* is still far from routine, mainly due to the small numbers of oocysts present in the samples. The method of repetitive nested polymerase chain reaction (PCR) of the small subunit (SSU) ribosomal ribonucleic acid (rRNA) gene, followed by sequencing, has become the benchmark for molecular genotyping of *Cryptosporidium* species from environmental samples (Ruecker et al., 2012; Chalmers and Katzer, 2013). Briefly this method uses slide scraping, oocyst destruction using multiple freeze/thaw cycles and deoxyribonucleic acid (DNA) extraction using a QIamp DNA purification kit. The primers and conditions for the PCR have been previously described by Xiao et al. (2001). Once amplified, the positive PCR products are sent off-site for sequencing. Species and genotypes are determined by phylogenetic analysis of the sequences.

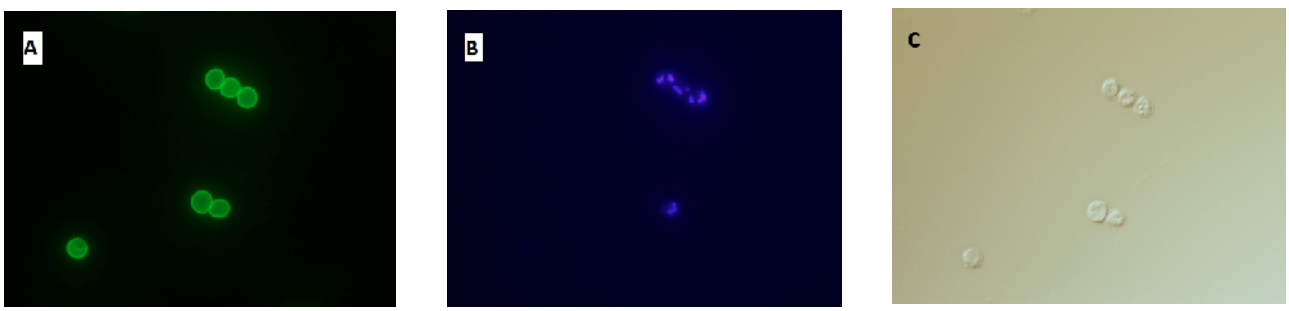


Figure 2.1. *Cryptosporidium* oocysts stained using (A) fluorescein isothiocyanate (FITC), (B) 4'6-diamidino-2-phenylindole (DAPI), and (C) visualised under differential interference contrast (DIC) microscopy.

2.2 Objectives

- To establish an Irish National Accreditation Board (INAB) ISO 17025-accredited facility for the detection and genotyping of *Cryptosporidium* oocysts in Irish drinking water supplies; and

- To promote the services of the CRL to relevant authorities not routinely involved in *Cryptosporidium* monitoring and to provide genotyping facilities to local authorities.

Figure 2.3. An IDEXX Filta-Max xpress® Pressure Elution Station with filters.

2.3 CRL Set-Up

2.3.1 Primary testing

A laboratory for primary testing of water samples for *Cryptosporidium* was established in the National Reference Laboratory (NRL) for Parasites at the Department of Agriculture, Food and the Marine (DAFM) Central Veterinary Research Laboratory (CVRL) Backweston Research Campus in Celbridge, Co. Kildare (Fig. 2.2).

Most of the equipment necessary for primary testing was already in place in the laboratory. Additional specialised equipment was purchased, including an IDEXX Filta-Max xpress® Pressure Elution Station which significantly increased the sample analysis capacity of the existing laboratory (Fig. 2.3).

2.3.2 Genotyping

The CVRL has a suite of accredited molecular diagnostic laboratories designed for a high throughput of samples, whilst minimising the risk of contamination. All stages of the *Cryptosporidium* genotyping process were carried out using this molecular suite of laboratories. To ensure the fidelity of the PCR diagnostic process, the molecular suite of laboratories included on the 'clean' pre-PCR side a DNA extraction laboratory and a DNA-free PCR set-up laboratory and the post-PCR facilities included amplification and analysis areas. Pre- and post-PCR areas are separated physically and are located in two different sections of the building. A system is in place that

Figure 2.2. The Central Veterinary Research Laboratory, Backweston Campus.

ensures that if analysts have been in the post-PCR areas they cannot return to the pre-PCR laboratories on the same day.

2.4 Validation and Accreditation

2.4.1 Primary testing

A validation protocol was prepared to validate the method for the detection and enumeration of *Cryptosporidium* species in drinking water. The protocol was based on repeatedly analysing filters spiked with known numbers of *Cryptosporidium* oocysts. These filters were prepared in the laboratory using a validation rig. The method was assessed for repeatability, reproducibility, recovery, precision, ruggedness and the limit of detection. As well as in-house validation, the CRL participated in a monthly external proficiency test scheme called Crypts (LGC Proficiency Testing Services, UK), which, in addition to recovery performance, examined the microscopy skills of the analysts.

A validation report that included results, analysis and performance of the validation protocol and the results of 10 rounds of external proficiency tests was submitted to the INAB in December 2011. The report concluded that the test method was fit for purpose. INAB carried out a site visit in May 2012 and the *Cryptosporidium* method was audited and accredited.

2.4.2 Genotyping

The molecular genotyping procedure takes place in the accredited suite of laboratories but, as yet, the method is not accredited. The methods established in the CRL are based on the current international benchmark methods and all quality controls are in place in order to ensure accuracy of results.

2.5 CRL Services

2.5.1 Primary testing

The primary testing method is accredited by INAB to ISO 17025. Since 2011, a number of local authorities that have supplies that are on the EPA RAL and that are not routinely involved in *Cryptosporidium* monitoring have submitted samples for primary testing to the laboratory. The results of this analysis provide vital information to local authorities on the levels of *Cryptosporidium* in their supplies and, additionally, data from these samples are used in the pilot scheme project (Chapter 3) to obtain an overall picture of the scale of the problem, the public health risk and the sources of *Cryptosporidium* contamination in these supplies.

In addition to analysing routine RAL samples, the CRL has been involved in a number of site-specific incidences investigating the source of *Cryptosporidium* in drinking water supplies. This service requires intensive monitoring of both raw and treated water of a supply for a short period of time.

The CRL has also supported a number of research projects in University College Dublin (UCD) by carrying out *Cryptosporidium* testing when required.

2.5.2 Genotyping

All filters submitted to the laboratory are initially processed by primary testing. All positive samples are then further analysed by genotyping. In addition, the CRL has been offering a referral service to other *Cryptosporidium* primary testing laboratories in Ireland for genotyping positive samples submitted to its laboratories by local authorities.

The CRL has also worked on a number of occasions with the HSE in outbreak investigations by genotyping human samples.

3 *Cryptosporidium*: Detection and Genotyping in Irish Drinking Water Supplies

3.1 Introduction

Waterborne cryptosporidiosis is a significant public health concern and has been reported worldwide. *Cryptosporidium* species are considered to be a threat to water supplies because they persist in the environment and are resistant to chlorine disinfection. They are believed to have a low infective dose and are also harboured by many animal species. Farm animals, humans and wild animals have been considered as major contributors to *Cryptosporidium* contamination of surface water (Meinhardt et al., 1996; Ono et al., 2001; Karanis et al., 2007). Furthermore, environmental contamination with oocysts of *Cryptosporidium* species that are not infectious to humans contributes to the difficulties in assessing the risk to public health from waterborne oocysts. Although all *Cryptosporidium* species can be found in water, only a few are important human pathogens. *Cryptosporidium parvum* and *C. hominis*, have been found to account for more than 90% of infections in human cases worldwide (Chalmers and Giles, 2010). Knowledge of their frequency and distribution facilitates identification of the most important transmission routes in an area and greatly aids determination of the source of future outbreaks.

Cryptosporidiosis poses a significant threat to public health in Ireland as indicated by several recent waterborne outbreaks. Several studies have detected *Cryptosporidium* species in Irish river basins (Chalmers et al., 1997; Skerrett and Holland, 2000; Lowery et al., 2001; Graczyk et al., 2004; Lucy et al., 2008). However, there is very little information about the occurrence and genotypes of *Cryptosporidium* oocysts in Irish drinking water catchments. The pilot study was designed to target supplies that were on the EPA RAL for lack of effective barrier to *Cryptosporidium* in order to gain an understanding of the occurrence, species and genotypes of *Cryptosporidium* in Irish drinking water supplies and to assess the public health risk.

3.2 Project Objectives

The project aimed to use the newly established CRL at Backweston for the following:

- To develop a database of the *Cryptosporidium* species in Irish drinking water supplies on the EPA RAL for lack of an effective barrier to *Cryptosporidium*;
- To assess the risk to public health from *Cryptosporidium* in these supplies;
- To offer a *Cryptosporidium* genotyping service to local authorities in Ireland to expand the knowledge on the types of *Cryptosporidium* species found in drinking water supplies; and
- To examine the relationship between turbidity in raw water and *Cryptosporidium* detection.

3.3 Materials and Methods

3.3.1 Scheme set-up

In January 2011, local authorities that had drinking water supplies on the EPA RAL due to lack of an effective barrier to *Cryptosporidium* or supplies that were designated high risk for *Cryptosporidium* were invited to participate in this pilot scheme. The benefits of participating were outlined, including availing of *Cryptosporidium* testing for supplies that were previously not routinely tested and, most importantly, the benefits of acquiring genotyping data on any *Cryptosporidium* isolates found in their supplies. In order to participate, the local authorities were required to cover the cost of sampling, including filters, and transport to the CRL.

3.3.2 Sampling

In general, samples were taken of treated water but, in some circumstances, raw water samples were analysed depending on the characteristics of the individual supplies tested and local authority requirements. Local authorities used IDEXX Filta-Max

or Filta-Max xpress® filters to filter approximately 1,000 l of water on-site over a 24-h period. The filters were transported to the CRL in Backweston without delay. All samples submitted to the CRL were accompanied by a sample submission form recording relevant information, including sample volume, location, date, etc. Local authorities were asked to comment on weather conditions preceding sampling and to note the turbidity readings at the time of sampling.

3.3.3 Drinking water catchment – supply types

Seven different supply types were targeted for pilot scheme testing:

1. Groundwater (GW);
2. Groundwater influenced by surface water in agricultural (GW-IS-AG) catchment;
3. Surface water upland (SW-U) catchment;
4. Surface water lowland agricultural (SW-L-AG) catchment;
5. Spring water (SPR);
6. Spring water influenced by surface water in agricultural (SPR-IS-AG) catchment; and
7. Groundwater spring water mix (GW-SPR).

Supplies designated GW-IS-AG were located in karst limestone areas where there was evidence of surface water infiltrating the groundwater, in particular during and just after heavy rain. These supplies were in areas where the predominant land use was agriculture, mainly cattle and sheep. Supplies that were designated SW-U were where the source waters were either rivers or lakes in mountain areas. SW-U supplies were in areas where there was no intensive agriculture but may have low numbers of grazing sheep, deer or other wildlife, and SW-L-AG supplies were usually river sources where the predominant land use was intensive agriculture, mainly cattle and sheep.

3.3.4 Turbidity – event sampling

In addition to the sampling carried out in the pilot scheme, one supply was selected for high-frequency monitoring to examine the relationship between rainfall levels, changes in turbidity and the detection of *Cryptosporidium* oocysts. The supply chosen was SW-L-AG in an area predominantly used for agriculture (Fig. 3.1). In addition, there were a number of WWTP discharge points into the supply. An automatic sample unit, which contained a solenoid valve that was triggered to open once a set turbidity level was reached, was installed on the raw water supply. This valve remained open for a preset period of time. Samples to obtain *Cryptosporidium* baseline levels and trigger event samples were taken from the same supply over a period of 7 months.

Figure 3.1. Cattle seen in close proximity to a drinking water supply. This supply was chosen to examine the relationship between turbidity changes in raw water and number and genotypes of *Cryptosporidium* detected.

3.3.5 Detection and enumeration

Analysis of all samples was carried out according to the Standard Operating Procedure (SOP) developed by the CRL for the detection and enumeration of *Cryptosporidium* in treated water by filtration, immunomagnetic separation and fluorescent antibody identification. This SOP is based on the US EPA Method 1622 (US EPA, 2005) and the UK method (UK Environment Agency, 2009). See Appendix 1 for the CRL SOP for this method. Briefly, filters were sent to the laboratory and oocysts eluted from the filter using an IDEXX Filta-Max xpress® Pressure Elution Station or automatic wash station in 0.01% PBS–Tween 20. Samples were concentrated by centrifugation and immunomagnetic separation was carried out using Dynabeads (Technopath) to isolate and concentrate the oocysts. The oocysts were stained using fluorescently labelled anti-*Cryptosporidium* antibodies and DAPI (Sigma–Aldrich). Identification and enumeration were carried out using three confirmative microscopic techniques: FITC staining, DAPI staining and DIC microscopy. Oocyst results were reported per 10 l of volume filtered.

3.3.6 Genotyping

3.3.6.1 Slide scraping and DNA extraction

Briefly, cover slips were removed from the slides using a sterile swab soaked in acetone. Oocysts were recovered from slides by repeated scraping of the surface area using an inoculating loop and three 60-µl volumes of QIAGEN ATL buffer. The recovered oocysts were subjected to eight freeze/thaw (liquid nitrogen/100°C heating block) cycles in order to release the DNA. The DNA was then purified using a QIAamp DNA Mini Kit (QIAGEN). Finally, the DNA was eluted in 50 µl of elution buffer and stored at –20°C prior to amplification.

3.3.6.2 Molecular genotyping

Molecular genotyping of recovered *Cryptosporidium* oocysts was carried out by repetitive nested PCR amplification of the SSU (18s) rRNA gene using the PCR primers under conditions described by Xiao et al. (2001) and Ruecker et al. (2011, 2012). Briefly, each isolate was amplified four times using two 45 cycles of nested PCR. Five microlitres of DNA were added to each of the four replicate tubes in the primary PCR. Four microlitres of DNA and 1 µl of *C. muris*-positive control DNA were added to a fifth replicate tube to test for inhibition. Then 2 µl of DNA from the primary PCR were added to the secondary PCR. Secondary PCR products were visualised on a 1.5% agarose gel stained with GelRed™ (Biotium). Positive PCR samples were purified using a PCR clean-up kit (QIAGEN QIAquick) and sent off-site for sequencing (Source Bioscience). Sequence analysis was carried out using Chromas Lite 2.1.1 (Technelysium PTY Ltd). Sequences were exported as FASTA-formatted files and manually edited at the 5' and 3' ends to allow comparison with a reference database generated from Genbank containing representative sequences from all the major species and genotypes. The edited sequences were aligned with the reference database using Clustal W (Thompson et al., 1994). Phylogenetic analysis was carried out on this alignment using BioNJ (http://phylogeny.lirmm.fr/phylo_cgi/index.cgi), and the evolutionary distances were calculated using the Kimura's two-parameter model (*Toxoplasma gondii* as the outgroup) with 1,000 bootstraps. Phylogenetic trees were visualised using TreeDyn. In addition, NCBI BLAST[2] analysis was carried out on all sequences from the pilot scheme.

3.4 Results

3.4.1 Cryptosporidium *occurrence in drinking water supplies on the EPA RAL*

A total of 560 samples were submitted to the CRL by seven local authorities between February 2011 and October 2013. The samples were taken from 65 different drinking water supplies spread throughout Ireland. More than 90% of supplies tested had no treatment in place other than chlorination and, therefore, there was little difference between raw and treated water in relation to *Cryptosporidium.* Sampling frequency for supplies varied greatly throughout the project, with 17 supplies tested only once; however, 20 supplies were targeted routinely over the course of the project and were sampled 10 times or more. The rest of the supplies were sampled between three and nine times each. Table 3.1 details the primary testing results of the samples submitted to the CRL for the

2. The National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST).

Table 3.1. Details of pilot scheme samples submitted to the *Cryptosporidium* Reference Laboratory (CRL) between February 2011 and October 2013.

	Year of sampling			Total
	2011	2012	2013	
Number of samples submitted	123	184	253	560
Total positive on primary testing (% positive)	63 (51%)	70 (38%)	66 (26%)	199 (36%)

3 years of the pilot scheme. In total, 199 (36%) samples were positive for *Cryptosporidium* by microscopy. Of the supplies tested, 57% were positive at least once over the 33 months of the study.

The supplies tested fell into seven different catchment types, as detailed in Table 3.2. None of the four GW supplies tested positive over the course of the study. Of the 12 GW-IS-AG supplies tested, 10 (83%) were positive at least once over the study, with *Cryptosporidium* detected in 45% of the samples tested. There were 34 SW-U supplies and 56% of these were positive at least once during the study, with *Cryptosporidium* detected in 34.7% of samples. All of the seven SW-AG supplies were positive over the study, with 39.7% of samples testing positive. Of the six SPR supplies tested, 50% were positive and both SPR-IS-AG supplies were positive at least once. Overall, 30% of samples from SPR supplies and 16.6% of samples from SPR-IS-AG were positive over the study period. Only one of the two GW-SPR supplies was positive. Over the course of the pilot scheme, additional samples were received from various local authorities for other reasons, such as catchment or outbreak investigations. These samples have been included in the pilot scheme overall numbers but have not been included in the analysis of data from the different catchment types.

3.4.2 Cryptosporidium *species and genotypes in drinking water supplies in Ireland*

3.4.2.1 Pilot scheme slides – method performance

From February 2011 to October 2013, all slides that tested positive by microscopy (n = 199) were processed for genotyping by repetitive nested PCR

Table 3.2. *Cryptosporidium* detection in drinking water catchments.

Source water	Catchment type code	Predominant catchment use	No. of supplies	No. of samples tested	% Positive
Groundwater	GW	NA	4	4	0
Groundwater influenced by surface water in agricultural catchment	GW-IS-AG	Agriculture – cattle/sheep	12	142	45.0
Upland surface water	SW-U	Forestry/Some sheep and deer/Wildlife	34	236	34.7
Lowland surface water in agricultural catchment	SW-L-AG	Agriculture – cattle/sheep	7	68	39.7
Spring water	SPR	NA	6	20	30.0
Spring water influenced by surface water in agricultural catchment	SPR-IS-AG	Agriculture – cattle/sheep	2	30	16.6
Groundwater/Spring water mixed	GW-SPR	NA	2	11	36.6

NA, not applicable.

sequencing. Table 3.3 details the efficiency of the slide genotyping method in relation to the number of oocysts per slide. The number of oocysts per sample ranged from 0 to 264 (0–2.4 oocysts/10 l), with 125 slides (63%) containing five or less oocysts. Genotyping resulted in 92 (46%) samples successfully genotyped to species/genotype level. Of the remaining slides (n = 107), 86 (80.4%) contained five oocysts or less. No inhibition was detected for the remaining 21 slides that were negative for PCR but contained greater than five oocysts. These samples were from 14 different drinking water (both raw and treated) supplies across the country. The process of repetitive nested PCR sequencing was repeated for these samples but was not successful. It was concluded that unknown environmental factors in these supplies must have affected the samples.

3.4.2.2 Referral slides – method performance

An additional 135 slides were submitted to the CRL as part of the slide referral service. These slides originated from five local authorities through three different primary testing laboratories. All slides submitted to the CRL under this service were positive for *Cryptosporidium* oocysts. Data on the number of oocysts per slide were supplied for 125 of the slides submitted. Table 3.3 outlines the number of samples genotyped in relation to the number of oocysts per slide. Ninety-one (73%) had five oocysts or less present per slide, with 60 slides containing only a single oocyst. Of the 135 slides submitted for typing, 55 (40%) were successfully genotyped (Table 3.3).

3.4.2.3 Cryptosporidium species/genotypes in pilot scheme slides

A summary of the *Cryptosporidium* species and genotypes detected in the pilot scheme project is given in Table 3.4.

Overall, eight species of *Cryptosporidium* and four genotypes were detected in the pilot scheme samples (n = 590) analysed in this project. Figure 3.2 shows the distribution of *Cryptosporidium* species in the seven catchment types targeted. *Cryptosporidium andersoni* was detected most frequently in 39 samples from 12 supplies, representing four catchment types (GW-IS-AG, SW-U, SW-L-AG, SPR-IS-AG). *Cryptosporidium ubiquitum* was the second most common species identified, detected 35 times in 16 supplies from three catchment types (GW-IS-AG, SW-U, SPR-IS-AG). *Cryptosporidium parvum* was detected nine times in six supplies from catchment type GW-IS-AG and in one supply from an SW-U catchment type. *Cryptosporidium bovis* was detected seven times in four supplies from two catchment types (GW-IS-AG, SPR). There were two isolates of *C. muris* detected in two supplies from two catchment types (GW-IS-AG, SW-U). Other *Cryptosporidium* genotypes detected included three isolates identical to isolate P156 (Robinson et al., 2011), one isolate identical to UK E7 (Robinson et al., 2011), two isolates nearly identical (1

Table 3.3. Efficiency of genotyping *Cryptosporidium* oocysts from slides.

No. of oocysts on slides	No. of pilot scheme slides	No. of pilot scheme slides genotyped (%)	No. of referral slides	No. of referral slides genotyped (%)
1	64	14 (22%)	60	10 (16%)
2	21	7 (33%)	15	8 (53%)
3	19	9 (47%)	9	3 (33%)
4	10	4 (40%)	5	4 (80%)
5	11	5 (45%)	2	2 (100%)
6–10	18	10 (55%)	7	4 (57%)
>10	56	43 (77%)	27	16 (59%)
Unknown	0	0	10	8 (80%)
Total	**199**	**92 (46%)**	**135**	**55 (40%)**

Table 3.4. Species/Genotypes of *Cryptosporidium* in high risk drinking water supplies.

Species/Genotype	No. of occurrences in supplies in pilot scheme (prevalence (%)[1])	No. of occurrences in referral samples (prevalence (%)[2])	Major known hosts	Association with human cryptosporidiosis[3]
Cryptosporidium andersoni	39 (6.6%)	37 (27.4%)	Cattle	Rarely associated
Cryptosporidium ubiquitum	35 (5.9%)	5 (3.7%)	Sheep, deer, rodents	Sporadic cases
Cryptosporidium parvum	10 (1.5%)	15 (11.1%)	Mammals, humans	Commonly associated
Cryptosporidium bovis	7 (1.1%)	4 (2.9%)	Cattle	Rarely associated
Cryptosporidium xiaoi	2 (0.3%)	0	Sheep	No association
Cryptosporidium muris	2 (0.3%)	1 (0.7%)	Rodents	Rarely associated
Cryptosporidium ryanae	0	2 (1.5%)	Cattle	No association
Cryptosporidium canis	0	1 (0.7%)	Dog	Sporadic cases
UK E7 genotype	1 (0.2%)	0	Unknown	No association
W19 genotype	1 (0.2%)	0	Unknown	No association
***Cryptosporidium* sp. P156**	2 (0.3%)	2 (1.5%)	Unknown	No association
SW 4 (05.1586.Va, Ass: HM015878)	2 (0.3%)	0	Unknown	No association
UK E6 genotype	0	1 (0.7%)	Unknown	No association
***Cryptosporidium* sp. 6876**	0	1(0.7%)	Canada geese	No association

[1,2]Prevalence is based on the number of occurrences of a particular species/genotype divided by the total number of water samples analysed in the pilot scheme (n = 590)[1] and slide referral service (n = 135)[2].
[3]As detailed by Chalmers and Katzer (2013).

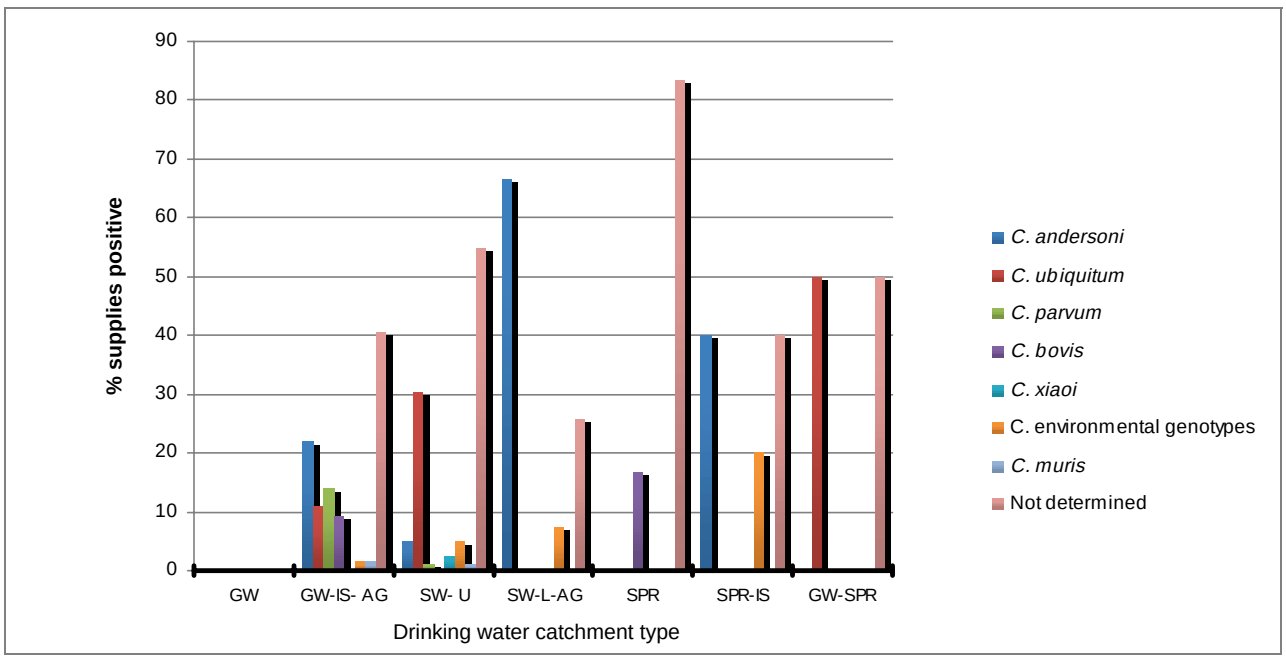


Figure 3.2. Distribution of *Cryptosporidium* species in drinking water catchment types.

bp difference) to SW4 (Nichols et al., 2010) and one isolate nearly identical (1 bp difference) to W19 (Chalmers et al., 2010). There were nine samples that contained mixed species/genotypes: C. *parvum* was found mixed twice with *C. ubiquitum* and once with *C. andersoni*; *C. ubiquitum* was also found mixed with *C. bovis* (n = 2) and *C. xiaoi* (n = 2); and *C. andersoni* was also found to be mixed with *Cryptosporidium* sp. P156 (n = 1) and *C. bovis* (n = 1).

3.4.2.4 Cryptosporidium *species/genotypes in referral slides*

A summary of the *Cryptosporidium* species and genotypes detected in the referral slides is outlined in Table 3.4. There were seven species of *Cryptosporidium* detected and three genotypes. *Cryptosporidium andersoni* was the most common, being detected in 27.4% of slides submitted followed by C. *parvum*, which was detected in 10.4% of the samples submitted. *Cryptosporidium ubiquitum*, *C. bovis*, *C. muris* and *C. ryanae* were detected in 3.7%, 2.9%, 0.7% and 1.5% of samples submitted, respectively. Three of the genotypes detected were identical to *Cryptosporidium* sp. P156 (Robinson et al., 2011), UK E6 (Chalmers et al., 2010) and *Cryptosporidium* sp. 6876 (Zhou et al., 2004). There were 10 samples that contained mixed species. *Cryptosporidium andersoni* was found mixed with the most species, including *C. parvum* (n = 2), *C. muris* (n = 1), *C. canis* (n = 1), *C. ubiquitum* (n = 1) and UK E6 genotype (n = 1). *Cryptosporidium parvum* was also found once mixed with *C. ubiquitum*. *Cryptosporidium ubiquitum* was also found mixed with both *C. bovis* (n = 2) and *Cryptosporidium* sp. P156.

3.4.3 Turbidity, rainfall and Cryptosporidium

One surface water supply was tested 25 times in 7 months to provide both baseline data through routine samples and trigger data through event samples on this supply. Samples were classified as event samples when rainfall was greater than 5 mm and or turbidity exceeded 5 NTUs in the 24 h prior to sampling. Samples were either taken manually or by automatic sampling for 24 h. A total of 11 routine and 14 event samples were submitted during the study period. The results of the comparisons between routine and event samples are shown in Table 3.5.

Cryptosporidium oocysts were frequently detected in this catchment in both routine and event samples, with 64% of total samples being positive. All samples taken in the spring months of March, April and May were positive (Fig. 3.3) and only one sample taken in September was positive (Fig. 3.4). Oocyst numbers ranged from 0 to 3.66 per 10 l, with the average concentration being 0.48 per 10 l. The average turbidity for this supply for the 7 months was 2.9 NTUs and the average daily rainfall was 1.9 mm. The wettest month was March, with 85 mm rain, and the driest was September, with 37.5 mm rain. *Cryptosporidium andersoni* was the only species detected in both routine and event samples in this supply over the 7 months of the study.

Figures 3.3 and 3.4 outline the relationship between turbidity, rainfall and *Cryptosporidium* in the surface water supply for the months of March and September when most monitoring was carried out.

3.5 Discussion

3.5.1 Genotyping – method performance

Overall, the genotype of isolates from 44% of slides processed was successfully determined by the methods used in the laboratory. The performance of the genotyping method improved over the course of the study (data not shown); however, the low numbers of oocysts present on the slides continued to be a significant obstacle throughout the study. Sixty-five per

Table 3.5. Comparison between routine and event samples.

Sample type	No. of samples	Turbidity[1] (NTU	Rainfall[1] (mm)	Positive samples (%)	*Cryptosporidium* oocysts per 10 l[1]
Routine samples	11	1.61 (3.02)	1.71 (2.54)	54	0.06 (0.2)
Event samples	14	7.77 (19.8)	5.87 (19.5)	71	0.81 (3.66)

[1]Data shown for turbidity, rainfall and *Cryptosporidium* oocysts are mean with maximum in parentheses.

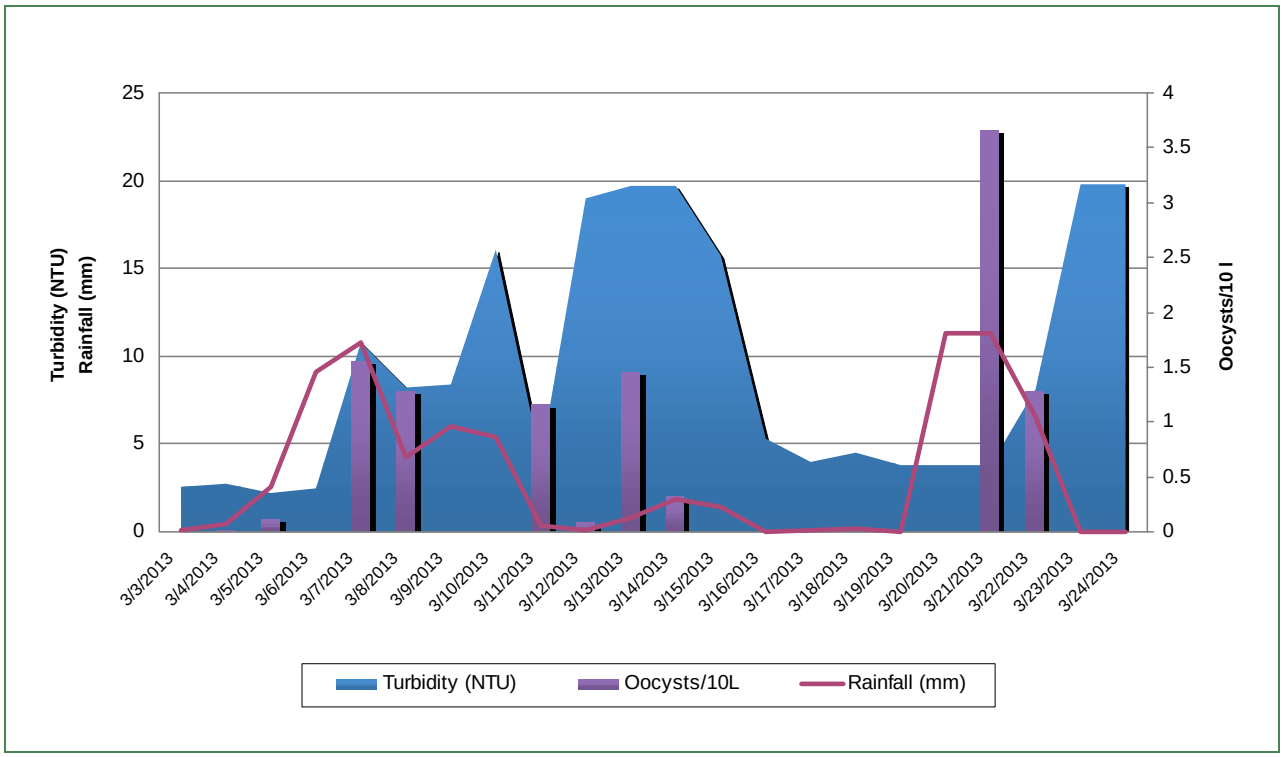


Figure 3.3. Relationship between turbidity, rainfall and *Cryptosporidium* detection in surface water supply in March 2013.

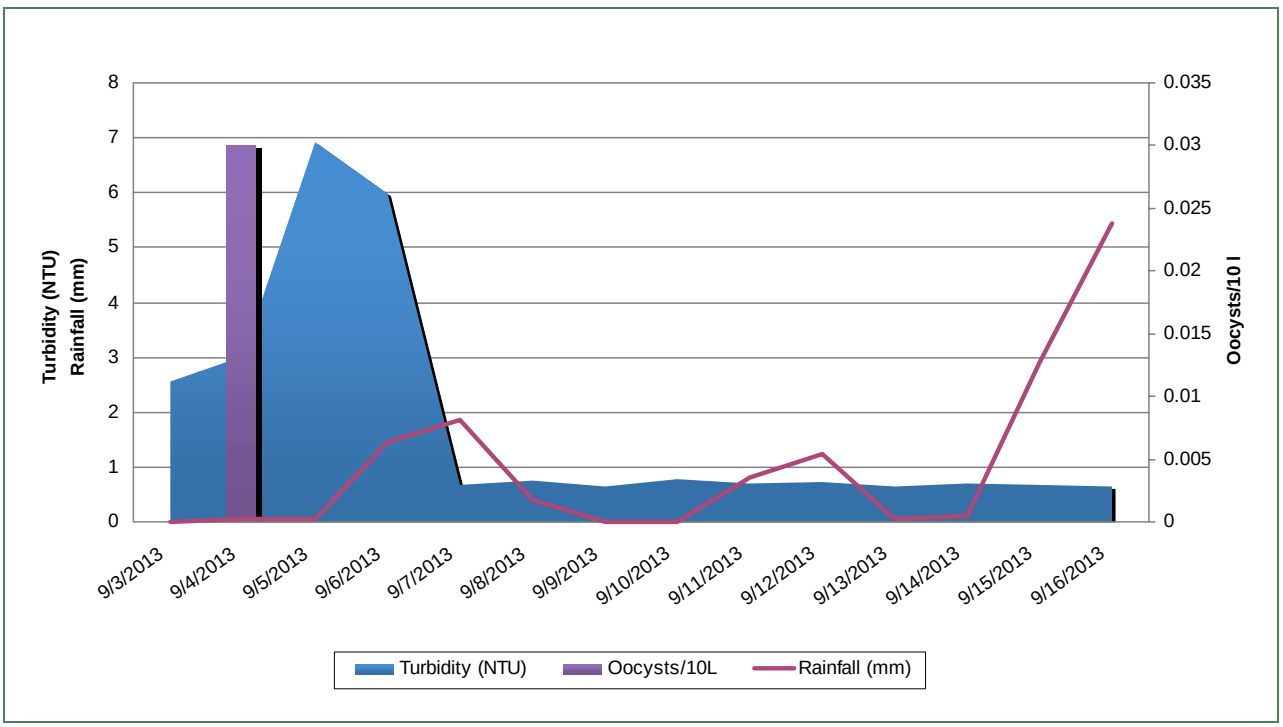


Figure 3.4. Relationship between turbidity, rainfall and *Cryptosporidium* detection in surface water supply in September 2013.

cent of the slides processed by the laboratory contained five oocysts or less and, while genotyping was successful for 30% of these slides, the issue of low oocyst numbers remains the single limiting factor in the successful determination of oocyst genotype from water slides. In addition, the methods used by referral laboratories in preparing the slides can have an impact on downstream molecular genotyping methods. Work carried out by Di Giovanni et al. (2010) has shown the effect that formalin-containing mounting media and fixing buffer has on the success rate of PCR and concludes that formalin-free reagents should be used when slide genotyping is to be performed. Work is ongoing in the CRL to improve the overall performance of the method in-house. It may be necessary for other primary testing laboratories to modify their in-house methods if downstream processing for molecular genotyping is to be carried out by the CRL.

3.5.2 Cryptosporidium *detection and genotyping in high risk supplies*

The pilot scheme's main aim was to investigate the prevalence of *Cryptosporidium* in drinking water supplies in Ireland that were on the EPA RAL for lack of an effective barrier for *Cryptosporidium* and to assess the public health risk associated with the oocysts detected in these supplies. Contamination of drinking water is common, with oocysts detected in 36% of the supplies sampled during the programme. This is not surprising as sampling was targeted at vulnerable supplies in rural areas where, in most cases, treatment was limited to chlorination. Overall, eight species and four genotypes of *Cryptosporidium* were identified, demonstrating that contamination is from multiple host species from both agricultural and wildlife origin but also some is from unknown sources. Although oocysts were detected in 36% of samples submitted to the laboratory, molecular analysis demonstrated that the species that pose a high risk to public health – *C. parvum* and *C. hominis* – were rare. In fact, *C. hominis* was not detected in any of the samples submitted and *C. parvum* was detected only in 1.5% of submitted samples. *Cryptosporidium parvum* was detected in only seven of the 65 supplies tested. Six of these supplies were groundwater supplies that were under the influence of surface waters from predominantly agricultural catchments. There was no *C. parvum* detected in the supplies that were sourced directly from surface water in predominantly agricultural catchments. However, 25% of the isolates from these supplies could not be genotyped, so genotypes other than those determined may have been present but not detected. While it is was not possible to determine if the *C. parvum* isolated in this study originated from a human or an animal source, the fact that no isolates of *C. hominis* were detected and yet there was widespread contamination with oocysts from other cattle species is an indication that livestock is the most likely source of the *C. parvum*. The majority of *Cryptosporidium* species found in the supplies tested appear to be of cattle or wildlife origin and are considered of low or no known risk to public health. Cattle, in particular adult cattle, appear to be the largest reservoir for environmental contamination, with a combined detection rate of 9.1%. *Cryptosporidium andersoni* was the most common species detected in 39 samples and it was detected frequently in both catchment types dominated by agricultural practices (GW-IS-AG and SW-AG). *Cryptosporidium andersoni* is commonly detected in adult cattle and has been rarely associated with human illness (Fayer, 2010; Chalmers and Katzer, 2013). Another cattle species, *C. bovis*, was also detected in this study but at a much lower frequency (1.1%) in four GW-IS-AG supplies and one SPR supply. *Cryptosporidium bovis* has been frequently isolated from post-weaned calves (Fayer, 2010). Age-related occurrence of *Cryptosporidium* species in cattle has been documented (Xiao, 2010) and the high prevalence of *C. andersoni* in comparison with other cattle species may be linked to different systems for management of young and adult cattle on farms in Ireland. However, this study has shown that contamination of drinking water supplies with oocysts originating from livestock is widespread, and of concern is the demonstrated route from farm to catchment. Effective livestock management practices are vital to ensure the security of these vulnerable catchments from contamination by pre-weaned calves, which could potentially have a significant impact on the public health risk associated with these drinking water supplies.

Cryptosporidium ubiquitum was the second most commonly detected species in the supplies tested and

the most common species detected in SW-U supplies. It infects the greatest number of host species, being commonly detected in domestic and wild ruminants, rodents, omnivores and primates (Fayer, 2010). Just over 71% of the *C. ubiquitum* detected in this study came from U-SW catchments. The most likely sources of contamination from *C. ubiquitum* in these catchments are sheep or deer. While it has been found in sporadic human cases worldwide, *C. ubiquitum* has never been associated with a waterborne outbreak and the public health significance of the detection of this species in the catchment remains unclear (Chalmers et al., 2010). The presence of the environmental genotypes in the drinking water catchment indicates contamination of surface waters from unknown wildlife sources.

This study has shown that *Cryptosporidium* contamination of drinking water catchments in Ireland is widespread in six of the seven catchment types found to be positive for oocysts. Of particular concern is the high rate of detection of *Cryptosporidium* oocysts in groundwater supplies that are influenced by surface waters. Overall, six different species of *Cryptosporidium* were found in these supply types, but the predominant species found in cattle were *C. andersoni, C. parvum* and *C. bovis*. These results indicate that these supplies are being routinely contaminated with oocysts from surface water. This surface water is having a significant impact on the quality of the groundwater in particular, as *C. parvum* has been detected in these supplies. Oocysts were also detected in all the spring-type catchments included in our study, indicating that these supplies are also vulnerable to contamination by *Cryptosporidium*. Both groundwater and spring supplies, therefore, can present hidden dangers in terms of *Cryptosporidium* risk, and attention must be given to both hydrogeology and surface catchment use when assessing the suitability of groundwater/spring supplies as drinking water sources. Surface water from upland supplies in this study has been shown to be contaminated with four different *Cryptosporidium* species and a number of genotypes of unknown origin. These data show that, although in some cases these supplies are considered pristine because the sources are above human habitation with no intensive agriculture, the presence of low numbers of grazing animals and additional wildlife can lead to contamination of these supplies. *Cryptosporidium ubiquitum* is emerging as a zoonotic pathogen, having been found in humans worldwide, primarily in industrialised nations (Li et al., 2014). As stated previously, the public health significance of the detection of this species is still unclear; however, this study has demonstrated that these upland surface water supplies are subject to environmental contamination with zoonotic species of *Cryptosporidium*.

3.5.3 Turbidity, rainfall and Cryptosporidium

The turbidity monitoring project set out to examine the relationship between rainfall events, changes in turbidity and *Cryptosporidium* oocyst concentration in a catchment system in order to provide information to design more-targeted-efficient sampling schedules. Currently, sampling schedules for *Cryptosporidium* do not take into account local weather conditions or changes in turbidity of the raw water. In many cases, sampling is random, taking place infrequently at any time of the year. The value of the data from this random sampling is questionable. A more targeted approach to sampling would give a better indication of the risk of the supply and add value to the data obtained. The results here have shown that there is a positive relationship between events in the catchment and the numbers of oocysts in the raw water supply, with oocyst numbers higher just after a rainfall event when turbidity has spiked. A monitoring programme designed to test at periods when oocysts are at their peak should be based on historical data of a catchment that take into account the events that may lead to a spike in the numbers of oocysts such as rainfall, agricultural practices, river flow and turbidity. Monitoring for triggers to events and timing sampling to events provide a more efficient and cost-effective sampling regime and, more importantly, provide more valuable data on the *Cryptosporidium* risk than from random sampling.

3.6 Conclusion

Historical monitoring data on individual supplies, including molecular profiling of species, provide vital information on the implementation of effective drinking water safety plans. The data generated can aid in the decision-making process in incidence response

investigations and can be used to implement effective targeted catchment management plans. Moreover, the data can also provide clarity on the risk of *Cryptosporidium* in individual supplies, providing evidence for the requisition of funding for upgrading of treatment works or provision of new treatment works.

4 A Survey of *Cryptosporidium* Monitoring in Irish Drinking Water Treatment Plants

4.1 Introduction

Under the Drinking Water Regulations 2007, WSAs are obliged to test treated drinking water for the presence of *Cryptosporidium* oocysts only if the parameter for *Clostridium perfringens* is breached. All other monitoring activity is discretionary. This survey was carried out in order to determine the extent of routine *Cryptosporidium* testing in drinking water in Ireland. From the responses and a comparison of monitored and unmonitored supplies, the criteria generally used to select supplies and define monitoring schedules were established and evaluated. Services and charges of commercial testing laboratories were compared. Finally, WSAs were asked whether they considered the existing guidelines for *Cryptosporidium* testing effective and how they thought they might be improved. A second survey was conducted to assess the genotyping practices currently carried out by local authorities. In addition, this survey aimed to estimate the future requirements for genotyping if services of a national reference facility were available. The following are the objectives for each survey.

4.1.1 Survey of Cryptosporidium *monitoring in Irish drinking water treatment plants*

- To determine the extent of routine *Cryptosporidium* testing in drinking water in Ireland;
- To establish and evaluate the criteria used to select supplies and define monitoring schedules;
- To compare services and charges of commercial testing laboratories; and
- To determine whether WSAs consider the existing guidelines for *Cryptosporidium* testing to be effective and how these could be improved.

4.1.2 Survey of Cryptosporidium genotyping requirements of local authorities

- To gather information on the genotyping practices/requirements within local authorities; and
- To determine the need for a national genotyping testing service for water.

4.2 *Methodology*

4.2.1 Cryptosporidium *monitoring in Irish drinking WTPs*

A brief survey designed using SurveyMonkey (https://www.surveymonkey.com) was sent to the senior scientist/engineer/scientific officer in charge of drinking water monitoring in each WSA during 2011 (Appendix 2). The survey, customised for each city/county council, listed up to 10 randomly selected supplies in the area (including seven public water supplies and three public or private GWSs). The first part of the survey sought information on *Cryptosporidium* monitoring in specific water supplies, such as reasons why a supply was or was not tested, the monitoring history, testing frequency and water type tested (raw or treated). The second part dealt with general *Cryptosporidium* testing issues, including commercial testing laboratories' contact details, the charge per sample and the information reported back to the WSA (e.g. oocyst numbers, viability, species identity). The survey concluded with an open question as to whether the respondent considered the existing guidelines for *Cryptosporidium* testing in drinking water to be adequate – as specified in the *Handbook on the Implementation of the Regulations of Water Services Authorities* (EPA, 2010a,b).

The online survey was followed by a brief telephone interview. Contact details and basic supply information, including the water supply type, source, volume and population size supplied, treatment type and in some cases the *Cryptosporidium* risk score,

were derived from the *Register of Drinking Water Supplies* provided by the EPA. Routinely monitored and unmonitored supplies were statistically compared with respect to their size, source water type and *Cryptosporidium* risk scores using χ^2 analysis. Differences were considered significant at $p < 0.05$. Many Irish water supply zones receive blended water from various different supplies, frequently ones situated in neighbouring counties.

4.2.2 Cryptosporidium *genotyping requirements of local authorities*

A brief survey designed using SurveyMonkey was again conducted in 2013 and sent to the senior scientist/engineer/scientific officer in charge of drinking water monitoring in each WSA. The survey aimed to gather information on the genotyping practices/ requirements within local authorities. Local authorities were asked a series of questions about their current testing schedules for primary testing. They were also asked about the number of positive samples they received annually and what percentage, if any, of these samples they had sent for genotyping over the previous 3 years. In addition, local authorities were asked why they did/did not get samples genotyped and, if there was a national reference facility, would they avail of this service and what would be the best estimate of the number of samples they would require typed annually.

4.3 Results

4.3.1 *Survey of* Cryptosporidium *monitoring in Irish drinking water treatment plants*

According to the study's survey, 227 public water supplies are currently monitored for *Cryptosporidium*. This represents roughly one quarter of all public water supplies (the total number of public water supplies in the *Register of Drinking Water Supplies* is 926, 30 short of the 956 reported in the EPA 2011 report (EPA, 2011)), but amounts to approximately 65.6% of the volume of drinking water provided by these supplies. While this is a very considerable proportion, it is important to note that monitoring frequencies are generally very low. Figure 4.1B shows that in 83% of monitored public supplies the final water is tested less than 10 times per year. Raw water monitoring is only performed in 9.6% of all public water supplies, generally at low frequencies (Fig. 4.1A).

4.3.1.1 Response rate

The response rate to the online survey was relatively high, with 67% of WSAs completing most or all questions.

A comparison between monitored and unmonitored supplies (Fig. 4.2A–C) showed that the former:

- Served larger numbers of people;
- Were predominantly sourced from surface water; and
- Were assigned mostly high or very high risk scores.

Monitored supplies included some of the largest supplies. Of the small number of large (serving 19,900–28,000 people) unmonitored supplies, most have low catchment risk scores (two are fed by groundwater) and/or effective treatment. In contrast the majority of small supplies (serving <1,000 people) do not monitor for *Cryptosporidium*.

Figure 4.2B shows that 50% of monitored supplies are fed by surface water, while over half of the unmonitored plants are supplied by groundwater. Blended water is more likely to be monitored. Finally, over 83% of monitored supplies are characterised by a high or very high overall *Cryptosporidium* risk score. The difference between monitored and unmonitored supplies was significant in relation to size ($\chi^2 = 76.55$, $df = 4$, $p < 0.0005$), source water type ($\chi^2 = 74.55$, $df = 3$, $p < 0.0005$) and risk score ($\chi^2 = 231.76$, $df = 3$, $p < 0.0005$).

Asked why certain supplies were monitored, the main reason the WSAs gave was that it was listed on the EPA RAL and lacked an effective barrier against *Cryptosporidium* oocysts (Fig. 4.3A). Under these circumstances, testing was perceived as providing some level of protection to the consumer. In addition, compliance with EPA guidelines and monitoring programmes agreed with the HSE were listed as important reasons. Other reasons included catchment management, treatment control, surface source water and treated water storage in an uncovered reservoir.

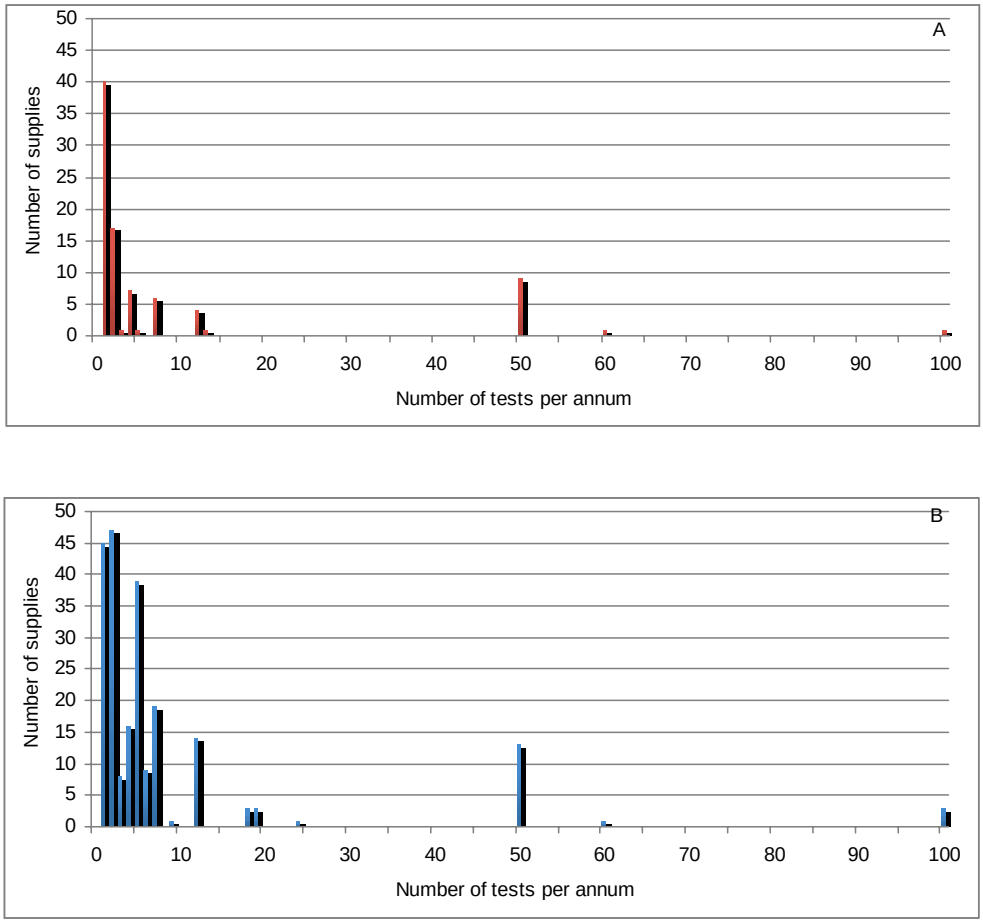
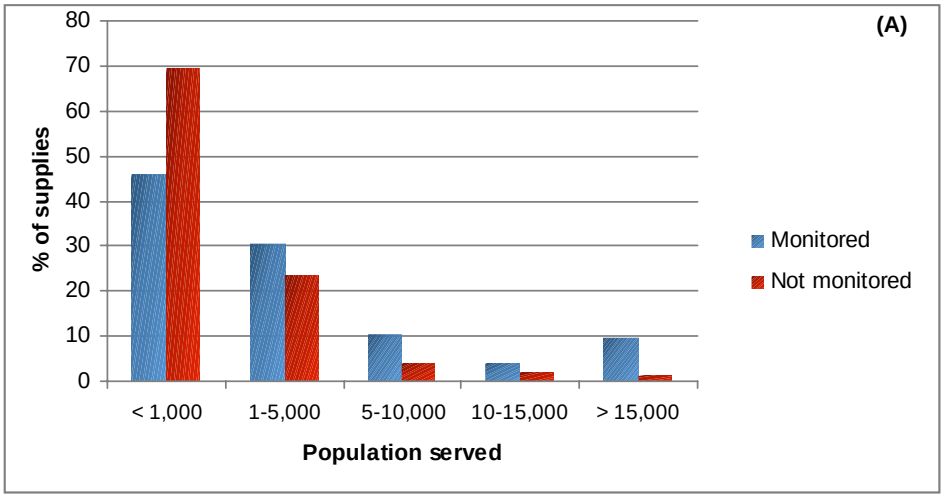


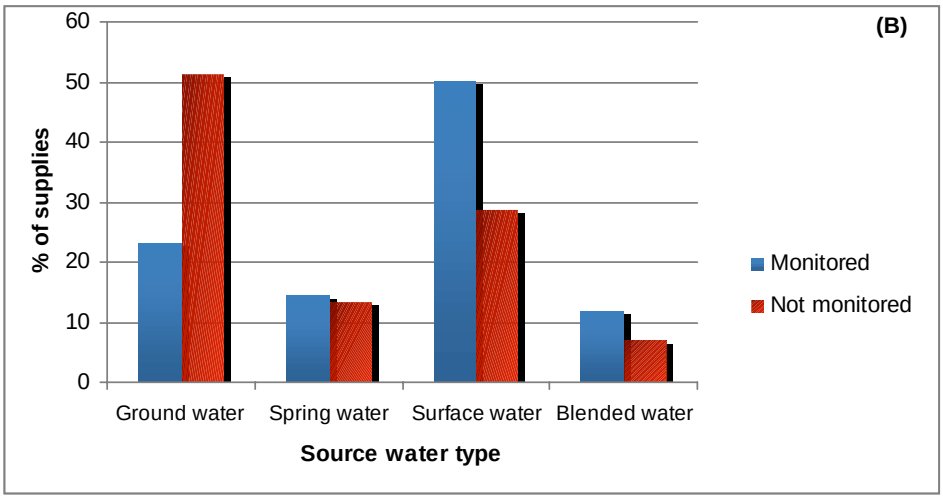
Figure 4.1. Monitoring frequencies of (A) raw and (B) treated water in public water supplies.

Monitoring programmes designed in conjunction with the HSE usually focused on larger supplies with high or very high risk scores, as reflected in the monitored supplies characteristics described above. Lack of resources was cited as the main reason why more supplies are not routinely monitored (Fig. 4.3B). Another important reason given was low risk scores, chiefly due to effective treatment in the plant and/or the fact that the supply was fed by groundwater. At least two WSAs used Geological Survey of Ireland (GSI) vulnerability ratings to assess the safety of their groundwater sources (Groundwater Protection Scheme/Source Protection Plans).

The annual crude incidence rates for cryptosporidiosis from 2006 to 2009 ((HPSC, 2007–2010a) were plotted against the approximate percentage of monitored drinking water volume in the various HSE areas (Fig. 4.4). The highest monitoring rate occurred in the east, where annual case numbers were negligible. In contrast, in the midlands, where relatively high infection rates are recorded, very little monitoring was carried out. In other areas, the monitoring effort reflected the typical annual incidence, with relatively high monitoring levels in the mid-west (with crude incidence rates ranging between 15.5 and 19.4%) and low monitoring levels in the north-east, where the incidence rate is relatively low (ranging from 6.1 to 8.4%).

Information on *Cryptosporidium* monitoring in GWSs was scarce. Generally WSAs advise the scheme operators to test for the parasite if significant levels of *Clostridium perfringens* exist in the treated water or if the HSE has alerted them to cryptosporidiosis cases in the community. Some public GWSs are monitored indirectly because they receive water from regularly tested public supplies. With two notable exceptions of public GWSs monitored directly on a monthly basis (both of which had very high *Cryptosporidium* risk





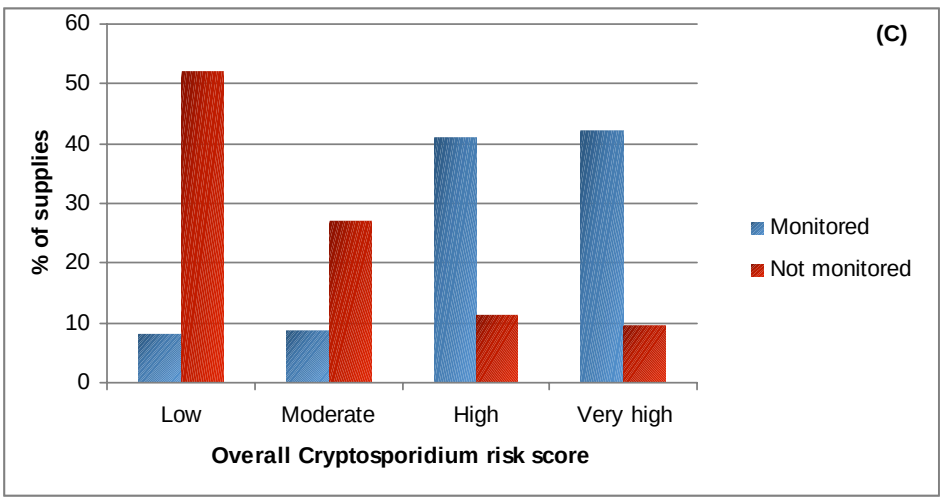


Figure 4.2. Population size (A), source water type (B) and *Cryptosporidium* risk scores (C) of monitored supplies versus those that are not monitored.

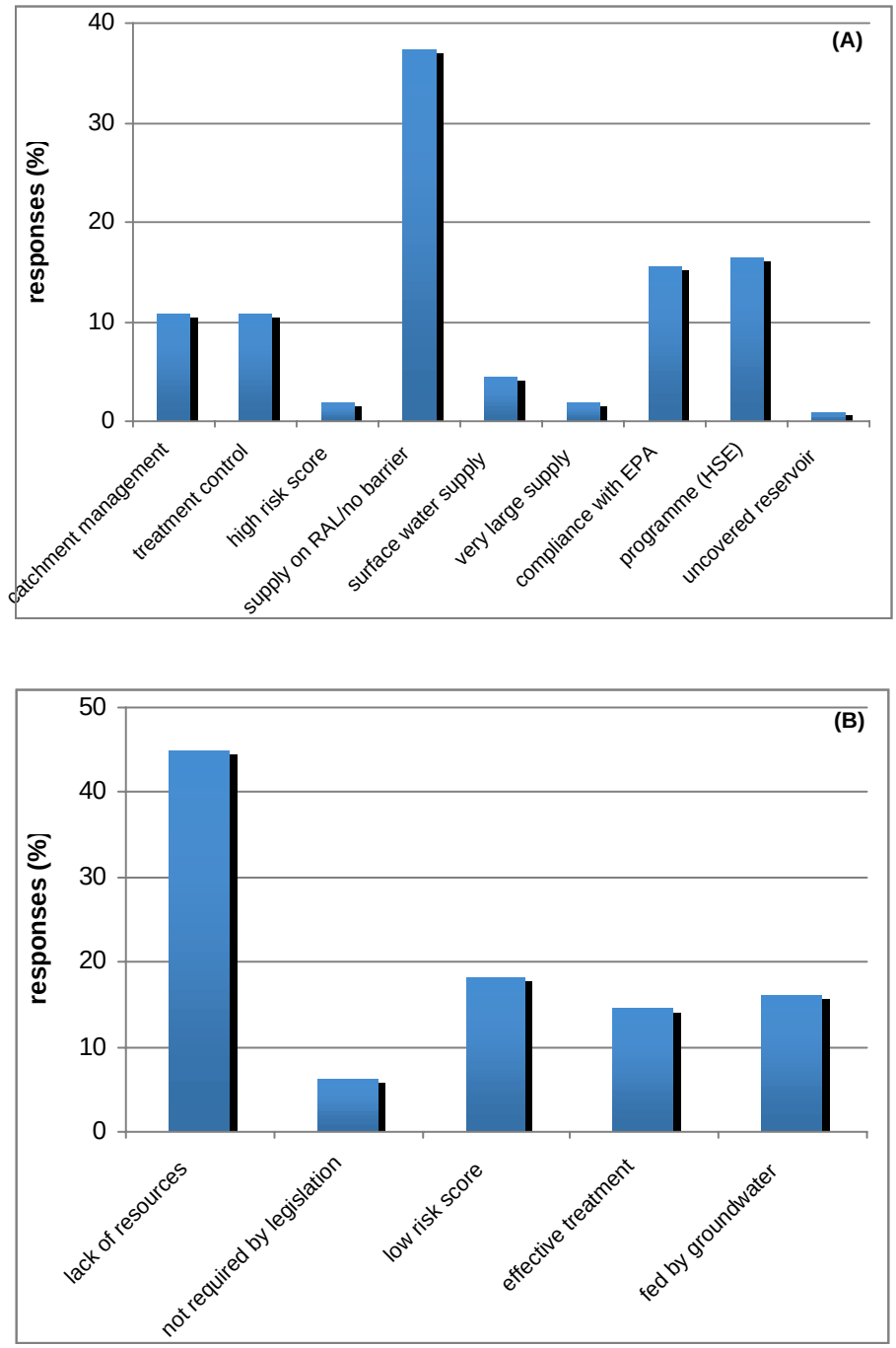


Figure 4.3. Reasons provided by Water Service Authorities as to why supplies are (A) routinely monitored and (B) not routinely monitored. RAL, Remedial Action List; EPA, Environmental Protection Agency; HSE, Health Service Executive.

factors), information derived from the WSAs indicated that none of the remaining public or private GWSs were monitored. According to the National Federation of Group Water Schemes, the *Cryptosporidium* risk in upgraded schemes is effectively managed by incorporating effective barriers in treatment, most commonly UV treatment systems. Further, GWSs (supplying between 100 and 1,500 houses) were assessed in relation to their *Cryptosporidium* risk scores (which to date have apparently not been reported to the WSAs or the EPA). Those with high or very high risk scores will be upgraded once funding becomes available under the Design, Build and Operate (DBO) Scheme.

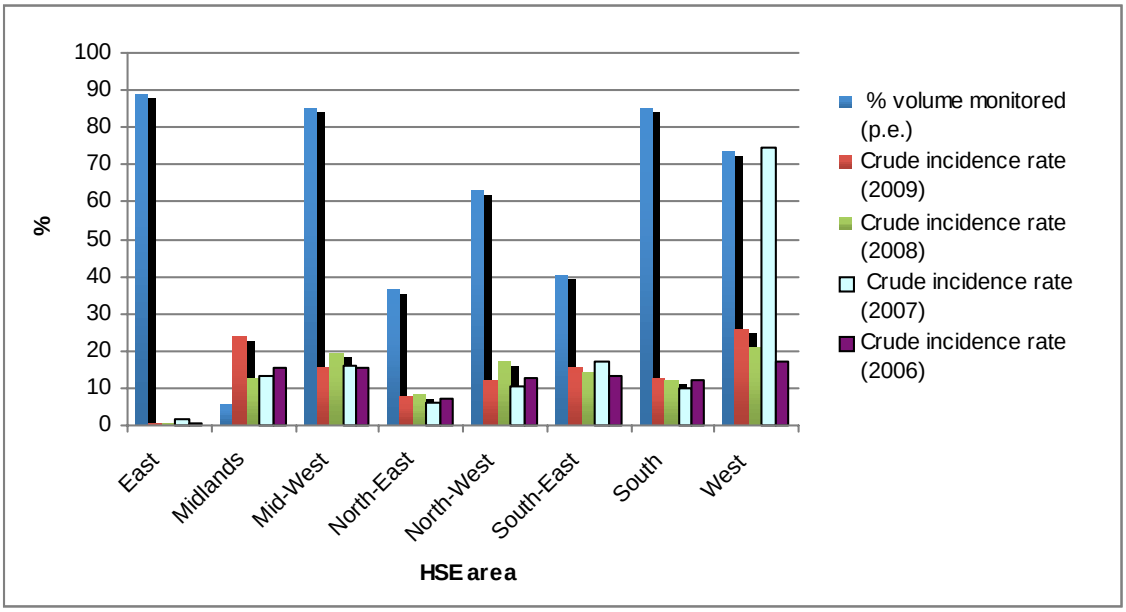


Figure 4.4. Routine monitoring and cryptosporidiosis incidence by Health Service Executive (HSE) area.

4.3.1.2 Evaluation of existing guidelines for Cryptosporidium *testing by WSAs*

Almost half (46%) of the respondents found the guidelines as specified in the *Handbook on the Implementation of the Regulations of Water Services Authorities* (EPA, 2010a,b) to be adequate. The remainder voiced the following criticisms and recommendations:

- **Guidelines:**
  More clearly specified guidelines and sampling schedules under legislation are required which should be determined by the source water type as well as the supply size. Recommended schedules should include sampling during and immediately after extreme weather events (particularly heavy rainfall). The *Handbook on the Implementation of the Regulations of Water Services Authorities* recommends monitoring for an initial period of 2 years. However, there is no guidance on how the monitoring data should be interpreted or evaluated or how sampling schedules should be modified following this initial period.

- **Indicator parameters:**
  Clostridium perfringens, widely recognised as a poor indicator for *Cryptosporidium*, should be replaced by turbidity as a suitable surrogate parameter for the possible presence of *Cryptosporidium* oocysts.

- ***Cryptosporidium* risk assessment:**
  Cryptosporidium risk scoring makes no provision for risk reduction due to ozonation or Acti-Flow treatment (a physical-chemical settling process combining micro-sand ballasted flocculation and lamella settling). Extreme weather events (flooding or freezing conditions), their potential effects on source water quality and the treatment process are not considered.

- **HSE:**
  The HSE should publish national guidelines clearly specifying the course of action to be taken when oocysts are detected, particularly when boil-water notices are issued or supplies closed. Currently, a uniform response is lacking and decisions often differ significantly in different counties. By liaising more closely with the HSE, WSAs could focus their resources on *Cryptosporidium* monitoring when cases of cryptosporidiosis are reported in the community.

- ***Cryptosporidium* genotyping:**
  The importance of identifying the oocyst species found in drinking water was emphasised. Guidelines for monitoring schedules, boil-water

notices and supply closures should be based on species identity as well as oocyst numbers. One respondent called for the establishment of a national *Cryptosporidium* genotyping facility.

- **Monitoring vs effective treatment:**
  Some respondents proposed that for well-maintained plants operated within their design parameters, the risk arising from *Cryptosporidium* was minimal even if they were supplied by surface water. Resources should be used to upgrade and maintain treatment plants rather than carry out routine *Cryptosporidium* monitoring.

- **Resources:**
  A centralised laboratory financed by all local authorities would reduce *Cryptosporidium* testing expenses. A number of respondents felt that monitoring guidelines did not consider the limited resources available to the WSAs.

4.3.2 Cryptosporidium *genotyping requirements of local authorities*

Overall, 64% (20/31) of local authorities responded to the survey. Nineteen responded online and one response was followed up by telephone. Their responses are briefly summarised in Figs 4.5 and 4.6.

The survey of *Cryptosporidium* monitoring in Irish drinking water treatment plants (WTPs) in 2011 indicated that final water is tested less than 10 times per year in 83% of monitored public supplies and raw water monitoring is only performed in 9.6% at generally low frequencies. However, the number of raw and treated water samples to be tested annually would increase to 1,437 if a genotyping service was available according to 85% of the WSAs (n = 17) in the 2013 genotyping survey. The importance of *Cryptosporidium* genotyping in drinking water was emphasised in the first survey report. All water authorities completing the survey in 2013 (n = 20) stated that genotyping of 247 samples is required annually (Fig. 4.5). The reasons provided by 65% of WSAs (n = 13) for not genotyping positive samples included costs, lack of genotyping service, a small number of oocysts on the slide or the fact that supplies were fed by groundwater. The main reason, however, was due to a low number of positives due to effective treatment in the plant (Fig. 4.6). Asked, why genotyping was carried out, eight WSAs said that the main reason was requests from the HSE and EPA to determine public health risk factors in addition to catchment management.

4.4 Conclusions and Recommendations

Under legislation, Irish drinking WTPs must be tested for *Cryptosporidium* oocysts if the concentration of *Clostridium perfringens* spores exceeds 0 in 100 l of treated water. It also recommends that supplies are tested if the turbidity of the water is greater than 1 NTU. This study's survey indicated that many WSAs are aware of the limitations of *Clostridium perfringens* as an indicator for the possible presence of *Cryptosporidium* oocysts and support its replacement with turbidity as a more suitable surrogate parameter. Furthermore, continuous turbidity monitors in many treatment plants already provide this information on a real-time basis as treated water leaves the plant. It is important to stress, however, that, while high turbidity indicates the possible presence of *Cryptosporidium* in the treated water, low turbidity does not guarantee its absence (Carey et al., 2004). Another water testing trigger should be the occurrence of *Cryptosporidium* cases in the community. In Ireland, cryptosporidiosis in humans is notifiable. As a result, local HSE officials should immediately notify WSAs of the exact location and extent of any outbreaks that occur in their functional area.

In addition to water testing because of other breached parameters or in response to HSE alerts, the *Handbook on the Implementation of the Regulations of Water Services Authorities* (EPA, 2010a,b) and the EPA Environmental Enforcement Network *Cryptosporidium* Working Group recommend the establishment of regular monitoring schedules. This study found that a considerable proportion of Irish drinking water plants are regularly tested for *Cryptosporidium*. The characteristics of these monitored supplies reflected the WSAs' focus on larger, mostly surface-water-fed supplies with high or very high risk scores. However, monitoring frequencies were generally very low. Considering the treated water volumes that many drinking WTPs produce, the

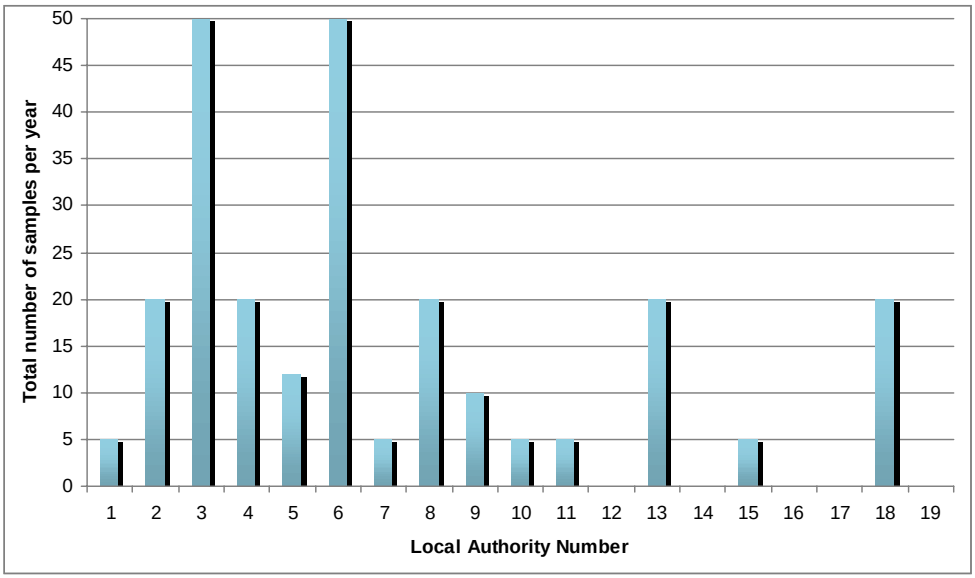


Figure 4.5. Genotyping frequencies of public water supplies annually if the service was available.

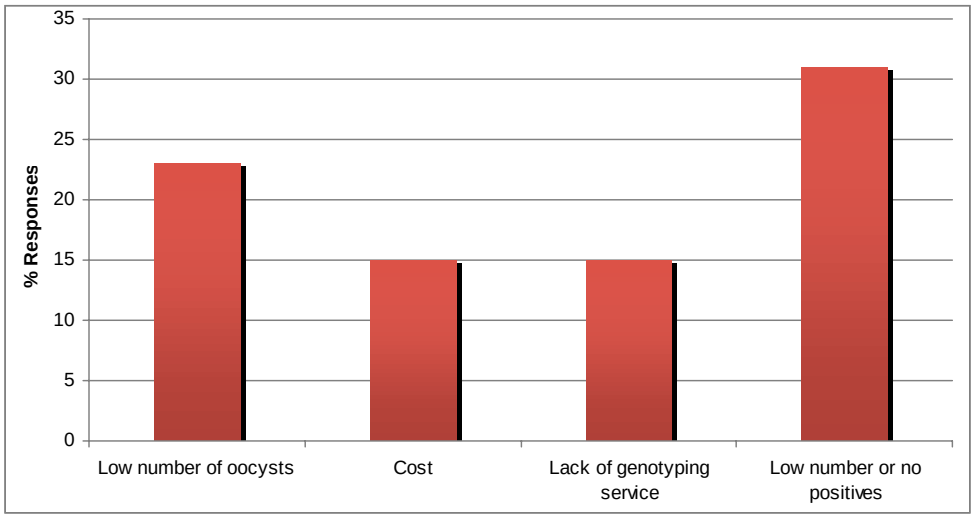


Figure 4.6. Reasons provided by Water Service Authorities for supplies not using genotyping services.

percentage of water tested each year was infinitesimal. On the other hand, due to the low but highly variable recovery rates associated with water testing, high sampling frequencies and indeed multiple samples have been recommended (Xiao et al., 2006). Given the limited resources available the cost for monitoring large numbers of supplies at a high frequency would be prohibitive. Instead, resources should focus on closely investigating a small number of supplies by frequent regular testing (including during and immediately after extreme weather events). Once the *Cryptosporidium* risk is clearly characterised, adequate treatment methods should be put in place. Subsequently, testing would only be carried out if the turbidity increased or if *Cryptosporidium* was reported in the community. Under these circumstances, water testing should include oocyst identification as well as enumeration.

While generally considered a useful tool, it was remarked that the *Cryptosporidium* risk assessment methodology, as outlined in the *Handbook on the Implementation of the Regulations of Water Services Authorities* (EPA, 2010a,b), does not provide for the reduction of risk resulting from certain water treatment

methods. It should be modified to take into account increased contamination risk during extreme weather, particularly flooding and freezing.

Finally, no national guidelines define the procedures to follow when *Cryptosporidium* oocysts are detected in treated drinking water. Instead, decisions are made on an ad-hoc case-by-case basis. Many WSAs felt that this approach was time-consuming and frequently inconsistent and suggested that guidelines should exist that clearly characterise the conditions that should trigger a boil-water notice and/or temporary closure of the plant, including the number of positive water samples in a given time frame (dependent on the size of the supply), oocyst concentrations and, importantly, species identity.

5 Evaluation of Emerging Waterborne Contaminants

5.1 Introduction

Safe drinking water availability is essential for the health and well-being of humans all over the world. Traditionally, the microbiological quality of drinking water attracted the greatest attention and this still continues to be the most important issue in large parts of the world. During the last decades, however, the focus on the chemical quality of drinking water has increased in line with increasing knowledge of chemical compounds and their possible toxic effects (Houtman, 2010).

This has led to a new term being used in relation to water quality – emerging contaminants. These contaminants can be broadly described as any chemical or biological substance with the potential to enter the environment and cause known or suspected adverse ecological and (or) human health effects (US Geological Survey, 2012). The occurrence of emerging or newly identified contaminants in Irish water supplies is of continuous concern for the health and safety of the consuming public (Bolong et al., 2009). This is in part due to their physical and chemical properties, which make them difficult to remove by conventional water treatment processes. These undesirable compounds are, therefore, being released, knowingly or unknowingly, into the aquatic environment (Bolong et al., 2009).

Contributing factors in their emergence include new chemical synthesis, bioaccumulation over several years, and changes in our own use and disposal of household and personal care products (PCPs). The development of new detection methods has also allowed identification of contaminants that were previously undetected due to their low levels. Over the last 40 years, instrument sensitivity has improved enormously, with a gain factor of 1,000 and even up to 100,000 being achieved (Houtman, 2010).

Sources of new emerging contaminants include, amongst others, pharmaceutical residues, man-made substances, as well as disinfection by-products, and all are threats to our water supply network. Revolutionary developments in resources and industrial technologies have produced more chemicals and compounds over the past number of years, which have consequently increased the number of compounds being identified as posing potential environmental threats, including threats to living organisms as well as to ecological systems. Pharmaceuticals and personal care products (PPCPs), surfactants, various industrial additives, and numerous chemicals purported to be endocrine disrupters are not metabolised and are discharged into sewers and WWTPs (Bolong et al., 2009). In 2010, a report by Rolf Halden of the Biodesign Institute, Arizona State University, noted that over 4,000 high production volume (HPV) chemicals in common usage have never been evaluated in terms of their ecotoxicity (their potential to adversely affect the environment) and the risks they may pose to humans (Arizona State University, 2010). Industrial and technological breakthroughs have outpaced the regulatory practices with respect to water quality (Bolong et al., 2009).

5.2 Objectives

In Ireland, all drinking water must comply with the European Communities (Drinking Water) (No. 2) Regulations, 2007, which have set standards for 48 individual microbiological, chemical and indicator parameters (Hayes et al., 2011).

Until 1 January 2014, WSAs from 34 city and county councils throughout Ireland were responsible for the production, distribution and monitoring of public water supplies. On this date the responsibility was assumed by Irish Water. The EPA is the supervisory authority in relation to public water supplies and the local authorities are the supervisory authority in relation to private water supplies. Both the local authorities and the EPA have enforcement powers under the regulations (HSE and EPA, 2011).

Many emerging contaminants remain unregulated and their numbers are ever increasing. In Europe, Article 16 of the Water Framework Directive (WFD)

(2000/60/EC) has set out strategies against the pollution of water, the first of which, through decision 2455/2001/EC, established a first list of priority substances. This list became an annex of the WFD. Substances were chosen based on the following specific factors:

- The effects of the substance on man or the environment;
- The exposure of man or the environment to the substance;
- The lack of data on the effects of the substance on man or the environment; and
- Other community legislation and/or programmes relating to the dangerous substance.

Following a comprehensive literature review, seven groups of emerging contaminants were chosen to be examined at two drinking water treatment plants (WTPs) in Ireland. The groups chosen are presented in Table 5.1. All were selected based on their applicability to Ireland's drinking water supplies and the likelihood of their occurrence due to agricultural practices, industrial activities, or as a result of water treatment processes.

Table 5.1. Emerging contaminants chosen for this project.

Group	Contaminant chemical name
Herbicides	Glyphosate
	Picloram
Molluscides	Metaldehyde
Endocrine disrupters	Oestrone
	Oestradiol
	Ethinyl oestradiol
	Nonylphenol
Perfluorinated chemicals	Perfluorooctanoic acid
	Perfluorooctanoic sulfonate
Disinfection by-products	Chlorite
	Haloacetic aid
	Trihalomethanes
Personal care products	Musk xylenes
Heavy metals	Uranium

5.3 Materials and Methods

5.3.1 Site selection

5.3.1.1 WTP1

This is a large drinking WTP, providing over 30% of the drinking water requirements for an urban region in the east of Ireland, with a throughput close to 165 Ml per day, and a maximum output of 175 Ml per day. The treatment processes include coarse screening, settlement following flocculant dosing, sand filtering, chlorination and fluoridation. Nearly 21% of the catchment served by WTP1 comprises large urban centres. These urban areas are a source of urban storm water run-off and associated pollutant loads. There are also a number of unsewered areas in the catchment. Agricultural and urban run-off is the most likely cause of poor water quality in the catchment area, which features livestock farming, including cattle and sheep, and also some forested areas. There is also a large WWTP upstream of the water treatment works. This plant was chosen due to the high volume of water treated and its proximity to an airfield where the presence of fire-fighting foams (a source of perfluorooctane sulfonate (PFOS)) might be detected.

5.3.1.2 WTP2

WTP2 extracts water from a river at a point before it enters the city it serves. It currently treats 45 Ml of water per day using a number of treatment processes, including pretreatment with caustic soda, coagulation with alum, and flocculation with polyelectrolyte. This is followed by sedimentation, fluoridation, filtration and addition of lime. The final step in the treatment is chlorination. The only additional treatment that occurs is chlorine boosting using chlorous acid at one of its three reservoirs. This plant was chosen as it is located downstream of another WWTP and the catchment is predominantly dominated by agriculture, including cattle, sheep and pig farming.

5.3.2 Sampling programme

The sampling programme lasted 10 months, from October 2010 to July 2011, and was designed so that one sample was taken from WTP1 every month and one from WTP2 every second month, giving 10 samples for WTP1 and five for WTP2. The sampling period allowed samples to be taken across the four seasons at random sampling times during each month.

This was aimed at trending any results of concern that may have occurred as a result of a change of season (rise/fall in temperatures, increased rainfall, and agricultural practices such as slurry spreading).

5.3.3 Sample collection

Samples were taken and stored in special bottles supplied to the local authorities and were delivered to the DAFM laboratory on the day of sampling. The samples were then shipped to Severn Trent Laboratories, UK, for analysis. The time between sample collection and receipt in the laboratory did not exceed 3 days on any occasion. This was imperative when analysing the samples for glyphosate as this analysis must be carried out within 4 days of sampling.

5.4 Results

5.4.1 WTP1

Graphs showing the sample results against existing guideline limits are presented (Figs 5.1–5.4) for those parameters where results were detectable, i.e. above the reported method detection limit. All remaining

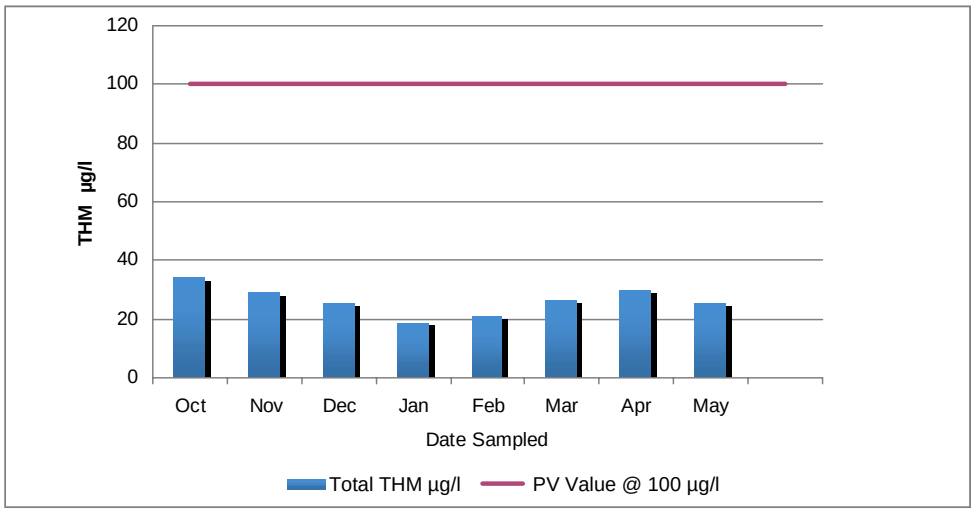


Figure 5.1. Trihalomethane (THM) values detected against the parametric value (PV) – Wastewater Treatment Plant 1.

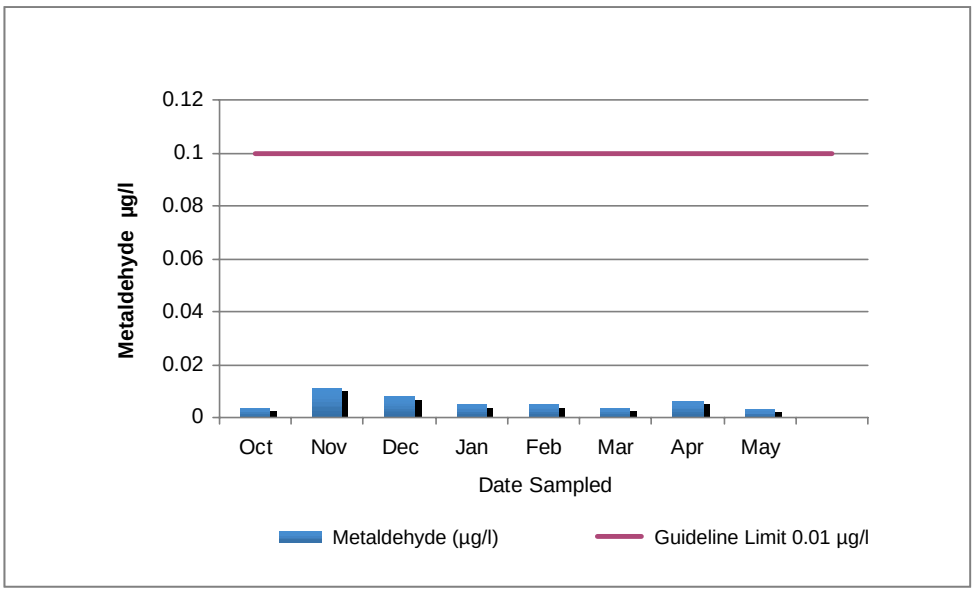


Figure 5.2. Metaldehyde values detected against the guideline limit – Wastewater Treatment Plant 1.

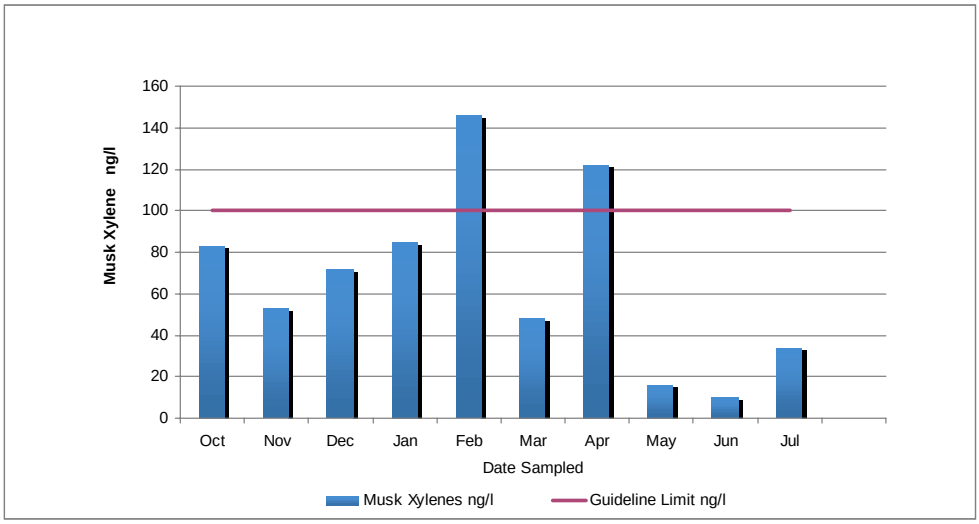


Figure 5.3. Musk xylene values detected against the guideline limit – Wastewater Treatment Plant 1.

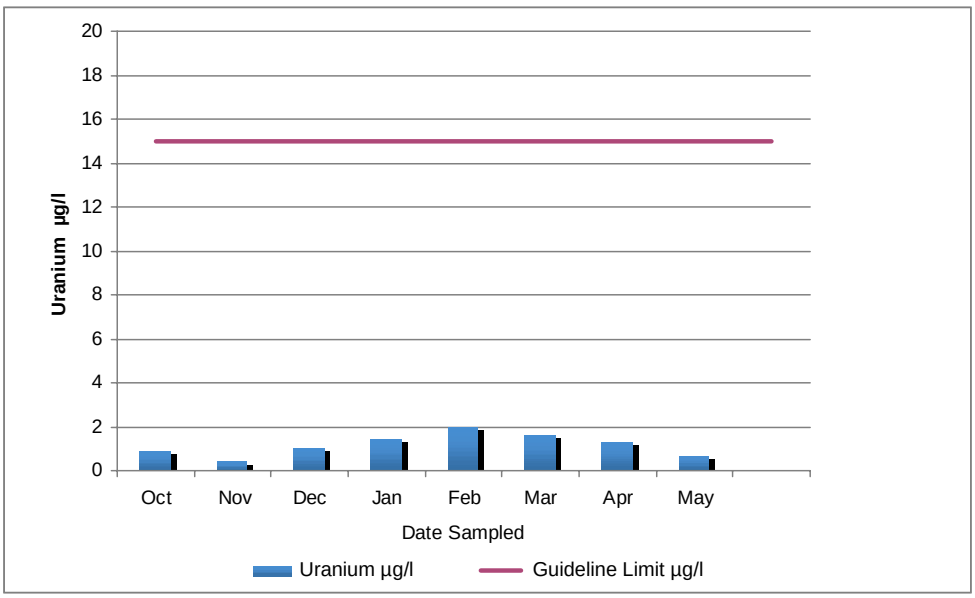


Figure 5.4. Uranium values detected against the guideline limit – Wastewater Treatment Plant 1.

emerging contaminant parameters were below the reported method detection limit and were also reported as being below the existing guideline limit (US Geological Survey, 2012).

5.4.2 WTP2

Sample results against existing guideline limits are presented for those parameters where the results were above the method reporting limit (Fig. 5.5). All remaining emerging contaminant parameters were below the reported method detection limit and were also reported as being below the existing set guideline limit.

5.5 Discussion

5.5.1 WTP1

Results for the molluscicides, steroid suite (endocrine disrupters – Table 5.1), perfluorinated chemicals, disinfection by-products and heavy metals were all below existing guideline limits. In two samples, musk xylene results were above the 0.1 µg/l surface water limit in February and April 2012, where values of 0.142

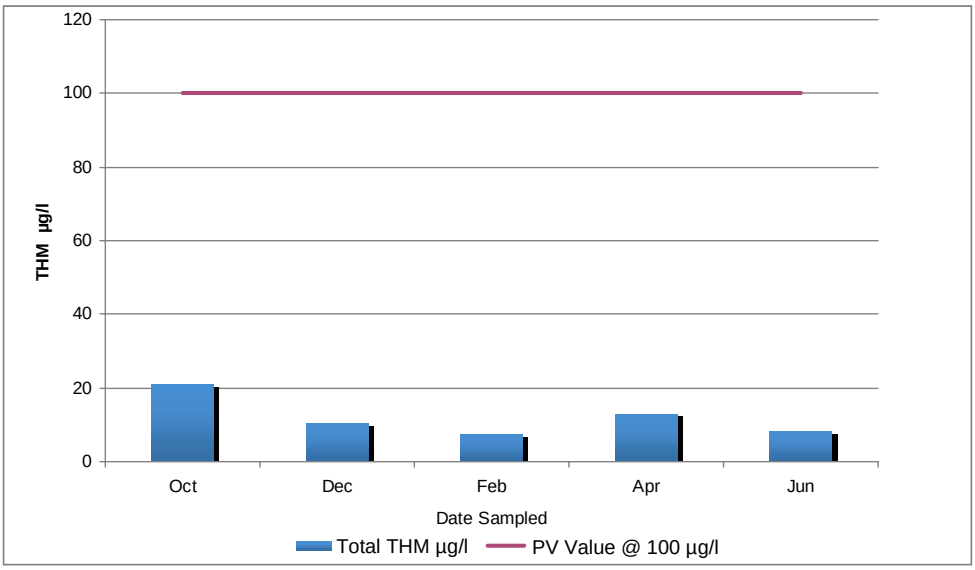


Figure 5.5. Trihalomethane (THM) values detected against the parametric value (PV) – Wastewater Treatment Plant 2.

µg/l and 0.122 µg/l, respectively, were recorded (Fig. 5.3). Galaxolide (HHCB[3]) is currently listed on EC 2008/105/EC Annex III as a substance subject to review for possible identification as a priority substance (European Parliament, 2008). It was the only musk xylene compound detected in all samples taken at WTP1; all of the other compounds (cashmeran, celestolide, phantolide, musk, traseolide, other musk xylenes, tonalide, musk moskene, musk tibetine and musk ketone) were below the method detection limit (<10 ng/l).

Galaxolide is a synthetic polycyclic musk fragrance introduced in the 1950s and is found in almost all scented consumer products, such as perfumes, soaps, cosmetics and detergents. The production of galaxolide has increased continuously over the last number of years as a result of the high cost and uncertain supply of natural musks.

After their use in private households, musk xylenes are discharged via the sewage treatment plants into the aquatic environment and, as they are lipophilic, they hardly degrade and have a tendency to bioaccumulate in fish and other aquatic organisms. In 2008, the European Commission conducted a risk assessment in accordance with Council Regulation EEC (793/93) on galaxolide as an existing substance (European Union, 2008). Concentrations of galaxolide were measured in a large number of rivers, streams, lakes and canals in Germany, the Netherlands, Switzerland, USA and Japan (European Union, 2008). In the study, levels of galaxolide were detected at up to 4.3 µg/l (Berlin). The risk assessment concluded that galaxolide's risk to the environment, human health (toxicity) and the consumer was such that at present there is no need for further information/testing and no need for risk reduction measures beyond those already applied. As a result of ineffective removal during wastewater treatment, galaxolide is almost always present at low levels in finished drinking water (European Union, 2008).

WTP1's Senior Executive Engineer reviewed the treatment processes carried out on those days where elevated galaxolide levels were detected (21 February 2012 and 25 April 2012). The review showed no evidence of changes in treatment processes and no other water pollutants were detected on those days. The weather conditions at the sampling time for the February sample were dry and mild, with a temperature of 13°C, while, for the April sample, the weather was wet, with a temperature of 8°C, which were typical of the time of year.

3. HHCB, 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta(γ)-2-benzopyrene.

5.5.2 WTP2

Results for herbicides, molluscicides, perfluorinated chemicals, disinfection by-products, PCPs and heavy metals were all below existing set guideline limits. In the June 2012 sample, one of the steroid suite of components, oestrone, was detected above the guide value of 7 ng/l set in the Netherlands, with a recorded value of 33.2 ng/l. Oestrone, also known as estrone (E1), and estradiol and estriol are known as estrogenic hormones. They are predominately female hormones, which are important for maintaining the health of the reproductive tissue, breasts, skin and brain (Ying et al., 2002). Oestrone, a naturally occurring hormone produced by the ovaries and adipose tissue, is the least abundant of the three estrogenic hormones. The amount of naturally excreted estrogens differs depending on age and gender, with the highest amount excreted during pregnancy. It is also naturally excreted by men. All humans, as well as animals, excrete hormone steroids from their bodies, which end up in the environment through sewage discharge and animal waste disposal. Oestrone, estradiol and estriol are biodegraded in sewage treatment works and the environment, although the speed of the degradation is often too slow to allow complete removal before reaching the watercourses. Oestrone, both as an estrogen and as a contraceptive, is of concern to the aquatic environment due to its endocrine disruption potential. This is because it may interfere with the reproduction of humans, livestock and wildlife (Ying et al., 2002).

Steroids have been detected in influents and effluents at sewage treatment plants and in surface water and groundwater at various levels (Ying et al., 2002). In 2003, the European Commission, in response to Council Directive 98/83/EC of 3 November 1998, reported on a study carried out on the fate of endocrine disrupters in the environment and focused on, amongst many others, natural and synthetic oestrogens. The project was carried out by the ESWE Institute for Water Research and Water Technology and the Fraunhofer Institute for Environmental Chemistry and Ecotoxicology in Germany (Wenzel et al., 2003). The aim of the project was to provide information on the exposure of endocrine disrupters through water intended for human consumption, using existing data from various European Union (EU) Member States and literature studies. Questionnaires were sent to national authorities of the EU Member States to gain a complete picture of the actual occurrence of endocrine disrupters and their potential threat to humans through drinking water. The study found that generally no oestrone was detected. Levels of 1 ng/l were reported, however, due to the treatment train of the waterworks investigated; the results were considered not plausible and required confirmation. However, Wenzel et al. (2003) found that the effects on humans due to the intake of steroid hormones in drinking water were unexpected and the rough estimation of the ingestion of estrogens in drinking water using a worst case scenario would contribute to a maximum of around 4% to the total exposure of an adult via mixed diet. The study concluded that, even if the highest concentration of an individual endocrine disrupting chemical reported for drinking water is considered for the assessment of the effects on humans, based on their knowledge, endocrine effects via consumption of drinking water are very unlikely (Wenzel et al., 2003). A review of the treatment processes carried out at WTP2 on the day when the elevated oestrone level was detected (27 June 2012) by the plant's Senior Executive Engineer showed nothing unusual about the treatment processes carried out on that day, nor were any other water pollutants detected on that day. The weather conditions at the time of sampling were dry, with a temperature of 13°C, which are typical for that time of year.

5.6 Conclusions and Recommendations

Emerging contaminants are any chemical or biological substance that has the potential to enter the environment and cause known or suspected adverse ecological and/or human health effects. Results from a 10-month project examining the presence of seven groups of emerging contaminants at WTP1 and WTP2 found that, overall, the majority of the substances were not present. The two contaminants that were detected (galaxolide at WPT1 and oestrone at WTP2) were at low levels. There was no seasonal variation in relation to the results and, in particular, disinfection by-product levels in all reported samples were less than the method limit of detection.

The project was a snapshot of two water treatment facilities and may not completely reflect the real presence of emerging contaminants in drinking water. This will be an area of importance for Ireland over the coming years and will require future extensive research across all WTPs.

6 Dissemination of Information

The project team was invited to a number of conferences and meetings over the 3 years of the project. Initially, presentations were focused on advertising the services of the CRL and informing the relevant authorities about *Cryptosporidium* in general. When the project was in its final year, the presentations included preliminary results and discussions regarding the project findings.

6.1 International

Carolyn Read and Jenny Pender presented the project's work at the Sino–European Symposium on Environment and Health in August 2013. The work on the emerging contaminants project and preliminary work on the *Cryptosporidium* pilot scheme project were presented.

6.2 National

Carolyn Read was invited to speak at the National Zoonosis Conference held in Dublin in June 2011. After the presentation, Carolyn participated in a panel discussion on *Cryptosporidium* in Ireland.

6.3 Regional

6.3.1 Safefood – Cryptosporidium *Knowledge Network*

Poster presentation at the Agri-Food and Biosciences Institute (AFBI) campus in Belfast at the inaugural meeting of the *Cryptosporidium* Knowledge Network.

6.3.2 Eastern Regional Zoonoses Committee (ERZC)

Carolyn Read was invited to present the current work and status of the *Cryptosporidium* Reference CRL at the ERZC meeting held at Backweston in April 2012.

6.3.3 EPA Water and Health-Networking Event, May 2012

Carolyn Read presented a short presentation on the CRL's functions and facilities at this networking event organised by the Science, Technology, Research and Innovation for the Environment (STRIVE) team for researchers working in the area where water quality and human health interface.

6.3.4 Kilkenny Water Quality Liaison Group Meeting, June 2012

Carolyn Read was invited to present at this meeting, which was co-ordinated by Kilkenny County Council.

6.3.5 South East Zoonoses Committee Meeting, April 2013

Jenny Pender was invited to present on the work under way in the CRL at the South East Zoonoses Committee Meeting in April 2013.

6.3.6 National Zoonoses Committee Meeting, June 2013

Carolyn Read was invited to present at this meeting. Outlined in her presentation were the preliminary results of the pilot scheme on the detection and species distribution of *Cryptosporidium* in drinking water supplies on the EPA RAL.

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Acronyms and Annotations

AFBI	Agri-Food and Biosciences Institute
BLAST	Basic Local Alignment Search Tool
CCL3	Contaminant Candidate List 3
CRL	*Cryptosporidium* Reference laboratory
CVRL	Central Veterinary Research Laboratory
DAFM	Department of Agriculture, Food and the Marine
DAPI	4'6-Diamidino-2-phenylindole
DBO	Design, build and operate
DBP	Disinfection by-products
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
DWI	Drinking Water Inspectorate
E1	Estrone
ENDWARE	European Network of Drinking Water Regulators
EPA	Environmental Protection Agency
EQS	Environmental Quality Standard
EQSD	Environmental Quality Standards Directive
ERZC	Eastern Regional Zoonoses Committee
EU	European Union
FITC	Fluorescein isothiocyanate
GCMS	Gas Chromatography Mass Spectrometry
GSI	Geological Survey of Ireland
GW	Groundwater catchment
GW-IS-AG	Groundwater influenced by surface water in agricultural catchment
GWS	Group water scheme
GW-SPR	Groundwater spring water mix catchment
HAA	Haloacetic acid
HHCB	1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclo penta(γ)-2-benzopyrene
HPSC	Health Protection Surveillance Centre
HPV	High production volume

HSE	Health Service Executive
IMS	Immunomagnetic separation
INAB	Irish National Accreditation Board
LCMS	Liquid Chromatography Mass Spectrometry
MCL	Maximum Contaminant Level
Ml	Megalitres
NCBI	National Center for Biotechnology Information
NRL	National Reference Laboratory
NTU	Nephelometric turbidity unit
PAH	Polycyclic aromatic hydrocarbon
PCP	Personal care product
PCR	Polymerase chain reaction
PFOA	Pefluorooctanoic acid
PFOS	Perfluorooctane sulfonate
PI	Propidium iodide
POP	Persistent organic pollutants
PPCP	Pharmaceuticals and Personal Care Products
PV	Parametric value
RAL	Remedial Action List
REACH	Registration, Evaluation, Authorisation and Restriction of Chemical Substances
rRNA	Ribosomal ribonucleic acid
SI	Statutory Instrument
SOP	Standard Operating Procedure
SPR	Spring water catchment
SPR-IS-AG	Spring water influenced by surface water in agricultural catchment
SSU	Small subunit
STRIVE	Science, Technology, Research and Innovation for the Environment
SW-L-AG	Surface water lowland agricultural catchment
SW-U	Surface water upland catchment
THM	Trihalomethane
UCD	University College Dublin
UK	United Kingdom
US	United States

US EPA	United States Environmental Protection Agency
UV	Ultraviolet
WFD	Water Framework Directive
WHO	World Health Organisation
WSA	Water Services Authorities
WTP	Water Treatment Plant
WWTP	Wastewater Treatment Plant

Appendix 1

Standard Operating Procedure:

Detection of *Cryptosporidium* spp. in Treated Water by Filtration, Immunomagnetic Separation and Fluorescent Antibody Identification

SOP: Detection of Cryptosporidium *spp. in treated water by filtration, immunomagnetic separation and fluorescent antibody identification*

1. INTRODUCTION/SCOPE

The purpose of this SOP is to describe the method used for the detection and enumeration of *Cryptosporidium* oocysts in drinking water. The method identifies genera of *Cryptosporidium* and not the species. It does not distinguish between viable and non-viable oocysts, origin of oocysts or public health risk associated with the presence of the oocysts in drinking water.

The method is based on the US EPA *Method 1622* (2005 revision of method first published in 1996) and the UK method: *The Microbiology of Drinking Water (2009) – Part 14 – Methods for the Isolation, Identification and Enumeration of* Cryptosporidium *Oocysts and* Giardia *Cysts*. This method is suitable for the detection and enumeration of *Cryptosporidium* oocysts in drinking water that has been filtered through IDEXX Filta-Max xpress® filters.

The limit of detection for this method is less than 0.01 oocyst/10 l. This is a performance-based method which has been validated by the US EPA *Method 1622: Cryptosporidium in Water by Filtration/IMS/FA.*

This method has been validated in drinking water.

2. HEALTH AND SAFETY

2.1. Personal safety equipment, laboratory coat and gloves must be worn at all times when handling samples. Laboratory staff should be made aware of the risks associated with *Cryptosporidium* and the potential for infection.

2.2. In the event of spillage please refer to Risk Assessments.

3. RESPONSIBILITY

3.1. Only suitably trained personnel can perform this method.

3.2. It is the responsibility of suitably trained staff to read and adhere to this SOP at all times.

4. IN-HOUSE DOCUMENTS REFERRED TO:

4.1. *Cryptosporidium* Worksheet

4.2. *Cryptosporidium* Ongoing Precision and Recovery Worksheet

4.3. Crypt Scheme Worksheet

4.4. Reagent Preparation Worksheet

4.5. Proficiency Test Evaluation Form

4.6. LIMS Operation Procedure

5. REAGENTS

Note: Product codes are specified where no other alternative exists.

5.1. Filta-max elution and concentration

5.1.1. PBS – Oxoid product code BR0014G or equivalent

5.1.1.1. Prepare 10 l batch by adding 100 PBS tablets to 10 l of reverse osmosis water and stir until completely dissolved.

5.1.2. Tween 20 – BIOXTRA Sigma–Aldrich product code p7949 or equivalent

5.1.3. PBS–Tween (PBS-T) 0.01%

5.1.3.1. Add 1 ml of Tween 20 to 10 l of PBS and stir for 15–20 min.

5.1.4. High vacuum silicon grease, product code 331353N or equivalent

5.2. Immunomagnetic separation (IMS)

5.2.1. Dynabeads anti-*Cryptosporidium* beads kit containing 10 × SL Buffer A, 10 × SL Buffer B and Dynabeads anti-*Cryptosporidium*, IDEXX product code 98-14288-00, stored at 2–8°C

5.2.2. Sodium hydroxide solution 1.0 M Sigma–Aldrich Product code 72082

5.2.3. Hydrochloric acid solution 0.1 M Sigma–Aldrich Product code 84428

5.3. Staining and mounting

5.3.1. Methanol, ACS Reagent Grade, Sigma–Aldrich Product code 179337 or equivalent

5.3.2. DAPI: 4'6-Diamidino-2-phenylindole dihydrochloride hydrate, Sigma–Aldrich Product code D9542

5.3.2.1. DAPI stock solution 2 mg/ml DAPI per millilitre of methanol

5.3.2.1.1. Weigh out 1 mg of DAPI and add it to 500 µl of methanol.

5.3.2.1.2. This stock solution is stored at 2–8°C and is stable for 1 month.

5.3.2.2. DAPI working solution

5.3.2.2.1. Add 50 µl of the DAPI stock solution to 50 ml of PBS.

5.3.3. EasyStain™ kit – 80 tests BTF Australia Product code EST-CG80. This contains EasyStain™, mounting medium, positive control and fixing buffer.

5.3.4. Microscope fluorescence immersion oil Fisher scientific product code MIC904-090F or equivalent

5.4. Quality control

5.4.1. EasySeed™ – 100 oocysts per vial – BTF Australia Product code ESCG100

OR

5.4.2. *Cryptosporidium parvum* oocysts – 100 flow cytometry sorted oocysts per vial – Scottish Water Scientific Services

5.4.3. PBS–Tween 0.05%

5.4.3.1. Prepare by adding 25 µl of Tween 20 to 50 ml of PBS.

6. EQUIPMENT

6.1. Equipment for Filta-Max xpress® Pressure Elution Station (PES) v2.0 – IDEXX

6.1.1 Filta-Max xpress® PES v2.0

6.1.2 Filta-Max xpress® filter housing with Swagelok quick-connect (QC) body and Swagelok quick-flow (QF) stem

6.1.3 Filta-Max xpress® outlet diverter assembly

6.1.4 Buffer reservoir bottles (one 10 l bottle and one 1 l bottle)

6.1.5 QC fitting

6.1.6 Corning Centrifuge tubes 500 ml – Sigma product code CLF431123

6.2 Equipment for immunomagnetic separation

6.2.1 Sample rotating mixer

6.2.2 Leighton tubes: Dynal L10. IDEXX Product code 98-14298-00

6.2.3 Magnetic particle concentrator for L10 tubes – Dynal MPC-6

6.2.4 Magnetic particle concentrator for 1.5 ml tubes – Dynal MPC-S

6.2.5 1.5 ml centrifuge tubes

6.2.6. MPC-6 mixer head – Techno-path product code 98-14309-00

6.2.7. Vortex

6.2.8. Pipettes – P1000, P200, P20

6.2.9. Disposable plastic pipettes 25 ml and 50 ml

6.2.10. Pipette controller

6.2.11. Incubator capable of 37°C

6.3. Equipment for staining and mounting

6.3.1. Epifluorescence microscope Olympus BX51

Note: All equipment for Olympus BX51 microscope supplied by Mason Technology

6.3.1.1. FITC fluorescence filter 488 nm

6.3.1.2. Mercury vapour lamp

6.3.1.3. UV filter for DAPI 340–380 nm

6.3.1.4. Differential interference contrast components:

6.3.1.4.1. Analyser slider – U-AN

6.3.1.4.2. DIC prism for 100× oil immersion – U-DIC100

6.3.1.5. Wide-field eyepiece with micrometer in 100 units: MICROSWH10X

6.3.1.6. Slide micrometer 1 mm in length: 02B00421/UKA

6.3.1.7. Objectives 20×, 40× and 100×

6.3.1.8. Fluorescent control slide by Immunoconcepts, Fannin Product Code 1900

6.3.2. Humid chamber – prepared by dampening the cloth in the magnetic immunostaining tray using reverse osmosis water

6.3.3. IDEXX single spot on slides

6.3.4. Cover slips

6.3.5. Nail varnish

6.4. General laboratory equipment

6.4.1. Refrigerator capable of 2–8°C

6.4.2. Wash bottles

6.4.3. Incubator capable of 37°C

6.4.4. Reverse osmosis water

6.4.5. Pasteur pipettes

6.4.6. Tally counter

6.4.7. Cotton buds

6.4.8. Pipette tips

6.4.9. Duran bottles

6.5. Quality control equipment

6.5.1. Ongoing precision and recovery control

6.5.1.1. IDEXX Filta-Max xpress® filter modules, IDEXX product code FMC 6001

6.5.1.2. IDEXX Filta-Max xpress® filter housing unit with Swagelok QC FMC 6007

6.5.1.3. Validation rig with injection port and flow control valve, Hydraulics Modelling Services

6.5.1.4. Septa-Red PTFE/white Si Septa, 12 mm, 100 pack, Aglient Technologies Product code 5183-4460

6.5.1.5. Syringes – BD Plastipak Luer Fitting 10 ml, Unitech Product Code 302188

6.5.1.6. Needles – BD 19 gauge, 2”

6.5.1.7. Filter housing opening tools – IDEXX

7. METHOD

7.1. Sample holding times

7.1.1. Filters should be stored at 2–8°C pending testing.

7.1.2. Elution and IMS must be completed within one working day.

7.1.3. Sample staining must be carried out within 48 h from IMS.

7.1.4. Slides can be read up to 7 days after staining. If they are not read immediately after staining, they must be stored in a humid chamber (prepared as per Section 6.3.2) in the dark between 1°C and 10°C until examination.

7.2. Sample elution using the IDEXX Filta-Max xpress® PES

7.2.1. Initial PES system check and purge

7.2.1.1. Place a blank filter into the IDEXX Filta-Max xpress® filter housing. This blank filter can be used for up to 1 month for system check and system purging.

7.2.1.2. Attach the outlet diverter fitting to the lid of the filter housing QF stem by pulling back on the collar of the diverter and pushing the fittings together.

7.2.1.3. Place a blank 500 ml Corning centrifuge tube in the collection vessel holder.

7.2.1.4. Remove the lid from the centrifuge tube.

7.2.1.5. Connect the filter housing QC body to the QC stem on the PES.

7.2.1.6. Ensure the air supply is connected and the air inlet valve is open.

7.2.1.7. Connect the buffer reservoir containing the PBS-T, check that the seal is tight and there is sufficient buffer present.

7.2.1.8. Press F1 on the control panel and allow an elution cycle to run through completely.

7.2.1.9. At the end of the elution cycle discard the liquid from the centrifuge tube and disconnect the filter housing from the PES.

7.2.1.10. Proceed with sample elution if no errors are generated during initial system check.

7.2.2. Sample elution

7.2.2.1. Label a 500 ml Corning centrifuge tube.

7.2.2.2. Place the sample filter bolt head down in the IDEXX Filta-Max xpress® filter housing. Tighten the lid until the two serial number tag holes are aligned. Tighten the lid an additional 1/8 of a turn beyond this point and then back again to align the holes for a complete seal.

7.2.2.3. Transfer any liquid in the sample transport container to the labelled 500 ml Corning centrifuge tube.

7.2.2.4. Place the labelled tube in the collection vessel holder in the PES.

7.2.2.5. Attached an outlet diverter to the lid of the IDEXX Filta-Max xpress® filter housing.

7.2.2.6 Place the assembled filter housing into the PES and close the door.

7.2.2.7. Press F1 on the PES.

7.2.2.8. Once the elution cycle is complete cap the centrifuge tube and proceed with centrifugation as in Section 7.3.

7.2.2.9. Disconnect the IDEXX Filta-Max xpress® filter housing from the PES by pushing the collar on the QC body upwards whilst pulling down on the filter housing.

7.2.2.10. Open the filter housing and discard the filter.

7.2.2.11. Wash the filter housing using equipment cleaning procedure outlined in Section 7.9.

7.2.2.12. After all samples are complete it is important to purge the buffer solution from the lines of the PES. Follow the steps outlined in Section 7.2.1 but replace the PBS-T buffer reservoir with the reverse osmosis water reservoir as in Step 7.2.1.7.

7.2.2.13. Disconnect the filter housing from the PES. The lines are now filled with reverse osmosis water.

7.3. Centrifugation

7.3.1. Balance the centrifuge tubes by addition of PBS-T where necessary.

7.3.2. Place the centrifuge tubes in the centrifuge.

7.3.3. Centrifuge the eluent in the 500 ml centrifuge tubes at 2,000 × *g* for 15 min. Allow the centrifuge to coast to a stop without braking.

7.3.4. Remove the supernatant from the tube using a 25 ml disposable plastic pipette and the electronic pipette controller or a 25 ml pipette attached to a flask and peristaltic pump taking care to avoid disturbing the pellet. Transfer the supernatant to a holding container for disposal.

7.3.5. Leave approx. 5–7 ml of supernatant above the pellet.

7.3.6. Estimate the pellet volume using the graduations on the side of the tube as a guide. Record the pellet volume on the *Cryptosporidium* Worksheet. If the pellet volume is less than 0.5 ml record the pellet volume as <0.5 ml. If the pellet volume is greater than 0.5 ml record the pellet volume to the nearest 0.5 ml.

7.3.7. If the pellet volume is less than 0.5 ml add reagent water to the centrifuge tube to bring the pellet volume approximately up to 8 ml mark on the tube.

7.3.8. Cap the tube and vortex until the pellet is completely resuspended. Proceed to Section 7.4.

7.3.9. If the pellet volume is greater than 0.5 ml the pellet must be separated into subsamples each equivalent to 0.5 ml of packed pellet volume.

7.3.9.1. Add reagent water to the centrifuge tube to bring the volume in the tube up the amount calculated using the formula below:

Total volume (ml) required = (pellet volume/0.5 ml) × 5 ml

7.3.9.2. If analysis of whole sample is required calculate the number of subsamples by dividing the total volume in the centrifuge tube by 5 ml and rounding up to the nearest integer.

7.3.9.3. Divide the total volume in the centrifuge tube by the number of subsamples to determine the total volume of resuspended concentrate per subsample.

7.3.9.4. Record all relevant calculations in the *Cryptosporidium* Worksheet including the number of subsamples processed.

7.3.9.5. If analysis of partial sample is required transfer 5 ml of resuspended concentrate to the L10 tube prepared in Step 7.4.1.3.

7.3.9.6. Proceed to IMS.

7.4. Dynal IMS procedure

7.4.1. Preparation of reagents

7.4.1.1. Remove the Dynabead anti-*Cryptosporidium* kit from fridge and allow contents to come to room temperature.

7.4.1.2. Add 1 ml of 10 × SL-buffer A to an L10 tube for each sample to be processed.

7.4.1.3. Add 1 ml of 10 × SL-buffer B to each L10 tube.

7.4.2. Oocyst capture

7.4.2.1. Transfer the resuspended concentrate from Section 7.3 to the prepared L10 tube using a Pasteur pipette.

7.4.2.2. Rinse out the centrifuge tube using 1 ml of reverse osmosis water and transfer this to the L10 tube.

7.4.2.3. Repeat rinse.

7.4.2.4. The total volume in the L10 tube should not exceed approx. 1 cm above the flat edge of the L10 tube.

7.4.2.5. Vortex the Dynabeads anti-*Cryptosporidium* reagent for approx. 10 s to ensure all the beads are resuspended before use.

7.4.2.6. Once vortexed immediately transfer 100 µl of the Dynabeads to the L10 tube containing the water sample concentrate. Cap the tube. The L10 tube should now contain the sample, Dynabeads and the mixed SL buffers.

7.4.2.7. Load the tubes into the MPC-6 (without the magnet in place).

7.4.2.8. Place the MPC-6 into the MPC-6 mixer head on the sample mixer and rotate at 15–25 rpm for at least 1 h at room temperature.

7.4.2.9. Prepare 1 × SL-buffer A. Approx. 1.5 ml are needed for each sample.

7.4.2.9.1. To prepare 1 × SL-buffer A add 1 part 10 × SL-buffer A supplied in the Dynal IMS Kit to 9 parts reverse osmosis water.

Note: A precipitate may have formed in the 10 × SL-buffer A during storage at 2–8°C so allow it to come to room temperature before use to ensure all of the precipitate has dissolved. Alternatively gently warming the buffer in a 37°C incubator may remove precipitation.

7.4.2.10. Remove the MPC-6 from the mixer head and place on the bench.

7.4.2.11. Ensure the flat side of the L10 tube is facing inwards towards the back.

7.4.2.12. Insert the magnet into the MPC-6 and lay it flat so that the flat side of the L10 tube is facing down above the magnet.

7.4.2.13. Gently rock the MPC-6 by hand end to end through approx. 90°. Continue this rocking action for at least 2 min with approx. 1 tilt per second. If the tilting action is interrupted for any reason for longer than 10 s remove the magnet, gently resuspend the beads and begin from Step 7.4.2.11 again. The maximum number of tubes that can be processed in this step at the same time is three.

7.4.2.14. Without removing the magnet immediately decant off the supernatant into a holding container for disposal, ensuring to do so away from the magnet side.

7.4.2.15. Remove the L10 tube from the MPC-6 and resuspend the sample in 0.5 ml of pre-prepared 1 × SL-buffer A (prepared as per Section 7.4.2.9). Mix very gently and ensure all the beads are resuspended. DO NOT VORTEX.

7.4.2.16. Transfer the suspension containing the beads to a labelled 1.5 ml centrifuge tube using a Pasteur pipette.

7.4.2.17. Rinse the L10 tube with another 0.5 ml of 1 × SL-Buffer A and transfer this to the labelled 1.5 ml centrifuge tube.

7.4.2.18. Repeat the rinse.

7.4.2.19. Place the 1.5 ml centrifuge tube into the MPC-S with the magnetic strip in place.

7.4.2.20. Without removing the magnet gently rock the MPC-S through 180^{o} for approx. 1 min. At the end of this step the beads should form a brown dot at the back of the centrifuge tube.

7.4.2.21. Immediately aspirate off the supernatant with a P1000 pipette taking care not to disturb the beads.

7.4.2.22. Using a P200 pipette ensure the complete removal of all the supernatant from the tube including from inside the lid.

7.4.3. Acid dissociation

7.4.3.1. Remove the magnetic strip and add 50 µl of 0.1 M HCl to the centrifuge tube and vortex vigorously for 30 s.

7.4.3.2. Tap the sample so that all the beads are in the acid at the bottom of the tube.

7.4.3.3. Allow to stand for 10 min at room temperature.

7.4.3.4. Vortex again for 30 s and tap the tube to ensure that all the beads are suspended at the bottom.

7.4.3.5. Prepare an IDEXX single spot on a slide by labelling it with the sample number and date.

7.4.3.6. Add 5 µl of 1.0 M NaOH to the centre of the spot on the slide.

7.4.3.7. Place the 1.5 ml centrifuge tube back in the MPC-S, insert the magnetic strip and allow to stand without moving for 20 s.

7.4.3.8. Using a P200 pipette carefully transfer the supernatant from the bottom of the centrifuge tube to the spot on the centre of the slide. Ensure all the liquid is transferred but avoid the spot of magnetic beads formed at the back of the tube.

7.4.3.9. Leave the slides to dry at room temperature overnight. Alternatively the slides can be placed on a tray in an incubator set to 37°C. Ensure the slide is completely dry before proceeding to sample staining.

7.5. Sample staining

Note: The sample must be stained within 72 h of application to the slide. Care must be taken at each of the staining steps to avoid disturbing the sample.

7.5.1. Fix the sample by gently adding 25 µl methanol and allow to air dry or dry in an incubator at 37°C.

7.5.2. Add 50 µl EasyStain™ (small brown bottle supplied with EasyStain™ Kit) to the sample and place in a humid chamber prepared as per Step 6.3.2.

7.5.3. Incubate at 37°C for 15 min.

7.5.4. Remove the EasyStain™ by gently tipping the slide on its edge and by placing the pipette tip near the side of the spot whilst applying gentle suction. Do not allow the pipette tip to touch the surface of the spot.

7.5.5. Apply 50 µl DAPI working solution (prepared in Step 5.3.2.2.1) to the sample and leave to incubate at room temperature for 2 min.

7.5.6. Gently remove the DAPI working solution as per Step 7.5.4.

7.5.7. Apply 300 µl of EasyStain™ fixing buffer (supplied with EasyStain™ Kit). Allow this to spill outside of the spot.

7.5.8. Incubate at room temperature for 2 min and gently remove the fixing buffer as per Step 7.5.4.

7.5.9. Placing a cotton bud near the edge of the spot tilt the slide to remove any remaining fixing buffer. Leave to dry at room temperature for approx. 2–3 min.

7.5.10. Add 5 µl of mounting medium (supplied with EasyStain™ Kit) to the centre of a cover slip that has been placed on tissue.

7.5.11. Upturn the slide and align the spot of mounting medium on the cover slip with the centre of the spot on the slide.

7.5.12. Gently lower the slide down to the cover slip so that the slide and cover slip are just touching. The cover slip will attach to the slide. Leave undisturbed for a few seconds to minimise unnecessary movement of cover slip.

7.5.13. Turn the slide the right way over again and place on the tissue to allow the mounting medium to spread across the well.

7.5.14. Seal all four sides of cover slip with nail varnish and leave to dry.

7.5.15. Record the date and time of staining completion on *Cryptosporidium* Worksheet.

7.5.16. Slides can be stored at this stage at 2–8°C until examination but must be examined within 7 days of staining. Allow the slides to equilibrate to room temperature before examination.

7.6. Microscopic examination

7.6.1. General notes

7.6.1.1. Using the manuals provided with the microscope all analysts must familiarise themselves with the workings of the microscope. This includes microscope operation and maintenance as well as mercury lamp replacement.

7.6.1.2. The fluorescent microscope is located in a dark-field fluorescent microscope room. Operating the microscope in near complete darkness greatly maximises the detection of fluorescence.

7.6.2. Scanning technique

7.6.2.1. Scan each slide in a systematic fashion. Starting on the right edge of the spot scan up and down counting oocysts in each field of view. When a vertical row has been completed identify an object or feature in the PTFE coating on the middle left side of field of view and move the slide across until this feature is on the right side of field of view. Proceed with scanning the next vertical row. Ensure the entire spot has been examined.

7.6.3. Counting

7.6.3.1. Use a hand-held tally counter to record the numbers of oocysts. Try and identify features that surround the oocyst to minimise the risk of duplicating the count.

7.6.4. Daily fluorescent assessment

7.6.4.1. A fluorescent control slide must be read before commencing reading of any samples to check for loss of output from the mercury bulb.

7.6.4.1.1. Set up the microscope for viewing at magnification of 20×.

7.6.4.1.2. Begin viewing at the +/– well and proceed to the 1+, 2+, 3+ and 4+ well, noting at which well positive staining of the microbeads is observed. The endpoint is the first well at which positive staining of the microbeads is observed.

7.6.4.1.3. The endpoint of positive staining should be 1+ for optimum fluorescence.

7.6.4.2. Acceptance criteria

7.6.4.2.1. If fluorescence is observed in the 1+ well microscopic analysis of samples can proceed.

7.6.4.2.2. If fluorescence is not observed in the 1+ well the alignment of the mercury bulb and the mercury bulb output must be examined. If necessary the bulb should be replaced. Repeat the daily fluorescence check before proceeding to microscopic analysis of the samples.

7.6.4.2.3. Record the daily FITC QC slide results as well as the mercury bulb lamp hours as shown on the lamp housing units on the form.

7.6.5. Positive and negative daily control samples

7.6.5.1. The preparation of a daily positive control slide works as a staining control to ensure the quality of the staining procedure; in addition the examination of the daily control slides also enables the microscopists to familiarise themselves with the staining characteristics of typical oocysts before commencing the examination of any field samples. This daily refresher allows the microscopists to adjust their eye to what to expect on the field samples.

Note: Environmental oocysts may look different from the quality control oocysts supplied with the kit but it is an important 'eye' training exercise to observe and size the positive control oocysts daily.

7.6.5.2. Examine daily positive and negative staining controls before proceeding with the microscopy of any samples. See Section 8.3 for preparation of controls.

7.6.5.3. Scan the positive controls slide using the 40× objective.

7.6.5.4. Examine the oocysts for typical FITC, DAPI and DIC characteristics.

7.6.5.5. Engage the 100× oil immersion lens and examine three oocysts in detail.

7.6.5.6. Record the staining characteristics, i.e. FITC (size/shape), DAPI and DIC, of three oocysts from the positive control samples on the *Cryptosporidium* Worksheet.

7.6.5.7. A positive oocyst has the following characteristics:

1. Round to ovoid in shape (FITC, DIC)
2. Green fluorescence with brightly highlighted rim (FITC)
3. Between 4 and 6 µm in diameter (DIC)
4. Contains one to four DAPI-stained sky blue nuclei or if no DAPI is observed then the presence of sporozoites is confirmed using DIC.

7.6.5.8. Once the three positive oocysts have been confirmed microscopic analysis of the samples can proceed.

7.6.5.9. If no oocysts are observed or cannot be confirmed analysis must stop and a full audit of the method must be undertaken. Analysis cannot proceed until a root cause has been determined and the appropriate corrective action implemented. Details of the audit and corrective actions mulct are recorded on the Non-Conformance Record.

7.6.5.10. Scan all fields of view in the negative staining control to ensure there has been no cross-contamination during staining. If no oocysts are observed microscopic analysis

of the sample may proceed. If oocysts are detected analysis must stop and a full audit of the method must be undertaken. Analysis cannot proceed until a root cause has been determined and the appropriate corrective action implemented. Details of the audit and corrective actions must be recorded on the Non-Conformance Record. Record results from the negative control slide reading on the *Cryptosporidium* Worksheet.

7.6.6. Examination of sample slides – step by step

7.6.6.1. Using the FITC fluorescence filter engage the 40× lens and scan the entire spot on the slide for *Cryptosporidium* oocysts. *Cryptosporidium* oocysts will appear as apple green fluorescent ovoid or slightly spherical objects of between 4 and 6 µm in diameter.

7.6.6.2. Once a suspected oocyst is observed which conforms to the basic FITC characteristics click on the tally counter to record it as a putative oocyst.

7.6.6.3. Switch to the DAPI (UV) filter to locate the sporozoite nuclei of the oocyst.

7.6.6.4. Proceed using the scanning technique described ensuring all fields of view have been examined. Record each of the putative oocysts on the back of the *Cryptosporidium* Worksheet. Each oocyst must then be confirmed using the 100× oil immersion lens.

7.6.6.5. FITC identification using 100× oil immersion lens

Note: Care must be taken to ensure that the oocysts are exposed to fluorescent light for minimum amount of time. Use the shutter to block off fluorescence while recording observations on the *Cryptosporidium* Worksheet.

7.6.6.5.1. Place a small drop of fluorescent immersion oil on the centre of the slide.

7.6.6.5.2. Engage the 100× oil immersion lens.

7.6.6.5.3. Locate each of the objects identified under 40× magnification.

7.6.6.5.4. Observe the FITC characteristics of the oocyst. The apple green fluorescence is often more intense around the edges with no visible breaks in the oocyst wall staining and a clear spherical shape. Some oocysts may exhibit a Pacman-like shape or appear fuzzy around the edges due to environmental exposure.

7.6.6.6. DAPI identification – presence of sporozoite nuclei

7.6.6.6.1. Switch to the UV (DAPI) filter and confirm the presence if any of the DAPI-stained sporozoite nuclei. Intact oocysts should contain up to four sky blue sporozoite nuclei of approx. 1 µm diameter.

7.6.6.6.2. Note the number and size of the sky blue nuclei and record on the *Cryptosporidium* Worksheet. Empty oocysts do not contain any sporozoites and therefore no sky blue nuclei. Empty oocysts can be confirmed by DIC.

7.6.6.7. DIC – morphology and size measurement

7.6.6.7.1. Engage the DIC optics and fine focus on the putative oocyst to confirm the size and presence of the sporozoites. If no DAPI-stained nuclei were observed under UV confirm the absence of sporozoites using DIC.

7.6.6.7.2. Look for the morphological characteristics atypical of *Cryptosporidium* oocysts including stalks, spores and large nuclei. The presence of these characteristics would indicate the object is not an oocyst.

7.6.6.7.3. Measure accurately the object using the eyepiece graticule. *Cryptosporidium* oocysts will be between 4 and 6 µm in diameter. Record any observations on the *Cryptosporidium* Worksheet

7.6.7. Confirmation of oocysts

7.6.7.1. Oocyst confirmation is based on taking into consideration the key findings from all three microscopy steps, FITC, DAPI and DIC.

7.6.7.2. A positive oocyst has the following characteristics:

1. Round to ovoid in shape (FITC, DIC)
2. Green fluorescence with brightly highlighted rim (FITC)
3. Between 4 and 6 µm in diameter (DIC)
4. Contains one to four DAPI-stained sky blue nuclei or if no DAPI is observed then the presence of sporozoites is confirmed using DIC.

7.6.7.3. Oocysts of the correct size and shape and FITC staining characteristics but have no sporozoites that can be confirmed by either DAPI or DIC are not confirmed as *Cryptosporidium* oocysts.

7.6.7.4. Once each putative oocyst has been examined and characterised under 100× oil immersion lens the final count of oocysts confirmed in the sample can be determined.

7.7. Calculation and reporting of results

7.7.1. Results are reported as number of oocysts per 10 l of sample.

7.7.2. To calculate the final result use the following formula:

$$\frac{\text{Number of confirmed oocysts on slide} \times 10}{\text{Initial sample volume (l)}} = \text{oocysts/10 l}$$

7.7.3. The limit of detection of the method is deemed to be 0.01 oocysts/10 l.

7.7.3.1. Where the concentration of oocysts is less than 0.01 per 10 l the result shall be reported as <0.01 oocysts/10 l.

7.7.3.2. Where the concentration of oocysts in the sample is greater than 0.01 the result shall be reported as the number of oocysts/10 l.

7.7.3.3. When no oocysts are detected in the sample the result shall be reported as <0.01 oocysts/10 l.

7.7.4. The total volume analysed and the final results are entered into the LIMS.

7.8. Slide storage

All slides must be stored in a refrigerator at 2–8°C after examination.

7.9. Equipment cleaning procedure

7.9.1. Rinse all components thoroughly in tap water and then immerse them in 6% (w/v) sodium hypochlorite solution for at least 30 min.

7.9.2. Remove all the components from the sodium hypochlorite solution and wash them thoroughly with tap water.

7.9.3. Using a bottlebrush and hot soapy water wash all components and then rinse with tap water.

7.9.4. Rinse all components once again with reverse osmosis water.

7.10. Disposal of waste buffer

7.10.1. Waste buffer and reagents must be decanted into a container containing 6% (w/v) sodium hypochlorite and left for 24 h before discarding.

8. QUALITY CONTROL AND METHOD PERFORMANCE

8.1. Weekly ongoing precision and recovery

Each week that the laboratory is testing a positive and negative control filter must be spiked and processed to act as a weekly method performance check. Results must be entered onto the OPR (Ongoing Precision and Recovery) Quality Control Chart.

8.1.1. Method – positive control

8.1.1.1. Unscrew the filter housing unit with Swagelok QC fittings.

8.1.1.2. Insert an IDEXX Filta-Max xpress® filter module (bolt head down) into the housing unit and secure the lid.

8.1.1.3. Attach the Swagelok QC fittings to either side of the filter housing unit.

8.1.1.4. Unscrew the injection port of the validation rig and carefully place a septum (red side towards injection hole) into the port.

8.1.1.5. Once inserted screw the injection port back on.

8.1.1.6. Open the inlet valve and allow the water to pump through the system (approx. 10 l). This helps ‘prime’ the filter whilst allowing the system to flush through highlighting if there are any leaks.

8.1.1.7. Decrease the water pressure to approx. 0.5 bar before proceeding to spike injection.

8.1.1.8. EasySeed™ spike preparation

8.1.1.8.1. Remove EasySeed™ spike from the fridge.

8.1.1.8.2. Add 2 ml of 0.05% (v/v) Tween 20 to the tube.

8.1.1.8.3. Replace the cap and vortex for 20 s.

8.1.1.8.4. Pull the spike into the syringe using the 19G needle (Microlance 19G × 2”).

8.1.1.8.5. Wash the spike tube again with 1 ml of PBS, vortex and pull into the same syringe.

8.1.1.8.6. Repeat the washing step three more times to ensure all the oocysts have been transferred.

8.1.1.8.7. Continue on to Step 8.1.1.10 for injecting spike.

8.1.1.9. Scottish Water spiking preparation

8.1.1.9.1. Remove Scottish Water flow cytometry sorted oocysts from the fridge.

8.1.1.9.2. Vortex the tube for 20 s.

8.1.1.9.3. Pull the spike into the syringe using the 19G needle (Microlance 19G × 2”).

8.1.1.9.4. Add 1 ml of 0.05% (v/v) Tween 20 to the tube, replace the cap and vortex for 20 s and pull into the same syringe.

8.1.1.9.5. Wash the spike tube again with 1 ml of PBS, vortex and pull in to the same syringe.

8.1.1.9.6. Repeat this wash step twice more.

8.1.1.9.7. Continue on to Step 8.1.1.10 for injecting spike.

8.1.1.10. Using the syringe and needle (19G × 2”) slowly inject the spike into the IDEXX Filta-Max xpress® filter module through the injection port on the rig. Do not apply any pressure on the syringe until the needle has been fully inserted.

8.1.1.11. Once the spike has been injected into the filter allow the syringe to back wash with water and then re-inject back into the filter. Repeat this two to three times to ensure the entire spike has been washed from the syringe.

8.1.1.12. Record the water meter reading.

8.1.1.13. Increase the water pressure to approx. 2 bar and allow at least 10 l of water to filter through.

8.1.1.14. Turn off the inlet valve.

8.1.1.15. Record the water meter reading. The total volume filtered must be recorded.

8.1.1.16. Uncouple the QC fittings by pushing the body sleeve towards the stem.

8.1.1.17. Unscrew the filter housing unit.

8.1.1.18. Remove the IDEXX Filta-Max xpress® filter module carefully without touching the foam discs.

8.1.1.19. Proceed to sample elution as per Section 7.2 and follow all steps to slide reading.

8.1.1.20. Record results on *Cryptosporidium* OPR Worksheet

8.1.2. Method negative control

8.1.2.1. Complete all steps from Section 7.2 to slide reading without an IDEXX Filta-Max xpress® filter module in place.

8.1.3. Acceptance criteria

8.1.3.1. Positive control

8.1.3.1.1. Record the number of fluorescent *Cryptosporidium* oocysts detected.

8.1.3.1.2. Calculate the *Cryptosporidium* recovery using the following formula:

$$\frac{\text{Number of oocysts detected} \times 100}{\text{Number of } \textit{Cryptosporidium} \text{ in spike}}$$

8.1.3.1.3. A pass is where the recovery is greater than 40% for the positive control.

8.1.3.1.4. If recovery for the positive control is less than 40% the method must be audited to include an assessment of the spike, reagents and procedure. Sample results must not be reported until a root cause has been determined and the appropriate corrective action has been implemented. Customers must also be informed if any of their sample results have been affected. Details of the audit and any corrective actions implemented must be recorded on the Non-Conformance Record.

8.1.3.2. Negative control

8.1.3.2.1. There should be no oocysts seen on the OPR negative control slide.

8.1.3.2.2. If oocysts are detected the method must be audited to include an assessment of the equipment wash procedure. Sample results must not be reported until a root cause has been determined and the appropriate corrective action has been implemented. Customers must also be informed if their results have been affected. Details of the audit and any corrective actions implemented must be recorded on the Non-Conformance Record.

8.1.3.3. Record all readings on the *Cryptosporidium* OPR Worksheet.

8.1.4. Construction of OPR Quality Control Charts

8.1.4.1. OPR recovery data should be plotted on a quality control chart in order to monitor the performance in the laboratory and to trigger investigations when performance changes.

8.1.4.2. Use data from 20 consecutive OPR results to establish a mean and standard deviation. Use these data to construct warning and response lines against which future quality control samples are compared.

8.1.4.3. The mean recovery is drawn as a horizontal line on the graph.

8.1.4.4. The warning line is ± 2 standard deviations.

8.1.4.5. The response line is ± 3 standard deviations.

8.1.4.6. Plot all recovery results obtained from the OPR exercise.

8.1.4.6.1. Note whether any of the following four response triggers has occurred:

1. The result falls outside the response line.
2. Two out of three results fall outside the same warning line.
3. Nine consecutive results fall on the same side of the mean.
4. Six consecutive results show a trend that rises or falls.

8.1.4.7. Response to triggers involves auditing the whole method including an assessment of spike, reagents and procedure. Details of the audit and any corrective actions implemented must be recorded on the Non-Conformance Record.

8.2. CRYPTS

8.2.1. Every month the laboratory participates in the *Cryptosporidium* proficiency test scheme (CRYPTS). The scheme is run by LGC in the UK in conjunction with the Scottish Parasite Diagnostic Laboratory in Glasgow. Each round has three components to it.

1. An IDEXX Filta-Max xpress® module spiked with an unknown number of *Cryptosporidium* oocysts that must be processed according to the SOP
2. A suspension of oocysts that are added directly to the IMS stage
3. A slide used to test the microscopists' *Cryptosporidium* identification skills.

8.2.2. Analysts must participate in at least four rounds annually.

8.2.3. Processing of CRYPTS filters

8.2.3.1. Carefully using the tools provided unscrew the filter housing and decant all the liquid into a prepared concentrator tube.

8.2.3.2. Make a note of the filter number on the CRYPTS Worksheet.

8.2.3.3. Proceed with filter processing as detailed in Section 7.2.

8.2.3.4. Record all details including final count of oocysts from filter on the CRYPTS Worksheet.

8.2.4. Processing of suspension

8.2.4.1. Prepare a Dynal IMS tube by following the instructions in Section 7.4.1.

8.2.4.2. Note the number on the lid of the suspension tube in the CRYPTS Worksheet.

8.2.4.3. Vortex the tube containing the suspension in order to resuspend any oocysts that may have settled during transport.

8.2.4.4. Carefully unscrew the cap and transfer the entire suspension into the prepared tube.

8.2.4.5. Add 1.5 ml of reagent water to the tube, cap the tube and vortex vigorously and add to the prepared tube using a pipette.

8.2.4.6. Repeat this wash three more times.

8.2.4.7. Ensure the volume in the L10 tube is approx. 1 cm above the lip on the flat edge.

8.2.4.8. Proceed with Step 7.4.2.5.

8.2.4.9. Record the count of oocysts on the CRYPTS Worksheet.

8.2.5. Slide examination

8.2.5.1. Remove the slide from slide holder and examine for the presence of *Cryptosporidium* oocysts as detailed in Section 7.6.

8.2.5.2. Record any observations and decisions regarding correct identification of oocysts on the CRYPTS Worksheet including the quality of the DAPI staining or any oocyst-like bodies (OLBs).

8.2.6. Reporting of CRYPTS results

8.2.6.1. Results of each round should be filled in on the CRYPTS electronic submission form. All details must be filled in including the unique analyst identification number.

8.2.6.2. Results are submitted to LGC at the following email address ccrypts@lgcgroup.com by the required date detailed on the round information sheet.

8.2.6.3. Once completed the slide and filter housing must be returned by courier to the Scottish Parasite Diagnostic Laboratory as detailed in each round information.

8.2.7. Analysis of results

8.2.7.1. Each month a participant report and a summary report are sent to the laboratory. The laboratory must then evaluate each result as detailed below to see if performance in the round has been satisfactory. The proficiency test evaluation form should be filled in once results from each round have been assessed.

8.2.7.2. Slide performance

8.2.7.2.1. Each slide is given an assigned value by the Scottish Parasite Diagnostic Laboratory. The difference between the analyst's result and the assigned value is reported. When assessing performance in the round all details should be taken into consideration including any notes that the analyst may have commented on.

8.2.7.2.2. The difference between the assigned value and the analyst's results should be no greater than $\pm$ 2 oocysts.

8.2.7.2.3. The laboratory's result should be plotted against the assigned value for each round. This will enable the trend in the laboratory's performance to be monitored and compared from round to round.

8.2.7.3. Suspension performance

8.2.7.3.1. The number of oocysts reported by the laboratory is reported as a percentage recovery of the assigned value of the suspension. The scheme also reports the mean, median, upper and lower quartile and standard deviation for the round from the results of all the participants in the scheme.

8.2.7.3.2. The laboratory should assess its performance in each round by comparing the percentage recovery achieved with the summary statistics of all the participants.

8.2.7.3.3. The suspension recovery should be within the upper and lower quartiles of the scheme.

8.2.7.3.4. The laboratory's recovery percentage should be plotted against the round mean each month. The graph should also include the upper and lower quartile recovery for each round for ongoing assessment of performance.

8.2.7.4. Filter performance

8.2.7.4.1. The number of oocysts reported by the laboratory is reported as a percentage recovery of the assigned value of the filter. The scheme also reports the mean, median, upper and lower quartile and standard deviation for the round from the results of all the participants in the scheme.

8.2.7.4.2. The filter recovery should be within the upper and lower quartiles of the scheme.

8.2.7.4.3. The laboratory's recovery percentage should be plotted against the round mean each month. The graph should also include the upper and lower quartile recovery for each round for ongoing assessment of performance.

8.3. Preparation of daily positive and negative staining controls

8.3.1. Prepare daily positive and negative staining controls in parallel to any sample staining to ensure quality performance of staining procedure.

8.3.1.1. Pipette 30 µl of positive control supplied with the EasyStain™ Kit onto the centre of the well in the spot on the slide. This is the positive control.

8.3.1.2. Pipette 30 µl of reagent water onto the centre of the well in the spot on the slide. This is the negative control.

8.3.1.3. Allow both slides to dry either at room temperature overnight or for faster drying incubate at 37°C.

8.3.1.4. Proceed with staining procedure as detailed in Section 7.5.

REFERENCES

IDEXX Laboratories, 2002. *IDEXX Filta-Max Xpress® v 2.0 Pressure Elution Station – Operator's Guide.* www.IDEXX.com

UK Environment Agency, 2009. *The Microbiology of Drinking Water – Part 14 – Methods for the Isolation, Identification and Enumeration of* Cryptosporidium *Oocysts and* Giardia *Cysts*. Environment Agency, Bristol, UK.

US EPA (US Environmental Protection Agency), 2005. *Method 1622:* Cryptosporidium *in Water by Filtration/IMS/FA*. US EPA, Washington, DC, USA.

Appendix 2

Survey Questionnaire of *Cryptosporidium* Monitoring in Irish Drinking Water Treatment Plants

1. Cryptosporidium monitoring in specific water supplies

1. Cryptosporidium risk

	very high (over 100)	high (76-100)	moderate (50-75)	low (less than 50)	I don't know
Supply 1	○	○	○	○	○
Supply 2	○	○	○	○	○
Supply 3	○	○	○	○	○
...	○	○	○	○	○

2. Is the supply being tested for Cryptosporidium oocysts?

	yes	no
Supply 1	○	○
Supply 2	○	○
Supply 3	○	○
...	○	○

3. What is the main reason why this supply IS being monitored for Cryptosporidium? (please select all responses that apply)

	Catchment management	Treatment control	Compliance with EPA guidelines	Alerts from the local authority/HSE representative
Supply 1	☐	☐	☐	☐
Supply 2	☐	☐	☐	☐
Supply 3	☐	☐	☐	☐
...	☐	☐	☐	☐

4. What is the main reason why this supply is NOT being monitored for Cryptosporidium? (please select all responses that apply)

	Low Cryptosporidium risk score	Treatment processes provide effective barrier	No Cryptosporidium sources in the catchment	Lack of resources
Supply 1	☐	☐	☐	☐
Supply 2	☐	☐	☐	☐
Supply 3	☐	☐	☐	☐
...	☐	☐	☐	☐

5. Monitoring history

	less than 1 year	1 to 3 years	more than 3 years
Supply 1	○	○	○
Supply 2	○	○	○
Supply 3	○	○	○
...	○	○	○

6. What type of water is being tested ? (please select all responses that apply)

	treated water	raw water (at the plant intake)	raw water (elsewhere in the catchment)
Supply 1	☐	☐	☐
Supply 2	☐	☐	☐
Supply 3	☐	☐	☐
...	☐	☐	☐

7. How often is the raw water tested for Cryptosporidium?

	weekly	monthly	only if other parameters are exceeded	none of the above (please specify below)*
Supply 1	○	○	○	○
Supply 2	○	○	○	○
Supply 3	○	○	○	○
...	○	○	○	○

*Please specify

8. How often is the treated water tested for Cryptosporidium?

	weekly	monthly	only if other parameters are exceeded	none of the above (please specify below)*
Supply 1	○	○	○	○
Supply 2	○	○	○	○
Supply 3	○	○	○	○
...	○	○	○	○

*Please specify

9. As far as can be foreseen, how long is the supply going to be monitored as specified in question 6?

	for the next 6 months	for the next 12 months	for the next 2 years	for the foreseeable future
Supply 1	○	○	○	○
Supply 2	○	○	○	○
Supply 3	○	○	○	○
...	○	○	○	○

2. General Cryptosporidium testing details

1. How is the water sample collected? (please select all responses that apply)

- ☐ Filtration using Envirocheck filters (Pall Life Sciences)
- ☐ Filtration using Filta-max filters (IDEXX)
- ☐ Filtration using Filtamax Express filters (IDEXX)
- ☐ Grab samples of 10 to 20 litres

Other (please specify)

2. Where are the filters/samples sent for analysis?

3. How are the samples sent?

- ☐ By post
- ☐ By courier

Other (please specify)

4. What is the charge per sample? (Please specify price relative to turnaround time)

	24 hours turnaround	48 hrs turnaround	3 to 5 days turnaround	5 to 7 days turnaround
Less than €150	☐	☐	☐	☐
€150 to 250	☐	☐	☐	☐
€251 to 350	☐	☐	☐	☐
more than €350	☐	☐	☐	☐

5. What type of information do you receive from the testing service? (please select all responses that apply)

- ☐ Oocyst presence/absence
- ☐ Oocyst numbers
- ☐ Oocyst viability
- ☐ Oocyst species identity

6. Would you be interested in receiving information on the species identity of any oocysts that are detected?

- ○ Yes
- ○ No

7. In YOUR opinion, are the existing guidelines for Cryptosporidium testing in drinking water* adequate?
(* as specified in the Handbook on the implementation of the Regulations for Water Service Authorities)

- ○ Yes
- ○ No
- ○ Don't know

If not, how do you think they could be improved on?

8. Do you have any further comments in relation to Cryptosporidium testing in drinking water?

AN GHNÍOMHAIREACHT UM CHAOMHNÚ COMHSHAOIL

Tá an Ghníomhaireacht um Chaomhnú Comhshaoil (GCC) freagrach as an gcomhshaol a chaomhnú agus a fheabhsú mar shócmhainn luachmhar do mhuintir na hÉireann. Táimid tiomanta do dhaoine agus don chomhshaol a chosaint ó éifeachtaí díobhálacha na radaíochta agus an truaillithe.

Is féidir obair na Gníomhaireachta a roinnt ina trí phríomhréimse:

Rialú: *Déanaimid córais éifeachtacha rialaithe agus comhlíonta comhshaoil a chur i bhfeidhm chun torthaí maithe comhshaoil a sholáthar agus chun díriú orthu siúd nach gcloíonn leis na córais sin.*

Eolas: *Soláthraímid sonraí, faisnéis agus measúnú comhshaoil atá ar ardchaighdeán, spriocdhírithe agus tráthúil chun bonn eolais a chur faoin gcinnteoireacht ar gach leibhéal.*

Tacaíocht: *Bímid ag saothrú i gcomhar le grúpaí eile chun tacú le comhshaol atá glan, táirgiúil agus cosanta go maith, agus le hiompar a chuirfidh le comhshaol inbhuanaithe.*

Ár bhFreagrachtaí

Ceadúnú

- Déanaimid na gníomhaíochtaí seo a leanas a rialú ionas nach ndéanann siad dochar do shláinte an phobail ná don chomhshaol:
- saoráidí dramhaíola (m.sh. láithreáin líonta talún, loisceoirí, stáisiúin aistrithe dramhaíola);
- gníomhaíochtaí tionsclaíocha ar scála mór (m.sh. déantúsaíocht cógaisíochta, déantúsaíocht stroighne, stáisiúin chumhachta);
- an diantalmhaíocht (m.sh. muca, éanlaith);
- úsáid shrianta agus scaoileadh rialaithe Orgánach Géinmhodhnaithe (OGM);
- foinsí radaíochta ianúcháin (m.sh. trealamh x-gha agus radaiteiripe, foinsí tionsclaíocha);
- áiseanna móra stórála peitril;
- scardadh dramhuisce;
- gníomhaíochtaí dumpála ar farraige.

Forfheidhmiú Náisiúnta i leith Cúrsaí Comhshaoil

- Clár náisiúnta iniúchtaí agus cigireachtaí a dhéanamh gach bliain ar shaoráidí a bhfuil ceadúnas ón nGníomhaireacht acu.
- Maoirseacht a dhéanamh ar fhreagrachtaí cosanta comhshaoil na n-údarás áitiúil.
- Caighdeán an uisce óil, arna sholáthar ag soláthraithe uisce phoiblí, a mhaoirsiú.
- Obair le húdaráis áitiúla agus le gníomhaireachtaí eile chun dul i ngleic le coireanna comhshaoil trí chomhordú a dhéanamh ar líonra forfheidhmiúcháin náisiúnta, trí dhíriú ar chiontóirí, agus trí mhaoirsiú a dhéanamh ar leasúchán.
- Cur i bhfeidhm rialachán ar nós na Rialachán um Dhramhthrealamh Leictreach agus Leictreonach (DTLL), um Shrian ar Shubstaintí Guaiseacha agus na Rialachán um rialú ar shubstaintí a ídíonn an ciseal ózóin.
- An dlí a chur orthu siúd a bhriseann dlí an chomhshaoil agus a dhéanann dochar don chomhshaol.

Bainistíocht Uisce

- Monatóireacht agus tuairisciú a dhéanamh ar cháilíocht aibhneacha, lochanna, uiscí idirchriosacha agus cósta na hÉireann, agus screamhuiscí; leibhéil uisce agus sruthanna aibhneacha a thomhas.
- Comhordú náisiúnta agus maoirsiú a dhéanamh ar an gCreat-Treoir Uisce.
- Monatóireacht agus tuairisciú a dhéanamh ar Cháilíocht an Uisce Snámha.

Monatóireacht, Anailís agus Tuairisciú ar an gComhshaol

- Monatóireacht a dhéanamh ar cháilíocht an aeir agus Treoir an AE maidir le hAer Glan don Eoraip (CAFÉ) a chur chun feidhme.
- Tuairisciú neamhspleách le cabhrú le cinnteoireacht an rialtais náisiúnta agus na n-údarás áitiúil (m.sh. tuairisciú tréimhsiúil ar staid Chomhshaol na hÉireann agus Tuarascálacha ar Tháscairí).

Rialú Astaíochtaí na nGás Ceaptha Teasa in Éirinn

- Fardail agus réamh-mheastacháin na hÉireann maidir le gáis cheaptha teasa a ullmhú.
- An Treoir maidir le Trádáil Astaíochtaí a chur chun feidhme i gcomhair breis agus 100 de na táirgeoirí dé-ocsaíde carbóin is mó in Éirinn

Taighde agus Forbairt Comhshaoil

- Taighde comhshaoil a chistiú chun brúnna a shainaithint, bonn eolais a chur faoi bheartais, agus réitigh a sholáthar i réimsí na haeráide, an uisce agus na hinbhuanaitheachta.

Measúnacht Straitéiseach Timpeallachta

- Measúnacht a dhéanamh ar thionchar pleananna agus clár beartaithe ar an gcomhshaol in Éirinn (m.sh. mórphleananna forbartha).

Cosaint Raideolaíoch

- Monatóireacht a dhéanamh ar leibhéil radaíochta, measúnacht a dhéanamh ar nochtadh mhuintir na hÉireann don radaíocht ianúcháin.
- Cabhrú le pleananna náisiúnta a fhorbairt le haghaidh éigeandálaí ag eascairt as taismí núicléacha.
- Monatóireacht a dhéanamh ar fhorbairtí thar lear a bhaineann le saoráidí núicléacha agus leis an tsábháilteacht raideolaíochta.
- Sainseirbhísí cosanta ar an radaíocht a sholáthar, nó maoirsiú a dhéanamh ar sholáthar na seirbhísí sin.

Treoir, Faisnéis Inrochtana agus Oideachas

- Comhairle agus treoir a chur ar fáil d'earnáil na tionsclaíochta agus don phobal maidir le hábhair a bhaineann le caomhnú an chomhshaoil agus leis an gcosaint raideolaíoch.
- Faisnéis thráthúil ar an gcomhshaol ar a bhfuil fáil éasca a chur ar fáil chun rannpháirtíocht an phobail a spreagadh sa chinnteoireacht i ndáil leis an gcomhshaol (m.sh. Timpeall an Tí, léarscáileanna radóin).
- Comhairle a chur ar fáil don Rialtas maidir le hábhair a bhaineann leis an tsábháilteacht raideolaíoch agus le cúrsaí práinnfhreagartha.
- Plean Náisiúnta Bainistíochta Dramhaíola Guaisí a fhorbairt chun dramhaíl ghuaiseach a chosc agus a bhainistiú.

Múscailt Feasachta agus Athrú Iompraíochta

- Feasacht chomhshaoil níos fearr a ghiniúint agus dul i bhfeidhm ar athrú iompraíochta dearfach trí thacú le gnóthais, le pobail agus le teaghlaigh a bheith níos éifeachtúla ar acmhainní.
- Tástáil le haghaidh radóin a chur chun cinn i dtithe agus in ionaid oibre, agus gníomhartha leasúcháin a spreagadh nuair is gá.

Bainistíocht agus struchtúr na Gníomhaireachta um Chaomhnú Comhshaoil

Tá an ghníomhaíocht á bainistiú ag Bord lánaimseartha, ar a bhfuil Ard-Stiúrthóir agus cúigear Stiúrthóirí. Déantar an obair ar fud cúig cinn d'Oifigí:

- An Oifig Aeráide, Ceadúnaithe agus Úsáide Acmhainní
- An Oifig Forfheidhmithe i leith cúrsaí Comhshaoil
- An Oifig um Measúnú Comhshaoil
- An Oifig um Cosaint Raideolaíoch
- An Oifig Cumarsáide agus Seirbhísí Corparáideacha

Tá Coiste Comhairleach ag an nGníomhaireacht le cabhrú léi. Tá dáréag comhaltaí air agus tagann siad le chéile go rialta le plé a dhéanamh ar ábhair imní agus le comhairle a chur ar an mBord.

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Climate - Water - Sustainability
Identifying pressures • Informing policy • Developing solutions

Towards Developing a Cryptosporidium Monitoring Protocol

Identifying Pressures

The prevalence and persistence of Cryptosporidium – particularly with respect to drinking water supplies – is one of the key environment and health issues. This project was established to initiate the development of a national Cryptosporidium monitoring protocol.

Informing Policy

- One of the key outcomes of this study was the establishing of methods, based on current international benchmark methods, for the genotyping of Cryptosporidium in drinking water and providing genotyping services to commercial primary testing laboratories and the Health Service Executive during outbreak investigations.
- By far the majority (~ 90%) of Cryptosporidium species found in Irish water supplies are considered of low or no known risk to public health.
- A survey on the occurrence of emerging contaminants in Irish water supplies found that, overall, the majority of the substances were not present.
- A survey of public and private water schemes indicate that roughly one quarter of all public water supplies monitored for Cryptosporidium. However, monitoring frequencies are generally very low – less than 10 times per year – and a lack of resources was cited as the main reason.

Developing Solutions

The establishment of a Cryptosporidium Reference Laboratory for the genotyping of Cryptosporidium isolates from water, providing an opportunity to initiate a comprehensive coordinated approach to sampling and analysis for Cryptosporidium in drinking water in Ireland.

Comhshaol, Pobal agus Rialtas Áitiúil
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