Persistence of *Cryptosporidium parvum* in Bovine Feces During Summer and Winter Exposure

by

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ABSTRACT

Grazing cattle on forested crown lands can be beneficial to the local economy but may also pose a risk to the drinking water extracted from the watershed. A major disturbance that the cattle present to watersheds is the deposition of protozoan pathogens such as *Cryptosporidium parvum* in their manure. Fecal pathogens deposited near riparian zones can be transmitted into water systems when disturbed by elevated river flow. Although cattle are out of pasture during spring freshet when water flow is high, their feces remain near rivers and streams. If *C. parvum* persists in feces beyond grazing season and into spring freshet, it may serve as a point source for disease.

Nested PCR targeting the *Cryptosporidium* 18S rRNA gene was used to assess the persistence time of *C. parvum* oocysts in bovine feces during both the winter and summer seasons in the Okanagan valley, British Columbia. Nested PCR was found to have a limit of detection less than one oocyst per reaction and was of comparable sensitivity to quantitative real-time PCR (3.2 oocyst LOD). Ruptured oocysts were not detectable by nested PCR,

Shading, precipitation, freezing/thawing events, temperature, and ultraviolent light were evaluated as factors associated with persistence time. In winter, heavily and moderately forested sites contained detectable *C. parvum* for less than 67 days, whereas lightly shaded sites showed persistence up to 106 days. Fecal pats were subjected to simulated river flow after winter exposure and no samples leached *C. parvum*. In summer experiments, feces in lightly forested plots contained no oocysts by 71 days of exposure, whereas heavily and moderately forested plots showed persistence up to 261 and 227 days, respectively. Freeze-thaw events as well as direct ultraviolet light exposure are supported as predominant factors against *C. parvum* persistence in winter and summer exposures. After three freeze-thaws, 59% of the oocyst population was lost and only 19.3% of the remaining oocysts contained intact cell walls. This research has application in grazing operations as it is provides unprecedentedly low detection limits for monitoring seasonal *C. parvum* persistence in different shading conditions.

PREFACE

All work presented in this document was conducted either in the Engineering, Management, and Education building or on campus land at the University of British Columbia, Okanagan. Permission for this study and the construction of plots containing biological specimens was granted by UBC Okanagan Office of Research Services and Campus Planning and Development [Campus Land Use Request #2014-001]. Approval was granted by Department Head Rehan Sadiq, Acting Associate Dean Dwayne Tannant and Research Services Associate Director Kristen Korberg.

Chapter 3: The limit of detection experiments in Chapter 2 were designed and performed by myself and all qPCR tests were performed in the lab of Dr. Mark Rheault, who assisted in initial instrument training.

Chapter 4: A version of Chapter 4 has been submitted to *Parasitology* on March 22, 2016 for review. The project was conceptualized and supervised by Dr. Deborah Roberts. I was responsible for experiment design, plot construction, data collection and analysis, flume construction and written compilation of research. Sample collection and processing was assisted by undergraduate research assistants Melissa Larrabee, Lindsey Hovey, Noah Dietrich, and Karen Reimann. Oocyst cell counting for the stock *C. parvum* solution was performed by Dr. Deborah Roberts. Manuscript proofreading was aided by Dr. Deborah Roberts, Melissa Larrabee, Lindsey Hovey, and Noah Dietrich. Three of the six microscopy images in Figure 4-3 are credited to Noah Dietrich, who also performed EPA Method 1623 on two of the three effluents generated during the flushing study.

Chapter 5: This work is an extension of Chapter 4 in that similar methodology was implemented to assess persistence of *C. parvum* during summer months. I was responsible for plot construction, experimental design, data collection and analysis, and written compilation of research. Summer sample collection and processing was not assisted by undergraduate researchers for this portion of the study with exception of fecal sample deposition onto the plots.

Funding for this project has been provided by the BC Ministry of Forests, Lands, and Natural Resource Operations and Agriculture and Agri-Food Canada and the BC Ministry of Agriculture through the Canada-BC Agri-Innovation Program under *Growing Forward 2*, a federal-provincial-territorial initiative. The program is delivered by the Investment Agriculture Foundation of BC. Opinions expressed in this document are those of the authors and not necessarily those of AAFC, the Ministry of Agriculture or the Investment Agriculture Foundation.

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GLOSSARY OF ABBREVIATIONS

ANCOVA: Analysis of covariance

BMP: Best management practice

BSA: Bovine serum albumin

CDW: Committee on drinking water

C_q: Quantification cycle

ddPCR: Droplet digital polymerase chain reaction

HF: Heavily Forested

IFA: Immunofluorescence assay

IFM: Immunofluorescence microscopy

IMS: Immuno-magnetic separation

LF: Lightly Forested

LOD: Limit of detection

LSD: Least Significant Difference

MF: Moderately Forested

nPCR: Nested polymerase chain reaction

OPG: Oocysts per gram

opPCR: Oocysts per polymerase chain reaction

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PI: Propidium Iodide

PMA: Propidium monoazide

qPCR: Quantitative real time polymerase chain reaction

RO: Reverse osmosis

RT-PCR: Reverse transcription polymerase chain reaction

TBE: Tris-borate-acetate buffer

US EPA: United States Environmental Protection Agency

UV: Ultraviolet

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I want to thank the undergraduate research assistants, Melissa Larrabee, Noah Dietrich, Lindsey Hovey, and Karen Reimann, for their aid on this project. I am grateful that they were always eager to help me extract pathogens from buckets of feces, and their high level of diligence and responsibility was a blessing on long processing days. Additionally, I want to thank Keith Duhaime and Rob Dinwoodie for their work in applying this research and linking my results to cattle management policy and risk analysis.

My enlightening experience at UBC-O also owes respect to the chemistry lab manager, Dr. Sandra Mecklenburg, who employed me as a teaching assistant for the entire duration of my degree. I have further realized my love for teaching science and my employment as a TA has reinvigorated my interest in teaching and community outreach. I have bolstered my knowledge of applied organic chemistry during this job and I have no doubt that this stronger foundation will serve me well in future biochemical research.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

This chapter will introduce the potential conflict between cattle grazing operations and *Cryptosporidium parvum* (Tyzzer, 1912) in local watersheds and the current gaps in understanding associated with prolonged *C. parvum* presence in bovine feces on rangelands. An overview of potential factors that influence *C. parvum* longevity as well as current methodologies used to detect and monitor *C. parvum* in feces will also be discussed.

1.1: Cattle grazing and *Cryptosporidium*

Cattle grazing operations on public land are common in agricultural economies as they provide ranchers an expanded feed and water resource for the herds. During these operations, cattle are permitted scheduled access to forage grasses and water from late June to September, allowing some degree of free range behavior (Borth, 2011). In addition to subsidizing ranching costs and improving livestock quality, responsible grazing practices can improve grassland ecosystems by lowering fire fuel-loads, reducing the spread of invasive weeds, and increasing vegetative diversity (Huntsinger et al., 2007). Despite the ecological and husbandry benefits, this practice has raised concerns with health and water quality officials since cattle can be hosts of the obligate enteric protozoan parasite *Cryptosporidium parvum* and may graze within close proximity to community watersheds (Graczyk et al., 2000; Walker, 1998). Many efforts are made to reduce the input of fecal *C. parvum* directly into streams and rivers, but these efforts cannot entirely prevent cattle defecation in surrounding riparian zones. Limited knowledge exists as to how long *C. parvum* remains in feces near riparian zones and whether their persistence will overlap with high river flow

events. Additionally, no field studies have been conducted as to whether different rangeland environmental conditions may foster or disfavor *C. parvum* decay.

As of 2015, 27 species of *Cryptosporidium* have been identified, but most have a narrow host range and pose minimal risk to public health (Ryan and Hijjawi, 2015). *C. parvum* is of particular interest to water quality and rangeland officials since it is capable of infecting both cattle and humans, with zoonotic transmission occurring by the fecal-oral route (Ryan et al., 2014). Lower host specificity coupled with the close interaction of humans and cattle in agricultural communities grant *C. parvum* greater opportunity to spread between both cattle and human populations (Miron et al., 1991). When ingested, *C. parvum* proliferate rapidly in the gut and are ultimately excreted in feces as extremely durable, quiescent oocysts capable of immediately infecting another host (Atwill, 1996; Walker, 1998). In a single bowel movement, as many as 10^8-10^9 oocysts can be excreted and yet it requires as few as 10-30 oocysts to instigate infection (Chappell et al., 1996; DuPont et al., 1995). Persons or animals unfortunate enough to ingest *C. parvum* often show symptoms of cryptosporidiosis as diagnosed by watery diarrhea, fever, vomiting, and painful cramping, all of which are particularly harmful or even lethal to immunocompromised, young, and elderly demographics (Carey et al., 2004; DuPont et al., 1995).

The prevalence and impact of cryptosporidiosis outbreaks makes *C. parvum* of great public health interest. Worldwide, *C. parvum* accounts for more than half of waterborne protozoan parasite outbreaks, with most of the reported outbreaks occurring in the United States and Canada (Putignani and Menichella, 2010). However, it is likely that the high prevalence of *C. parvum* outbreaks in these nations is due to more frequent and accurate reporting and diagnosis of cryptosporidiosis and not due to higher incidence. In Canada

alone, 3.5% of treated water samples, 4.5% of raw water samples, and 6.1% of raw sewage samples (from 72 water municipalities) were found to contain *Cryptosporidium* oocysts (unspeciated) (Wallis et al., 1996).

Feces-contaminated drinking water is often hypothesized as the most common method of *Cryptosporidium* transmission and despite actual genotypic evidence, feces from sheep and/or cattle are historically implicated as the source of the contamination (Fayer et al., 2000). Cattle ranching operations came under close investigation especially after the massive outbreak in 1993 in Milwaukee, WI, in which over 403,000 became symptomatic of cryptosporidiosis and 104 people died after ingesting *Cryptosporidium* oocysts in contaminated municipal drinking water (Hass and Rose, 1994; MacKenzie et al., 1994). The cost of the outbreak, including work-force absenteeism, was estimated at $96.2 million, causing great strain on the local economy (Corso et al., 2003). A panel appointed by the Wisconsin Division of Health noted that cattle were previously observed along the rivers that flow into the Milwaukee harbor and their feces could have been transported by spring rains and snow runoff (MacKenzie et al., 1994). Although it was later determined that human excrement and *C. hominis* were the cause of the outbreak (Peng et al., 1997), questions remained as to the possibility of *C. parvum* transport during spring freshet. Indeed, most outbreaks of *C. parvum* occur during spring and coincide with large runoff events (Sopwith et al., 2005). Responsible cattle grazing operations seek to limit the potential of cattle directly defecating into water resources, but these mitigation strategies cannot prevent cattle from defecating near the streams and rivers where flushing potential is high. Additionally, poor storage and/or application of manure used near sensitive waterways can become a source of pathogen transmission if disturbed by rainfall or other flushing events. This risk has been

previously assessed and the results predominantly impact agricultural sectors and dairy farms that generate and apply bulk fertilizer within a confined region (Jenkins et al., 1999; Pachepsky et al., 2006).

Concern remains as to whether grazing cattle feces located near dormant streams could retain *C. parvum* long enough to pose a risk when rivers and streams swell during the spring. The duration that these oocysts remain a risk to water quality and public health has not been previously investigated under field conditions that include actual seasonal exposure, different exposure conditions, and occurrence within individual fecal pats. The connection between grazing practices and cryptosporidiosis risks remains poorly understood and information on the long-term risks from cattle feces near watersheds will address the concerns as to whether resurgence of *C. parvum* during spring freshet is a plausible event (Tate et al., 2000).

1.2: Preventative and responsive treatment of *C. parvum* in cattle

1.2.1: Preventative strategies

In an attempt to prevent the spread of *C. parvum* from cattle to watersheds, several best management practices (BMPs) have been employed by cattle ranchers and through rangeland management. One of the methods that ranchers use to minimize environmental loading of *C. parvum* is the removal of young or sick cattle from grazing pasture as it is widely recognized that most oocyst shedding occurs in calves and diarrheic cows. From collective multi-national studies, *C. parvum* was observed in 22% to 40% of diarrheic calves and in 8% to 11% of non-diarrheic calves (Garber et al., 1994; National Health Monitoring System., 1994; Reynolds et al., 1986). From these infected calves, *C. parvum* shedding was often as high as ~10^{10} oocysts cow^{-1} day^{-1} (Uga et al., 2000). Calves are therefore typically

held from pasture until at least four months of age, at which point diarrhea and average shedding concentration of *C. parvum* are greatly reduced (Uga et al., 2000). While these efforts greatly reduce the incidence of *C. parvum* on rangeland, *Cryptosporidium* (unspeciated) is still found on rangeland (Forest Practices Board, 2012). Oocyst shedding can occur in calves even after four months and 13% of mature British Columbia beef cattle have been observed to shed *C. parvum* as well, albeit at a lower loading than calves (9,200 oocysts cow^{-1} day^{-1}) (Atwill et al., 2003; McAllister et al., 2005). Despite measures to quell oocyst predominance on grazing land, some sick cattle may still roam.

To deter deposition of fecal *C. parvum* directly into waters and streams, additional BMPs can be implemented in which exclusion fencing, partial fencing and off-stream watering discourages cattle from walking or resting in the water (Borth, 2011). One of the objectives of these BMPs is to minimize cattle presence within 5 meters of streams or rivers in an attempt to reduce the potential leaching or physical transport of *C. parvum* into riparian zones (Borth, 2011). Exclusion fencing completely impedes cattle access to sensitive water systems and while useful in areas of riparian importance it is expensive and highly impractical to implement at every site that grazing cattle visit in the rangeland (Forest Practices Board, 2012). Partial fencing, also known as 'nose-holes', involves wooden barriers that have enough space for a cow's head to reach stream water and off-stream watering involves pumping water to a reservoir away from the stream. Off-stream watering has been highly successful in reducing cattle traffic near riparian zones and could be an excellent means towards water source protection in the future (Alberta Agriculture and Food, 2007). Although off-stream watering does assist in withdrawing cattle from sensitive water resources, grazing cattle are still attracted to cool areas near water during the summer and

fall. In open areas where fencing and off-stream watering are impractical, silvopasture and management of grass stubble height can provide natural sieving of oocysts that may have leached from fecal pats and can accordingly limit oocyst transport in meadows (Borth, 2011). These BMPs may impede oocysts from directly entering the water, but feces are still found at the edges of riparian zones as personally observed while field sampling in Okanagan watersheds. Some of the fecal pats tested for *Cryptosporidium* near partial fencing were positive for *Cryptosporidium* as noted by rangeland auditors (Forest Practices Board, 2012). I also found positive samples throughout the year during bimonthly water and fecal testing (unpublished data). The species of the *Cryptosporidium* could not be assessed for all samples at this site, but for the few samples that sequenced successfully, only *C. andersonii* was found.

Despite BMPs and the absence of cattle on grazing land for many months prior to spring freshet, cryptosporidiosis outbreaks still occur during the freshet. Even with BMPs in a watershed, the potential of *C. parvum* persisting in feces for long durations near riparian zones remains. After fall grazing season is over, oocysts deposited as late as September may endure throughout the winter and into spring when transport risk of pathogens by snowmelt and rainfall is heightened (Simon et al., 2013). Additionally, during summer grazing scheduling, any extended persistence of *C. parvum* near rivers and lakes could pose risk to downstream water quality in the event of summer precipitation or river swells. Insight into how long *Cryptosporidium* remains detectable in feces throughout natural winter and summer exposure will address the validity of these concerns and may assist in efficient application of BMPs and herd scheduling to mitigate *C. parvum* transport into community watersheds.

1.2.2: Responsive strategies

In events where *Cryptosporidium* does enter drinking water intakes, treatment facilities are one of the last barriers between pathogen-laden water and potential cryptosporidiosis outbreaks. Unfortunately, standard water treatment procedures are generally ineffective at disinfecting *Cryptosporidium*. Due to the durable nature of *Cryptosporidium*'s disulfide and glycoprotein reinforced cell wall (Jenkins et al., 2010), conventional water treatment approaches such as chlorine disinfection, coagulation/flocculation, and filtration often fall short. As per the "Interim Enhanced Surface Water Treatment Rule" announced by the United States Environmental Protection Agency (US EPA), at least a 2 $\log_{10}$ (99%) reduction of *Cryptosporidium* is required prior to discharge of water from a treatment facility (Betancourt and Rose, 2004). In Canada, guidelines established by the Federal-Provincial-Territorial Committee on Drinking Water (CDW) require at least a 3 $\log_{10}$ (99.9%) disinfection of *Cryptosporidium* in drinking water (Health Canada, 2014). Microscopy-based detection of even non-viable oocysts in drinking water can result in non-compliance with water treatment criteria due to the difficulty in confidently assessing oocyst morphology (LeChevallier et al., 1991). A 2 $\log_{10}$ disinfection of *C. parvum* requires 7200 mg min L^{-1} chlorine, nearly 180,000 times as much concentration or contact time required for comparable disinfection of *E. coli,* making chlorine disinfection impractical (Rochelle et al., 2005). Coagulation and flocculation can provide as high as a 2 $\log_{10}$ disinfection but depends greatly on the turbidity of the water to aid in co-sedimentation; the use of aluminum sulfate [$Al_2(SO_4) \cdot 14\ H_2O$], ferric chloride [$FeCl_3 \cdot 6\ H_2O$], or polyaluminum hydroxychlorosulfate polymer as flocculants in samples with low turbidity yields less than a 1-$\log_{10}$ reduction of *Cryptosporidium* oocysts (Dugan et al., 2001).

Filtration using granulated activated carbon and/or slow sand filters has provided a 2 to 2.5 $\log_{10}$ removal of oocysts in a majority of 66 tested US water treatment facilities and these facilities have been prompted to bolster their water treatment operations by including a disinfection procedure (LeChevallier et al., 1991). The small size of *Cryptosporidium* (~5μm) often warrants expensive micro- or ultra-filtration procedures such as diatomaceous earth or synthetic membranes in order to obtain consistently adequate disinfection (>4 $\log_{10}$) of *Cryptosporidium* (Jacangelo et al., 1995). Conventional downstream water disinfection approaches are often unreliable in removing *Cryptosporidium* alone and a proper disinfection protocol requires a multi-tiered treatment process (Betancourt and Rose, 2004; Corona-Vasquez et al., 2001). The use of ultraviolet (UV) light has been shown to be a cost-competitive alternative to conventional disinfection approaches such as chlorination and is highly effective at killing waterborne oocysts after reducing water turbidity (>4 $\log_{10}$ at 41 mJ cm^{-2}) (Bolton et al., 1998). However, only ~20% of water treatment facilities use UV light for disinfection (Trojan UV, 2016) and there is little regulatory incentive for upgrading a treatment facility unless it demonstrates failure and consistent non-compliance. In light of all of these water treatment solutions, it is important to note that the largest outbreaks of cryptosporidiosis are due to nonexistent or inadequate treatment of upstream *Cryptosporidium* contaminated water prior to discharge for human consumption (Fayer et al., 2000). While water treatment is essential for providing clean drinking water, it is safer to encourage preventative strategies for the reduction of *C. parvum* upstream instead of placing the burden onto treatment facilities.

Due to the risk of widespread outbreak and the difficulty in adequately disinfecting the oocysts, range management officials and ranchers are encouraged to employ preventative

strategies to mitigate *C. parvum* spread from cattle into riparian systems. The efficacy of current preventative strategies are currently founded on the assumption that either cattle are not carrying *C. parvum* into the watershed due to effective BMPs or that any *C. parvum* present in summer- and fall-deposited feces will die during the winter. Information as to whether the oocysts actually die during summer or winter exposures, as well as which environmental factors greatly affect oocyst persistence, will finally provide clarity to these assumptions.

1.3: Factors influencing *C. parvum* persistence

Research assessing the influence of environmental factors has been predominantly performed with isolated oocysts in buffered solutions under controlled laboratory conditions (Table 1-1). While useful in assessing individual factors that impair oocysts, these test conditions do not provide an accurate assessment of *C. parvum* persistence in the field where the oocysts are in bovine feces exposed to collective environmental exposures and microbiotic activities. Furthermore, the impact of these variables on fecal oocysts varies seasonally. During the summer, UV light and high temperatures are likely to be predominant influences on persistence whereas in winter, freezing temperatures may be the greatest factor.

In laboratory tests, UV light has been demonstrated as especially effective in killing *Cryptosporidium*, with even low pulses of polychromatic UV light (7.5-11 mJ cm^{-2}, comparable to sunny exposure) yielding 2 log (99%) disinfection of *C. parvum* (Mofidi et al., 2001). UV light penetrates the oocyst cell wall and irradiates DNA to form cyclobutane pyrimidine dimers and 6'- 4' photoproducts, which if not repaired can impede transcription of necessary housekeeping genes and/or stimulate apoptosis (Sinha and Hader, 2002). Although *Cryptosporidium* hosts the genes encoding for these proteins, it does not initiate a

repair response to DNA damage (Rochelle et al., 2005). As a result, mild UV doses can render the oocysts non-viable.

Table 1-1: Selected factors associated with *C. parvum* death and reported resistances in different testing environments.

Factor	Resistance	Environment	Reference
Low temperature	Inactivation of oocysts after 775 hrs at -22°C	Water, soil	(Robertson et al., 1992)
Freezing temperature	Inactivation after overnight freezing at -70°C	Water	(Fayer et al., 1991)
High temperature	Inactivation of oocysts after 1 minute at 72.4°C	Water, soil	(Fayer et al., 1996)
Salinity	100% persistence at 12 wks at 30 ppth salinity, 10°C	Water	(Fayer et al., 1998)
Desiccation	3% persistence after 2 hours of desiccation	Fixed slide	(Robertson et al., 1992)
UV light	1% persistence after 7.5-11mJ cm^{-2} medium pressure UV light	Water	(Mofidi et al., 2001)

Cryptosporidium oocysts show exceptional tolerance within a relatively wide temperature range. Oocysts retain infectivity towards mice for several months at temperatures ranging from 4°C to 25°C with the colder temperatures in this range favoring longer persistence time (Fayer et al., 1996; Pokorny et al., 2002). Oocyst infectivity has been observed as long as six months at 4°C in aqueous 2.5% potassium dichromate and three months in phosphate-buffered saline (Sherwood et al., 1982). As temperatures increase beyond 25°C, oocysts lose infectivity within 5 days at 37°C (Sherwood et al., 1982) and 1 minute at 72.4°C (Fayer et al., 1996). This reduction in infectivity is also observed for freezing temperatures; oocysts have been shown to remain infectious for up to 31 days at -22°C (Robertson et al., 1992) but those subjected to overnight freezing at -70°C were non-

viable after thawing (Fayer et al., 1991). Notably, the simple process of freezing and thawing is highly effective at rupturing oocysts and can be used in extracting cellular DNA, RNA or protein (Jiang et al., 2005).

During the Okanagan winter, low temperatures and thermal cycles near 0°C will be driving factors in oocyst decay. Desiccation and UV light are less likely to influence survivability since heavy snow accumulation minimizes potential desiccation of fecal pats and reflects as much as 80% incident UV light (World Health Organization, 2016). Compared to winter conditions, oocysts in summer-deposited feces are subject to greater (>10mW/cm^2) UV light exposure, high ground temperatures, and potentially prolonged desiccation. Furthermore, natural barriers such as fecal crusting (Sinton et al., 2007) and debris can further shield oocysts from direct UV light exposure and have even been shown to act as a cryoprotective matrix for *Cryptosporidium* (Kim and Healey, 2001). Vegetation can also shield fecal pats from high temperatures and UV exposure, making these areas potential havens for persistent oocysts.

Persistence times of *C. parvum* in environmentally-exposed feces are likely to differ from laboratory-controlled tests and will be contingent on both local climate as well as natural shading conditions that alter the influences of UV light, temperature fluctuations, and precipitation. The effects of shading factors on *C. parvum* are not well studied, leading to an incomplete understanding of *C. parvum* survival in natural exposures. This limited knowledge makes it difficult for cattle ranchers to accurately employ preventative strategies for mitigating the spread of *C. parvum* into community watersheds and downstream drinking water sources.

1.4: *C. parvum* isolation and detection methods

Evaluating *C. parvum* persistence remains difficult due to extraneous fecal material, durable oocyst cell walls, and inadequacies in current detection methods. Choosing proper fecal clean-up and detection methodologies is therefore critical in accurately observing persistence.

1.4.1: Oocyst and DNA extraction from feces

Bovine fecal samples consist of a large proportion of grass, rocks, and other fecal detritus that requires removal prior to oocyst detection. Several methods exist for complete isolation of oocysts from fecal samples, including floatation (e.g. NaCl, cold sucrose, $ZnSO_4$), sedimentation (e.g. formalin-ethyl acetate), and gradient centrifugation (e.g. CsCl, Sheather's, Percoll) (Jiang et al., 2005; Kostrzynska et al., 1999). Each of these isolation methods involves several centrifugations and solvent separation steps, resulting in oocyst recoveries consistently lower than 20% and accordingly poor limits of detection (Kuczynska and Shelton, 1999). Antibody-based immunomagnetic separation (IMS) has demonstrated highly variable oocyst recoveries (between 0% and 83%) in cattle excrement containing over 100 oocysts per gram, making it too inconsistent for use in detection of low concentration *Cryptosporidium* in environmental samples (Bukhari et al., 1998; McCuin and Clancy, 2005). Isolation of *Cryptosporidium* oocysts from feces is therefore avoided except in studies where cell integrity or oocyst purity is essential, such as oocyst morphology, excystation, and infectivity.

In cases where cell morphology is not of concern, oocyst isolation can be bypassed. Instead, direct extraction of nucleic acids can be performed on the oocysts, yielding genomic signatures that can be amplified using PCR (polymerase chain reaction) for visualization or

quantification. Bypassing oocyst isolation circumvents the aforementioned losses incurred during washing, pelleting, and resuspension of fecal samples. One of the concerns with skipping these clean-up steps is that fecal material may impair downstream nucleic acid isolation, amplification, and detection (LaMontagne et al., 2002; Liang and Keeley, 2011; Tsai and Olson, 1992). However, specialized kits for DNA and RNA extraction in feces have been developed to remove common inhibitors such as bilirubin, bile acids, and fulvic/humic acids which can impair downstream analysis (Jiang et al., 2005; Rump et al., 2010). Working with *C. parvum* requires additional diligence when using these extraction kits. Preliminary rapid thermal shocks at extreme high and low temperatures or longer bead-beating times are required in order to adequately rupture the durable oocyst cell wall (Carey et al., 2004; Chung et al., 1998; Kostrzynska et al., 1999).

1.4.2: *Cryptosporidium* detection

Accurate detection of the viability of *Cryptosporidium* is not straightforward due to its ability to form dormant oocysts and its dependence on a host environment to excyst and initiate reproduction. The statuses of "viable" and "infective" are used interchangeably in the literature and there is a lack of agreement as to whether microscopy-based viability assays are an adequate surrogate for animal infectivity studies (Belosevic et al., 1997). Whereas viability is generally used to designate a living cell, it is becoming recognized that this does not always indicate that the cell has the ability to illicit a disease response in a host (Neumann et al., 2000). A review by (Bukhari et al., 2000) compared popular *Cryptosporidium* viability assays and found that despite rigorous control measures, high levels of variability were observed between reported viability and infectivity, often with a high degree of false negatives (up to 86%). The consequence of false negatives can be drastic

in public health applications, since dissemination of only a few dozen oocysts can spur successive infection into another host(s), potentially resulting in *in vivo* proliferation up to final concentrations of 6×10^7 oocysts/g feces (Uga et al., 2000).

Alternatively, detection of *C. parvum* can be performed by assaying all oocysts present, including viable, infectious, and even non-viable. While this approach will likely over-report the number of infectious oocysts present in a sample, false positives serve as a safety margin when performing environmental monitoring and water quality risk assessment. Of course, a careful assessment of detection methodologies is required to ensure that the assay does not also yield positives for innocuous remnants of lysed *Cryptosporidium* oocysts.

Detection methods for infectious oocysts (section 1.4.2.1) as well as methods for total oocyst presence (section 1.4.2.2) will be reviewed below to illustrate the decision process as to which detection approach is best suited for a study on *C. parvum* persistence in fecal samples. The criteria for a successful detection method will be based upon practicality, low detection limit, and safe reporting (i.e. fewest false-negatives).

1.4.2.1: Persistence of infectious oocysts

Animal infectivity models

Animal infectivity models are generally touted as the "gold standard" for evaluating infectivity of oocysts. However, this method requires high doses of *C. parvum* (1000 oocysts/mouse) to obtain 100% infectivity and consistent results among trials (Peeters et al., 1989). Environmental samples may therefore have an insufficient number of oocysts to permit infectivity assays. Additionally, animal infectivity studies are highly demanding in laboratory materials and resources and are therefore only suited to small sample sizes (Carey et al., 2004). Due to the high number of samples (>600) required in longitudinal

environmental studies, animal infectivity models cannot be performed both ethically and to an extent required for statistical accuracy.

In vitro excystation

In vitro excystation proves useful for obtaining general measurements of viable oocysts in large samples, but the excystation efficiency (67-73%) is insufficient for both routine medical and environmental sampling in which both low detection limits and high accuracy are essential (Carey et al., 2004). A review of neonatal mice studies in which mice were fed both excysted and non-excysted oocysts concluded that *in vitro* excystation should not be used as an acceptable indicator of *C. parvum* infectivity due to the poor reproducibility and high proportion of false negatives (Neumann et al., 2000). Unreliable excystation is heavily attributed to the difficulty in accurately mimicking the complex cellular signaling mechanisms associated with stimulating infectivity (Kato et al., 2001). This method also requires oocyst isolation from fecal samples and therefore is subject to losses in oocysts during processing and higher detection limits.

Fluorescence microscopy

Fluorescence microscopy is a popular technique that can provide quantitative estimates of both living and dead *C. parvum* oocysts using fluorogenic dyes. Vital and non-vital fluorogenic dyes involve direct staining of isolated oocyst cell features using chromophores such as antibodies tagged with fluorescein isothiocyanate (FITC), or membrane permeable or impermeable dyes including 4'- 6'-diamidino-2-phenylindole (DAPI) or propidium iodide (PI). The stained features are visualized using fluorescence microscopy and viable and non-viable cells are typically counted manually (U.S. EPA, 2005). Although this assay has potential merit in differentiating viable and non-viable

oocysts, both related literature and my own preliminary research have shown that the recovery and detection of trace oocysts in environmental samples can be difficult via vital dye and immunofluorescence assays (IFAs) due to opaque detritus and dye cross-reactivity (Jenkins et al., 1999; Robertson et al., 1998). When working with specimens containing concentrations of below 200 oocysts/g, losses can occur during washing and processing stages associated with staining, yielding recovery values as low as 31-42% with coefficients of variation ranging as much as 10-50% (Davies et al., 2003). In addition to processing losses, accurate detection of viable and non-viable oocysts is difficult due to accompanying fecal microbes such as *Giardia lamblia* and algal species that can yield false positives due to similar physical and staining characteristics (Rodgers et al., 1995). *G. lamblia* cysts have similar fluorescence staining properties and morphology to *Cryptosporidium* oocysts, and are often differentiated from the latter solely by its slightly larger size (8-12µm × 7µm compared to 5-6µm × 5-6µm) and more ovoid appearance (Skotarczak, 2009). When working with environmental samples containing both microorganisms, accurate detection depends on the consistency and experience of the observer. Additionally, fluorescence microscopy does not easily allow for DNA-based speciation of the observed oocysts, thus making it difficult to verify the cell species, or even genus. However, IFA is useful for observing morphological changes in pure oocyst populations, as discussed in section 4.2.3.

Reverse transcription-PCR

Reverse transcription polymerase chain reaction (RT-PCR) is one of many PCR variations involving amplification of genetic information within a sample. Traditional PCR utilizes heat-stable polymerase enzymes to replicate targeted sequences flanked by deliberately designed oligonucleotide primers. The sample is subjected to thermocycles

during which the polymerase anneals to the target single-stranded DNA at the primer site (~60-70°C), replicates the target sequence using available free nucleotides (~72°C), and then separates the new strand from the old strand (>90°C). Repetition of this process results in exponential amplification of the target DNA in a sample (e.g. 35 cycles on one DNA strand can theoretically generate $2^{35} = 3.4 \times 10^{10}$, copies).

RT-PCR differs from conventional PCR in that small quantities of RNA are first reversely transcribed into more stable, complementary DNA (cDNA) using the enzyme, reverse transcriptase. The cDNA is then amplified in the same manner as standard PCR. Since RT-PCR can detect whether a cell is actively transcribing DNA into RNA, it can provide information as to the metabolic activity of the organism, and by proxy, its viability. In order to increase the concentration of RNA from dormant oocysts, samples are often heated as an effort to promote excystation and transcription of heat-shock related genes (Garces-Sanchez et al., 2013). RT-PCR shows promise in water samples, providing detection of viable *C. parvum* oocysts using beta-tubulin, hsp70 mRNA, or 18S SSU rRNA as a marker for living oocysts (Javier et al., 2009; Liang and Keeley, 2011; Widmer et al., 1999).

Like many methods, the issue of adequate RT-PCR amplification arises when working with soil or fecal samples: PCR inhibitors, such as even trace amounts of humic acids, can result in severely dampened RNA amplification and an underestimation of viable *C. parvum* (Ijzerman et al., 1997; Liang and Keeley, 2011). Additionally, mRNA is relatively unstable when compared to other biomolecules such as DNA, and is especially sensitive to the ubiquitous number of RNases present in soil and feces (Liang and Keeley, 2011). Furthermore, high temperatures (95°C) incurred during the thermal shock processing stages used to lyse oocyst cell walls (section 2.1.6) can result in RNA degradation and accordingly

poor viability reports (Fontaine and Guillot, 2003).

These downsides should not entirely discourage use of RT-PCR in assaying viable *C. parvum* oocysts. After proper optimization and preliminary testing, RT-PCR has been performed on fecal samples but detection limits are approximately 5500 oocysts/mL in fecal matrices (Garces-Sanchez et al., 2013) and 150 oocysts/g soil in soil matrices (Liang and Keeley, 2011). As a result, it is inadequate for sensitive environmental monitoring of feces.

Propidium monoazide PCR

Propidium monoazide (PMA) is a unique example of vital dyes that can be incorporated with PCR-based assays to yield amplification of only viable oocysts. Like PI, PMA preferentially intercalates into the DNA of cells with ruptured or weakened cell membranes. But unlike PI, it contains a photo-active (λ_{max} 460nm) azide moiety that cross-links with other adjacent PMA (Nocker et al., 2006), rendering the DNA of dead cells insoluble and providing amplification of only living cells (Brescia et al., 2009). Unfortunately, because PMA-PCR relies on photo-activation its application is limited to translucent samples. The method is currently ineffective at differentiating live and dead *Cryptosporidium* oocysts in turbid environmental samples even when sample cleanup is performed (Brescia et al., 2009).

1.4.2.2: Persistence of total infectious and non-infectious oocysts

In contrast to assaying solely infectious or viable oocysts, persistence can be reported in terms of total detected oocysts, which includes both potentially non-viable and viable/infectious forms not physically or chemically ruptured in the environment. The benefit of this approach is that it provides a higher degree of caution in reporting *C. parvum* contamination levels in high-risk studies, such as those concerning municipal drinking water.

In situations involving drinking water quality and public health, it is often prudent to err on the side of caution. Regarding *C. parvum*, it may therefore be insufficient to report only viable oocysts, especially when the quality of detection methods for viable oocysts remains spurious (section 1.4.2.1).

Accordingly, this study has focused on detecting total presence and persistence of *C. parvum* rather than only viable or infectious *C. parvum*. Ideally, both approaches would be pursued to provide accurate quantification of the total as well as viable oocysts in a sample. The most common options for the detection of total *C. parvum* presence are fluorescence microscopy and PCR-based DNA assays. As previously noted, fluorescence microscopy is useful for general quantification of cells and staining notable cellular features but erroneous results can arise due to identification error, processing losses, and substantial background noise generated from residual fecal detritus. Molecular detection techniques such as quantitative real-time PCR (qPCR), droplet digital PCR (ddPCR), and nested PCR (nPCR) provide a very low oocyst detection limit, high specificity, and also allow for species verification by nucleotide sequencing (Morgan et al., 1998). An overview of the PCR-based assays used for the study of *C. parvum* persistence will be introduced below.

Quantitative real time-PCR

qPCR differs from standard PCR in that the concentration of DNA amplicons can be measured after each elongation cycle, as commonly determined by fluorescence generated by dsDNA-binding dyes or using Förster Resonance Energy Transfer (FRET)-based ssDNA-binding hydrolysis probes that fluoresce upon dissociation during strand replication. Samples with a higher concentration of DNA reach an established fluorescence threshold sooner than samples with little to no DNA. The number of cycles required to reach this predetermined

threshold (C_q value) can be compared to a previously developed standard curve as a means of indirect quantification.

qPCR is recognized as a more rapid analytical method than conventional or nested PCR since it does not rely upon end-point analysis, such as gel electrophoresis and DNA staining, after amplification. In buffered solutions, qPCR can detect as few as 2.5 oocysts using the 18S rRNA locus as a target sequence and can be performed within three hours (Yang et al., 2014). However, in feces, PCR inhibitors can affect qPCR amplification as evident by higher amplification efficiency obtained in diluted fecal samples: fecal samples spiked with *C. parvum* DNA gave ~50% lower sensitivity compared to a 10X-diluted fecal sample spiked with the same concentration of *C. parvum* DNA (Yang et al., 2014). qPCR is particularly susceptible to PCR inhibitors and competing DNA in feces due to potential interference with fluorescent probes as well as interference with replication machinery (Skotarczak, 2009). Specifically, at concentrations of 500ng humic acid substances, nearly all fluorescence generated from qPCR dsDNA binding dyes ResoLight, SYTO82, SYBR Green I, and EvaGreen, was quenched resulting in amplification plots significantly lower than actual amplicon production (Sidstedt et al., 2015). However, hydrolysis probes were not greatly quenched by humic acids (Sidstedt et al., 2015). The efficacy of qPCR using dsDNA dyes is therefore reliant on how well PCR inhibitors can be removed from a DNA sample. In samples known to have high DNA concentration, such as DNA from cell cultures, the effect of inhibitors can be lessened by dilution of the DNA sample. However, samples with very low DNA such as those encountered during persistence monitoring cannot be diluted due to the potential loss of target DNA sequence. In environmental samples containing a plethora

of non-target DNA and PCR inhibitors, PCR detection quality and specificity diminishes (Goode et al., 2002).

Additives such as 5% dimethyl sulfoxide (DMSO) have been shown to relieve inhibition, but optimization must be performed as too much DMSO can inhibit polymerase activity as much as 50% (Gefland and Innis, 1990). Bovine Serum Albumin (BSA) has also been used to relieve inhibition, and is occasionally implemented in PCR master mixes (Bessetti, 2007; Kreader, 1996). Despite addition of these additives, residual inhibitors still impair amplification, especially in samples with small concentrations of DNA.

Droplet digital PCR

During droplet digital PCR (ddPCR), the sample of interest is fractionated into thousands or millions of water-in-oil droplets, each serving as a partitioned PCR environment containing the target template (Hindson et al., 2011). The effect is comparable to performing thousands of PCR replicates, which yields greater resolution, 7-fold higher day-to-day reproducibility, and a 37-86% lower coefficient of variation compared to either conventional or qPCR on low template samples (Baker, 2012; Hindson et al., 2013). In contrast to qPCR, ddPCR provides end-point absolute quantification of template copies and accordingly does not depend on a standard curve.

Tested on *Cryptosporidium* oocysts, ddPCR showed comparable sensitivity to qPCR, detecting as few as 2 oocysts per reaction in buffered solutions (Yang et al., 2014). In feces, ddPCR outperformed qPCR and was shown to be less susceptible to inhibitors: ddPCR provided efficient amplification of 1fg of template in up to 9.3ng/μL humic acid whereas qPCR was reduced to 20% efficiency in 5.6ng/μL humic acid (Hoshino and Inagaki, 2012; Yang et al., 2014). For comparison, soil and fecal samples can contain as much as 700-

3,300ng humic acid or humic-acid-like substances per 1 μL of sample (Tebbe and Vahjen, 1993).

ddPCR is gaining popularity in laboratories as an efficient method for obtaining quantitative genetic data. Unfortunately, the relatively high costs of ddPCR make it currently impractical for high-throughput analysis when alternative quantitative approaches such as qPCR exist. In 2014, the average cost of running 46 samples (in duplicate and excluding controls) on 96-well ddPCR, was $566 (or $12.30/sample) (Yang et al., 2014). The cost of running the same number of samples using qPCR (in duplicate and including standard curve samples) was $190 ($4.13/sample), making qPCR 3-times less expensive. The latter estimate is comparable to the qPCR costs observed during the present study ($4.62/sample). Additionally, the average sample turnaround time of ddPCR was reported as twice as long as that of qPCR.

The applications of ddPCR continue to grow and competitive pricing is making ddPCR costs more reasonable for high-throughput quantitative analysis. Due to the high number of samples analyzed in this persistence study, ddPCR was ruled out as an analytical approach. However, ddPCR would be useful as a means to accurately quantify qPCR standards used for qPCR. ddPCR was not performed in this study, but its potential application in evaluating *C. parvum* persistence in samples containing high concentrations of inhibitors was worth consideration.

Nested PCR

To further increase the specificity and sensitivity of PCR in high-complexity environmental samples, a second round of amplification can be performed within the target sequence. In nested PCR (nPCR), a preliminary set of primers are first used to amplify a

sequence of interest, as per conventional PCR. The product now contains an elevated concentration of target DNA, but in samples with high levels of competing DNA or inhibitors a single amplification of the target sequence may have been inadequate for detection by staining methods (Paul et al., 2009). To bolster the relative concentration of the target sequence, a small (1µL) aliquot is withdrawn from the primary PCR solution and subjected to a second round of PCR using primers that anneal within the preliminary PCR amplicons (Figure 1.1) (Goode et al., 2002).

Compared to other *C. parvum* detection techniques such as qPCR, RT-PCR, ddPCR, and IFM, nPCR provides some of the highest sensitivity and specificity without incurring high sample processing costs (Morgan and Thompson, 1998; Morgan et al., 1998; Yang et al., 2014; Yu et al., 2009). A single oocyst detection limit has been observed in many nPCR studies, making it one of the most precise molecular detection techniques for *C. parvum* in environmental matrices (Coupe et al., 2005; Yu et al., 2009). The high precision is most likely due to the longer history of nPCR for detection of *C. parvum*. IFM or other staining methods provided only 83.7% sensitivity compared to nPCR (Paul et al., 2009). In contrast with RT-PCR, qPCR, and IFM, nPCR encounters less issues with environmental inhibitors, making it an attractive technique when working with fecal samples (Higgins et al., 2001). One of the often cited problems with nPCR is the increased potential for false positive results. While doubling the number of PCR reactions increases sensitivity, it also increases the potential of PCR contamination and therefore requires diligent use of controls (Persing, 2010). Another setback for nPCR is the approximately 8-hours required to process a 96-well plate through two PCR cycles, perform electrophoretic separation, and stain the DNA. This is more than twice the time required to obtain quantitative data via qPCR. Despite the long

processing time, nPCR remains the least expensive molecular detection technique for *Cryptosporidium* at $4.39/sample (including controls and replicates). The cost of a conventional PCR instrument is also considerably less expensive than either qPCR or ddPCR instruments, although the costs for these quantitative instruments are becoming increasingly competitive (Baker, 2012).

Within the last decade, nPCR has been declared the gold-standard in molecular detection methods for *Cryptosporidium* oocysts in feces and soil due to its repeatability and widespread clinical and research application in patient diagnostics, environmental monitoring, and source-tracking (Coupe et al., 2005; Hadfield et al., 2011; Jiang et al., 2005).

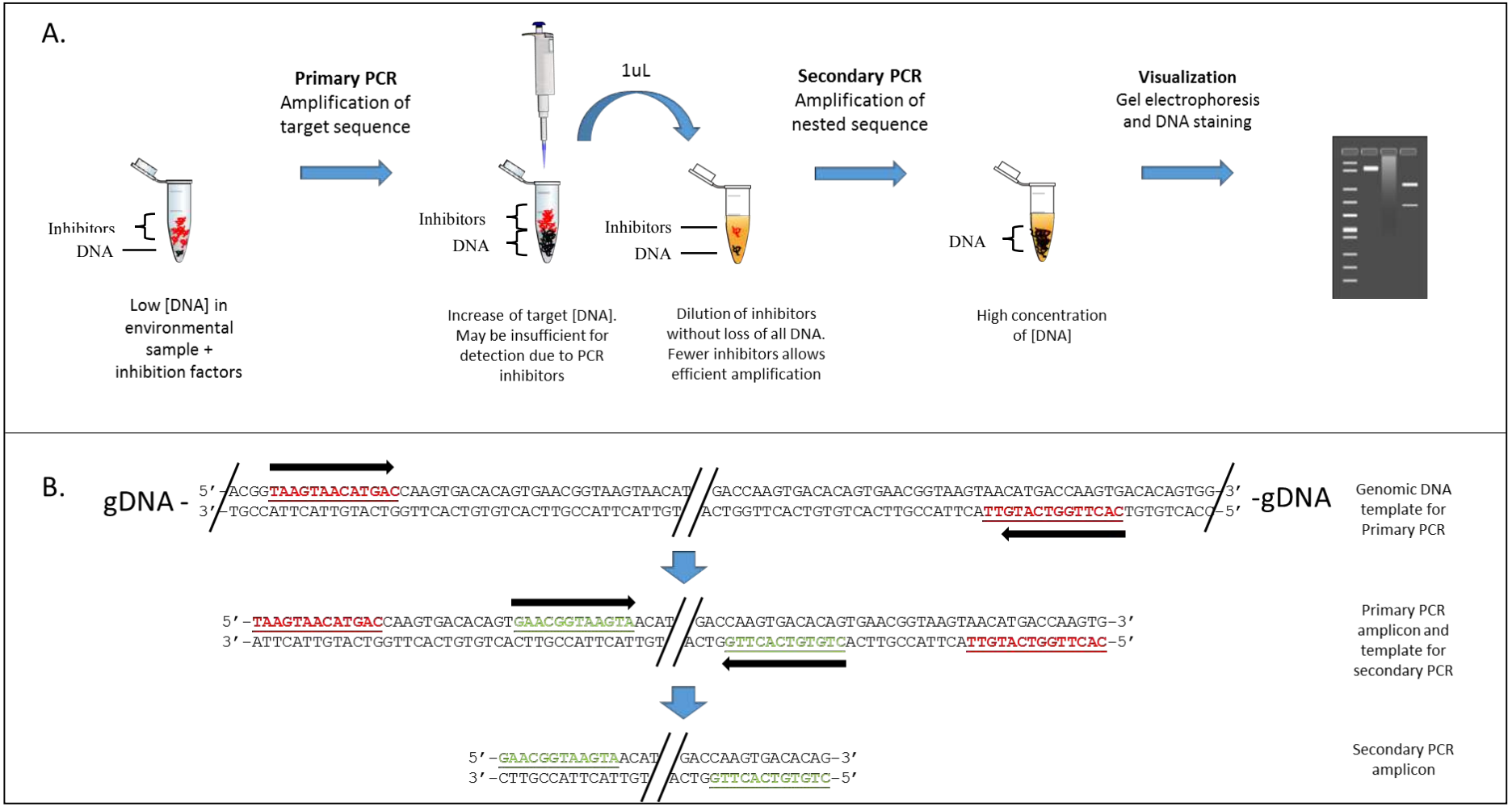


Figure 1-1: Nested PCR schematic including A) procedural overview in which red clusters represent inhibitors and black clusters represent DNA; and B) primer nesting.

1.4.3: Research objectives

From the literature review and concerns raised by local agricultural ministries and ranchers, it was determined that insufficient knowledge exists as to the duration that *C. parvum* will persist on grazing land and whether variations in shading and seasonality may influence persistence time. The following objectives address the knowledge gaps and the use of nested PCR as a method for environmental monitoring.

1. Evaluate nested PCR as a method for measuring oocyst persistence in bovine fecal samples and compare nested PCR with alternative molecular detection techniques such as qPCR. (Chapter 3)
2. Provide information as to the maximum time that *C. parvum* remains detectable on Okanagan grazing land during winter and summer exposure conditions. (Chapter 4 and Chapter 5)
3. Identify influence(s) for shading conditions and key environmental factors on *C. parvum* persistence during winter and summer exposures. (Chapter 4 and Chapter 5)

The objectives outlined above were achieved by performing the following experiments:

1. Developing and validating a method for extraction of *Cryptosporidium* DNA from fecal samples, comparing nPCR to qPCR. The methodology for this is explained in section 2.1.4-2.1.10, and the results and discussion are discussed in Chapter 3.
2. Exposing *Cryptosporidium* in fecal pats to Okanagan winter field conditions and observing whether oocysts leach after winter exposure. The methodology for this is explained in section 2.1.1-2.1.8. 2.1.10, 2.1.11, 2.1.13 and results and discussion can be found in Chapter 4.
3. Visualizing freeze thaw damage. The methodology for this is explained in section 2.1.12 and the results and discussion can be found in Chapter 4.
4. Exposing *Cryptosporidium* in fecal pats to Okanagan summer field conditions. The methodology for this is explained in section 2.1.1-2.1.8. 2.1.10, 2.1.11, and results and discussion can be found in Chapter 5.

CHAPTER 2
METHODS AND CONTROLS

This chapter serves as a repository of the methods and controls used for all research related to this thesis. Methods and controls for the winter (Chapter 4) and summer (Chapter 5) studies are similar and were therefore combined here to minimize repetition. Unless otherwise stated, methods and processing controls are identical between the two seasonal studies. In addition to the methods listed here, a brief overview of the methods used for each study will be reintroduced at the beginning of each subsequent chapter.

All chemical recipes can be found in Appendix A.

2.1: Methods

2.1.1: *C. parvum* oocysts

Bovine calf-derived *C. parvum* oocysts (Iowa isolate) in phosphate-buffered saline (PBS) were purchased from Waterborne Inc. (New Orleans, LA), generated by passage through infected calves and extracted/purified from feces using diethyl ether followed by a sucrose and Percoll ™ density gradient centrifugation. Stock oocyst concentration was determined by Petroff-Hauser chamber as $1.23 \times 10^5 \pm 2.6 \times 10^4$ oocysts/mL (n = 6). Oocyst stocks were maintained in the lab at 4°C for less than 6 months prior to use.

2.1.2: Feces

Over 15 gallons of bovine feces from different cattle herds were provided by Longhorn Farms in Kelowna, BC. Fecal material was partially de-watered using a 20/40 mesh sieve to permit proper molding of fecal patties. Absence of *C. parvum* in feces was verified by several trials with nested PCR.

2.1.3: Sampling plot development

Permission for placing *C. parvum* spiked bovine feces on University of British Columbia Okanagan land was granted from the University of British Columbia Office of Research Services after a thorough biosafety review. Areas with light (L), moderate (M), and heavy (H) natural shading conditions were chosen to emulate general conditions observed in Okanagan, BC Crown Land (Figure 2-1). Relative shading was characterized using ultraviolet light intensity as measured at 1 PM over several days with a General Tools and Instruments UV513AB digital UVAB monitor (L: 6-10+ mW cm^{-2}, M: 3-7mW cm^{-2}, H: 0-2 mW cm^{-2}). During the summer persistence study, a tarp was used to provide additional shading at the heavily forested plot. This was done to mimic the very high shading observed in one of the field sites that contained persistent *C. parvum* in sampled feces. For both studies, plots were established within 25m of each other and enclosed with 4m × 4m snow fencing. All plots had slope between 5-10%.

2.1.4: *C. parvum* oocyst spiking and sample plotting

A spiking volume of 100µL (~12,000 oocysts/kg feces) was used in the persistence studies in effort to emulate low fecal seeding comparable to ~9200 oocysts cow^{-1} day^{-1} as previously reported in field studies (Atwill et al., 2003). This low seeding was also chosen in interest of minimizing the amount of biohazardous material in the environmental plots. Eleven seeded fecal pats were placed in each of the plots ~0.5-0.7m apart. At time points of 0, 1, 2, 3, 4, 5, 6, 7, 14, 21, 28, 41, 67, 106, and 137 days, three fecal patties from each plot were chosen for sampling using a random number generator. Approximately 30-50g were cut from the periphery of the fecal pats and placed in sterile Whirl-Pack™ sampling bags to be

stored at 4°C prior to processing. Environmental detritus (pine needles, snow, twigs) were replaced if disturbed during the sample collection process.

2.1.5: Fecal pelleting

Portions (20.5 ± 0.5g) of the sampled feces were suspended in 50mL 1x PBS in 50mL conical centrifuge tubes and rested for 10 minutes to hydrate. Samples were then mixed by vortexing for 15 seconds and sieved through a tea-strainer (~ #40 mesh) to remove detritus. The filtrate was suspended in up to 50mL of 1x PBS and centrifuged at 3,000 × g for 10 minutes with a Sorvall Legend RT+ Centrifuge (Thermo Scientific) with Hareaus 75006445 4 × 750mL swinging bucket rotor (Thermo Scientific). The supernatant was discarded and the pellet was resuspended to 15mL in 1x PBS followed by centrifugation at 3,000 × g for 10 minutes. The supernatant was discarded and the pellet was mixed with a final volume of 5mL 1x PBS. Three 1.4mL aliquots were collected from each pellet resuspension and treated as pseudo-triplicates for the remaining processing stages.

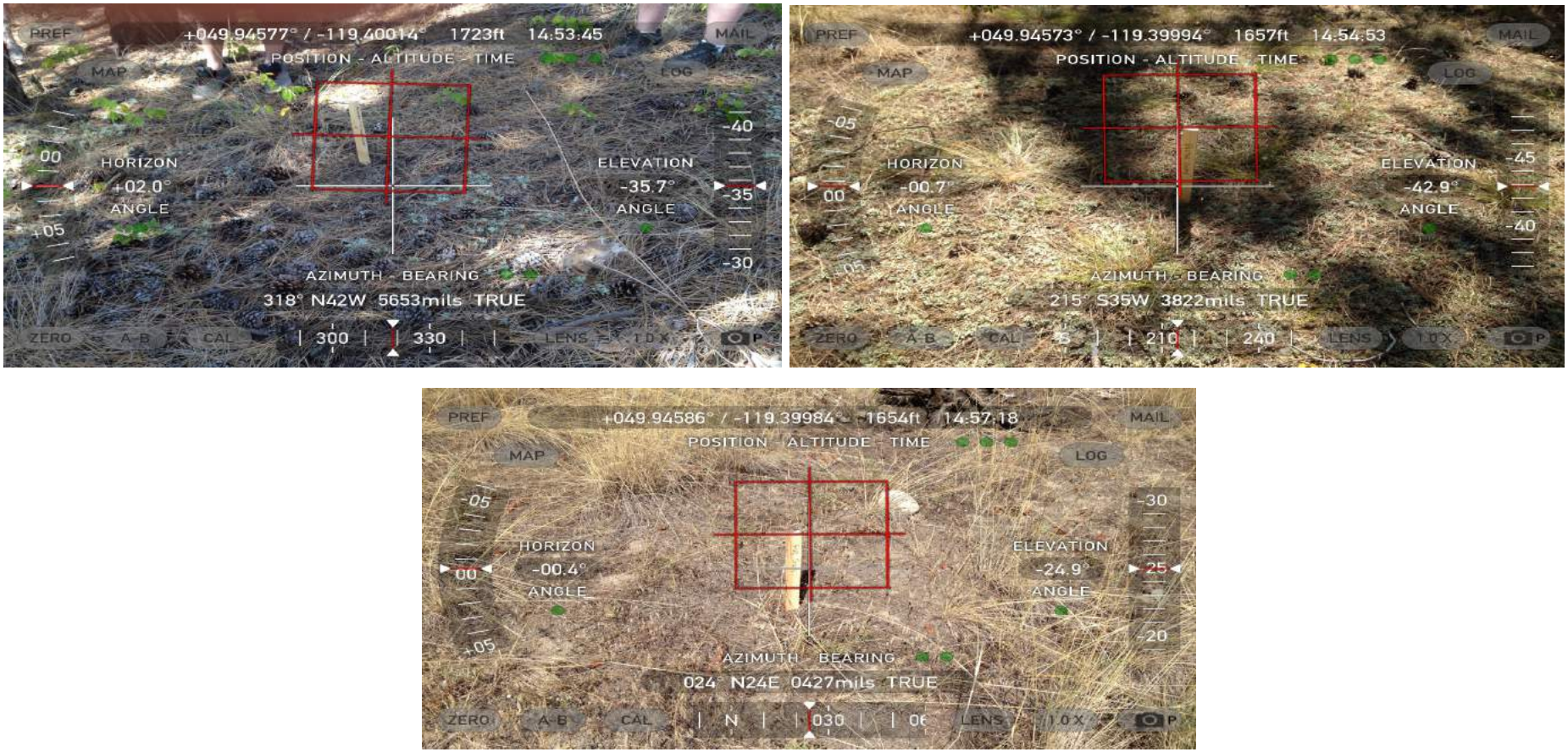


Figure 2-1: Theodolite images of plots used during the winter and summer for persistence study. Clockwise from top left: Heavily forested, moderately forested, lightly forested.

2.1.6: Oocyst thermal shock

All pelleted fecal subsamples were subjected to thermal shock to weaken the active oocyst cell walls prior to DNA extraction. A total of five freeze-thaw cycles (Jiang et al., 2005) of 15 minutes at -80°C in a freezer followed by 15 minutes at 65-70°C in a metal bead bath were conducted on each sample.

2.1.7: DNA extraction

DNA extraction on 600µL of each thermally shocked fecal sample was performed using a Mo Bio PowerSoil™ DNA extraction kit involving physical shearing of oocysts by bead beating, sodium dodecyl sulfate (SDS) cell lysis, and DNA precipitation and elution. The PowerSoil™ kit was chosen over the PowerFecal™ kit based on advice from a MoBio representative, fewer processing steps, and comparable results between the two kits during preliminary trials. Bead beating was performed using a Mo Bio VortexGenie 2 ™.

2.1.8: Nested PCR

DNA amplification was performed with an Eppendorf MasterCycler EPgradient S thermocycler using a nested PCR protocol with external primers amplifying the extended gene target and internal primers selective for an 18S rRNA gene segment in *Cryptosporidium* spp. as discussed by (Yu et al., 2009). Primers were also chosen based on supporting literature confirming the successful use of the method for environmental samples (Coupe et al., 2005; Sturbaum et al., 2001).

The external PCR run consisted of 10 minutes at 94°C followed by 35 cycles of 94°C (45 seconds), 55°C (45 seconds), and 72°C (60 seconds) using a 50µL reaction volume containing 5µL sample DNA as template, 200µg/mL Bovine Serum Albumin (BSA), 200nM primer, 4mM $MgCl_2$, 20µL of 5-Prime™ MasterMix, brought up to volume with

diethylpyrocarbonate (DEPC)-treated sterile water. Internal amplification was performed with identical thermocycling parameters as external PCR except with 25 instead of 35 cycles, 1μL of the external PCR product as template for internal PCR amplification, and with the internal primers from (Yu et al., 2009). The primer sequences for the 18S rRNA gene external and internal PCR runs and the reaction conditions were as follows:

External Forward Primer (1): 5'-TTCTAGAGCTAATACATGCG-3'

External Reverse Primer (2): 5'-CCCATTTCCTTCGAAACAGGA-3'

Internal Forward Primer (3): 5'-GGAAGGGTTGTATTTATTAGATA-3'

Internal Reverse Primer (4): 5'-AAGGAGTAAGGAACAACCTCCA-3'

Table 2-1: Reagent volumes and concentrations used for nested PCR during persistence studies.

External PCR		
Component	**Volume (μL)**	**Final Concentration**
DEPC-treated PCR-grade water	14	-
5Prime™ PCR Master Mix (2.5x)	20	1X
Bovine Serum Albumin (2500ug/mL)	4	200μg/mL
External Forward Primer (1) (10mM)	1	200nM
External Reverse Primer (2) (10mM)	1	200nM
$MgCl_2$ (25mM)	5	2.5mM
Sample DNA	5	-

Internal PCR		
Component	**Volume (μL)**	**Final Concentration**
DEPC-treated PCR-grade water	22	-
5Prime™ PCR Master Mix (2.5x)	20	1X
Internal Forward Primer (3) (10mM)	1	200nM
Internal Reverse Primer (4) (10mM)	1	200nM
$MgCl_2$ (25mM)	5	2.5mM
External PCR Product	1	-

The nPCR methodology and its application in evaluating *C. parvum* persistence in feces is discussed in detail in Chapter 3.

2.1.9: qPCR

qPCR was first attempted using the nested internal primers mentioned in section 2.1.8, but amplification was inconsistent. Instead, primers flanking the alleles of LIB13 (unknown function) were used and found successful and reproducible using a modified protocol from (Hadfield et al., 2011). Modifications include the use of SsoFast EvaGreen Supermix and use of only 45 cycles instead of 55. Thermocycling was performed with a BioRad CFX96 Touch™. Threshold cycles (C_q) were manually set in the log-linear phase. Custom primers were obtained through ThermoFisher.

Forward Primer (1): 5'-TCCTTGAAATGAATATTTGTGACTCG -3'

Reverse Primer (2): 5'-TTAATGTGGTAGTTGCGGTTGAAC -3'

Table 2-2: Reagent volumes and concentrations used for qPCR during persistence studies.

qPCR		
Component	**Volume (µL)**	**Final Concentration**
PCR-grade water	9.5	-
Bio-Rad SsoFast EvaGreen Supermix	12.5	1X
LIB13F Forward Primer (22500nM)	1	900nM
LIB13R Reverse Primer (22500nM)	1	900nM
DNA	2	-

The qPCR methodology and its application in evaluating *C. parvum* persistence in feces is discussed in detail in Chapter 3.

2.1.10: Gel electrophoresis and visualization

Gels were made using 1% wt/vol of PCR-grade agarose in 1x TBE and cast in a FisherBioTech, Electrophoresis System Midi-Horizontal FB-SB-1316 using two 20-well combs. 18µL of each nested PCR product were mixed with 2µL of 5x bromophenol blue

loading dye prior to injection into wells. Band sizes were estimated using 1kb plus ladder reference (Invitrogen, Lot No. 1513825). The gel was run in 0.5X TBE running buffer at 115V for 90 minutes. Staining was performed after electrophoresis using a 1.0x solution of SYBR Safe™ DNA stain (Life Technologies) in reverse osmosis (RO) water for 20 minutes. Stained DNA was visualized in an AlphaImager EC light cabinet at 365nm. For the first two samples, gel bands at ~850bp were excised and purified using Illustra GFX PCR DNA and a Gel Band Purification kit and submitted for capillary sequencing at the University of British Columbia, Okanagan, Fragment Analysis DNA Sequencing Service (FADSS). Reported sequences were analyzed for species identity using the NCBI BLASTn tool and had 98% identity with the expected *C. parvum* 18S rRNA gene derived from the oocyst spiking solution. Sequence polymorphisms between bovine *C. parvum* can range as much as 2 to 3.2% depending on geographical variation (Pereira et al., 1998).

2.1.11: Meteorological data

Temperature, relative humidity, and rainfall data were obtained from the closest meteorological data collection facility, Kelowna International Airport (1.87 km from study plots). Fecal crusting and patty hardness were observed based on the difficulty of extracting a sample.

Monitoring of the summer plots was performed using EL-USB-2 (DataQ Instruments) for recording hourly temperature, relative humidity, and dew point in each of the shading conditions. Ultraviolet readings were obtained during each sampling day using a UV513AB digital UVAB monitor (General Tools and Instruments). Gravimetric weights were taken by drying a known mass of feces from each sample at 100°C for 24+ hours.

2.1.12: Morphological characterization of freeze-thawed oocysts

Aliquots of 5µL *C. parvum* stock solution were subjected to either 0, 1, 2, or 3 freeze-thaw cycles at -80°C/70°C. Samples were diluted 50-fold, vortexed for 30 seconds and 30µL of the diluted sample was placed on a 2-well SuperStick microscope slide (Waterborne Inc., New Orleans, LA). Oocyst staining was performed using a fluorescein isothyocyanate (FITC)-conjugated monoclonal antibody (MAb) and 4'-6'-diamidino-2-phenylindole (DAPI) as per instruction in an A400FLR-1X Crypt-a-Glo™ G/C Direct Comprehensive kit (Waterborne Inc., New Orleans, LA. Revision 1/2010) with modifications of 20µL absolute methanol instead of 40µL as well as use of an enclosed slide warmer at 37.1°C (Boekel Scientific Model 240000). Slide viewing was performed using Zeiss Axioimager M1 microscope with epifluorescence and bright field optics. Oocyst examinations were performed using the 100X objective lens.

2.1.13: Flushing study

Fecal pats were collected from the winter sampling site after the winter persistence study was complete in order to evaluate if any *C. parvum* did indeed remain and whether the oocysts could be flushed out and be detected in the leachate by either nPCR or IMS/IFM methods as per EPA Method 1623. Two (~650g) fecal pats from each plot remaining from the winter persistence study were placed on a trough measuring 107 cm long × 56 cm wide set at a 5-degree angle and subjected to recirculated flushing with 15 L RO water for 5 hours using a submersible aquarium pump (Figure 2-2). Flow was set to 1700mL/min and diffused through eight horizontal 1-cm holes above the fecal pats in an effort to mimic low flow rates observed at the riparian edges in upper Crescent Creek, Vernon, BC. The recirculated effluent was filtered through an EnviroChek™ 0.5µm membrane filter to collect any

potential *C. parvum* oocysts. Elution of the filter was performed using 500mL of elution buffer shaken at 800rpm on a designated laboratory shaker. The eluent was centrifuged at 3,000 × g for 15 minutes to collect the pellet which was then resuspended in 15mL of 1x PBS. The solution was then processed in accordance with EPA Method 1623 using immunomagnetic separation (IMS) followed by immunofluorescence microscopy (U.S. EPA, 2005). A 2mL portion of the IMS was subjected to thermal shock and DNA extraction methods followed by nPCR (section 2.1.6-2.1.8).

2.1.14: Statistical analysis

Several statistical interpretations were attempted for the winter and summer persistence data. It was ultimately discovered that due to limitations in experimental design that several statistical analyses could not be performed due to violations of the assumptions of normality and equal variances. A discussion of these violations and the different approaches that were considered in this study can be found in section 6.4. Also in section 6.4

Figure 2-2: Flushing device for testing oocyst leaching from winter-exposed fecal pats.

is a discussion of an alternative experimental design that would facilitate accurate statistical interpretation.

Persistence data were represented in terms of the number of fecal pats that contained at least one sub-triplicate positive for *C. parvum*. Because the data were in the form of binomial proportions, traditional calculation of confidence intervals or standard error were inadequate and gave artificially low values (Appendix D). Instead, binomial proportion confidence intervals were calculated using the Agresti-Coull formula

$$p \pm z \sqrt{\frac{1}{(n+z^2)} * \left(\frac{X+\frac{1}{2}z^2}{n+z^2}\right) * \left(1-\frac{X+\frac{1}{2}z^2}{n+z^2}\right)}$$

where p is the proportion of fecal samples positive for *C. parvum*, z is the critical value for a 90% confidence interval (z = 1.645), X is the number of samples positive for *C. parvum*, and n is the number of trials (n = 3). Limits of the confidence intervals were capped at 3/3 (maximum persistence) and 0/3 (minimum persistence). While there are several formulas for binomial proportion confidence intervals, the Agresti-Coull was used for its relative ease in computation, flexibility in interval coverage, and use in data with low sample sizes (Agresti and Coull, 1998). Other methods such as Jeffrey's and Wilson's confidence interval approximations were performed and similar results were obtained.

Plots of the persistence data and calculations of confidence intervals were performed using EXCEL version 15.14.

2.2: Controls

The following controls were implemented during the winter and summer persistence study. Field controls were performed to identify whether nPCR amplified non-target oocyst lysates and also to provide a controlled reference for environmentally exposed fecal pats. Processing controls were performed to identify potential process errors and false negative or false positive results.

2.2.1: Field Controls

DNA persistence

Samples (600g) of autoclaved bovine feces were spiked with either 60µL of $1.23x10^5$/mL live *C. parvum* oocysts from stock (no treatment), 60µL of *C. parvum* oocysts subjected to 5 thermal cycles (20 minutes at -80°C, 20 minutes at 70°C), or with DNA extracted from 60µL of *C. parvum* oocysts. Samples were stored covered and at room temperature. Two samples (20.5 ± 0.5g) were withdrawn at 1, 48, 96, and 168 hours after spiking. For the untreated oocyst test, three samples were withdrawn during each time point. Samples were processed as described above with each sample split into pseudo-triplicates for a total of either six or nine samples. This control was implemented to assess if nested PCR positives could arise from either already ruptured oocysts or residual oocyst DNA.

Environmental

Three 1000g ± 50g samples of feces were spiked with 100µL of $1.23x10^5$/mL live *C. parvum* oocysts and stored at 4°C in a fridge. Triplicate samples of 20.0 ± 0.5g were withdrawn at time points of 1, 2, 3, 4, 5, 6, 7, 14, 21, 28, 41, 67, 106, 137, and 165 days. Each sample was subjected to the same processing as the environmental samples.

2.2.2: Processing controls

DNA extraction controls

DNA extraction success was based upon gel electrophoresis results in which presence of a strong band was expected for the positive control at ~850bp and no band for the negative control. Positive Control: 550µL of water containing 50µL of thermally shocked $2.5x10^4$/mL

live *C. parvum* oocysts. Negative Control: 600µL of water. Both samples were brought through DNA extraction and subsequent processing stages.

Nested PCR controls

Positive and negative controls were implemented during the nested PCR protocol to verify PCR success. Both samples were brought through PCR and subsequent processing stages. Positive Control: Same reagent concentrations as nested PCR but used DNA samples that previously gave positive gel band results at ~850bp and were verified as *C. parvum* via capillary sequencing. Negative Control: Same reagent concentrations as nested PCR but used 5µL of sterile DEPC-treated water instead of DNA.

Gel electrophoresis controls

Positive and negative controls were implemented during gel electrophoresis to verify gel staining. Positive Control: Post-PCR product previously determined to give positive *C. parvum* gel band. Negative Control: 18µL of DEPC-treated sterile water mixed with 2µL of 5X bromophenol blue loading buffer.

CHAPTER 3
PCR DETECTION LIMITS

3.1: Summary

Although nPCR was used as the primary means of *C. parvum* detection for both winter and summer persistence studies, qPCR was also evaluated as an alternative molecular detection assay. Limit of detection (LOD) tests were performed for both nPCR and qPCR in spiked fecal and PBS matrices in an effort to assess the effect of natural PCR inhibitors on the amplification processes and whether qPCR could be used in future oocyst monitoring studies. Although qPCR was not pursued during the summer study due to funding and time constraints, the qPCR experiments did provide insight into the utility of the two molecular detection methods for fecal *Cryptosporidium* studies. This chapter addresses the efficacy of nPCR and qPCR in detecting *C. parvum* in environmentally exposed fecal pats and concludes with a rationale for the use of nPCR during the summer study.

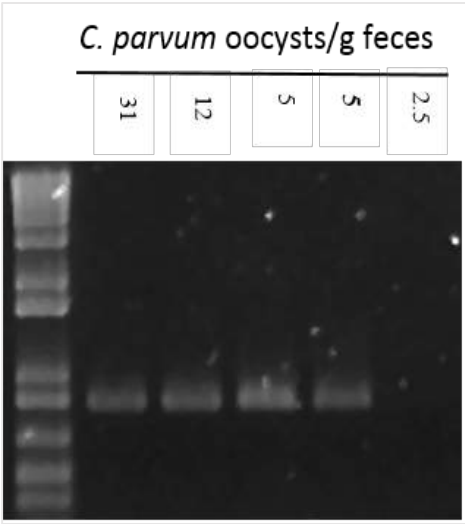
Methodology Overview:

For nPCR LOD tests, DNA extraction, nPCR, and gel electrophoresis were performed as per methods outlined in section 2.1.5-2.1.8. For qPCR LOD tests, fecal samples were spiked with different dilutions of *C. parvum* stock and subjected to DNA extraction and qPCR (section 2.1.9). Several primers were tested for qPCR, including both custom and literature β-tubulin gene primers as well as literature LIB13 gene primers. Amplification and melting curve analysis on the primers were performed to assess primer specificity.

3.2: Results

Detection by nested PCR

The efficacy of nPCR in this study depends on both its detection limit and specificity for intact *C. parvum* oocysts. Limit of detection (LOD) was determined for both oocysts/g (opg) feces and DNA equivalence in oocysts per nPCR reaction (opPCR). Volumes of 0µL, 20µL, 40µL, 100µL and 250µL of a 1.23x $10^5 \pm 2.56 \times 10^4$ *C. parvum* oocysts/mL were each spiked into separate 1000g ± 20g feces and thoroughly homogenized to give approximate concentrations of 0, 2.5, 5, 12, and 31 oocysts/g (opg) feces. 20.50 ± 0.50g samples were withdrawn in triplicate and processed as discussed above. Samples containing 40µL (5 opg) of *C. parvum* showed bands at ~826bp whereas samples spiked with lower volumes did not yield consistently observable bands (Figure 3-1). Accounting for sub-sampling and aliquots used during processing (Appendix B), a 40µL spiking volume approximately yields the DNA equivalence of 0.45 ± 0.12 oocyst per PCR (opPCR). The low LOD of ~0.5 oocysts



oocyst spiking Volume (uL)	opg	opPCR
0	**0**	**0**
20	**2.5**	**0.22 ± 0.06**
40	**5**	**0.45 ± 0.12**
100	**12**	**1.13 ± 0.29**
250	**31**	**2.84 ± 0.73**

Figure 3-1: Nested PCR amplification of *Cryptosporidium* 18S SSU rRNA from feces spiked to concentrations of 2.5, 5, 5 (replicate), 12, and 31 oocysts/g (opg), corresponding to final equivalents of 0.22, 0.45, 0.45, 1.13, and 2.84 oocysts/ PCR reaction (opPCR), respectively.

corresponds to approximately three rDNA gene copies (Blancq et al., 1997) and is at the theoretical limit of PCR detection as noted by MIQE guidelines (Bustin et al., 2009). Higher concentrations, up to 31 opg (2.84 opPCR), were tested and gave successful amplification as well, supporting the low detection capabilities of nPCR. Comparatively low *C. parvum* detection limits (1 oocyst), as achieved by nPCR, have been previously observed in buffer matrices (Coupe et al., 2005; Sturbaum et al., 2001; Yu et al., 2009). Detection of single oocysts has been further confirmed by studies in which one oocyst was micropipetted into PCR tubes, with 67 of 75 samples (89.3%) yielding positive results (Sturbaum et al., 2001). The results observed in the current study show that comparably low detection limits can be obtained using nPCR in environmental samples containing common PCR inhibitors such as humic substances (Tsai and Olson, 1992).

Successful amplification of such a low concentration of the *C. parvum* 18S rRNA gene target in a fecal matrix was likely facilitated by a combination of using a DNA extraction kit that removes PCR inhibitors (Mo Bio Powersoil™), introduction of BSA to minimize PCR inhibition (Kreader, 1996), and the secondary amplification process.

Detection by qPCR

qPCR was discussed as an alternative to nPCR after the winter study had begun and therefore any implementation of qPCR would occur during the proposed summer study. The internal primers used during nPCR were not used for qPCR due to the relatively large amplicon length (826bp) and the recommendations for qPCR amplicons smaller than 200bp in order to achieve greatest amplification efficiency. However, the proposed limitations on amplicon size, especially when using dsDNA binding dyes, appear to be heavily anecdotal and debated. Regardless, qPCR primers were chosen from literature based on their reported

low limit of detection and selectivity. The objective of this test was to find qPCR primers, regardless of gene target, that could provide comparatively low detection limits to those obtained by nPCR.

Primers flanking a 157bp fragment of the β-tubulin gene (Accession No. Y12615.1) were first tested based on literature supporting their use in low level detection and qPCR-based speciation of *Cryptosporidium* (Tanriverdi et al., 2002). Primer characteristics were analyzed using Primer3Plus and were found to have relatively low melting point, low GC %, and moderate propensity for self-annealing (Table 3-1) in accordance with the recommendations reported by Integrated DNA Technologies (IDT) (Prediger, 2016). It was observed after several trials using both standard PCR and qPCR that non-specific amplification and/or primer dimerization occurred. Using standard PCR and gel electrophoretic separation of the resulting amplicons, gel bands were visible at 2000bp and <200bp even in the no-template-control (NTC). Melting curve analysis for the custom designed β-tubulin primers showed scattered melt peaks with no defined maxima, and the β-tubulin primers from (Tanriverdi et al., 2002) revealed two melt peaks with maxima at 73°C and 79°C (Figure 3-2). Melt curve analysis of the NTC shows a sharp single peak maximum at 73°C, further suggesting that amplification and dimerization of the primers may be occurring (Applied Biosystems). Optimization using different concentrations of BSA, primer, and template as well as different PCR annealing temperatures and polymerase types were performed without success and no further attempts were performed using these primers.

Due to non-specific amplification, primer dimerization, and spurious results observed for the β-tubulin primers, new primers targeting the *C. parvum* LIB13 (unknown function)

Table 3-1: Characteristics of primers tested for qPCR of *C. parvum* DNA.

		Sequence	Amplicon Size	Primer Length (bp)	Tm (C)	GC (%)	ANY	SELF	ΔG
	Recommended		70-150	18-30	60-64	40-60			
(Tanriverdi et al., 2002)	β-tub Forward	ATGCTGTAATGGA TGTAGTTAGACA	157	25 (#552-576)	57.0	36	3	2	6.4
	β-tub Reverse	GTCTGCAAAATAC GATCTGG		20 (#609-709)	55.8	45	4	2	8.2
Custom Design	β-tub Forward	CTGGCTATTGGGG AACTTGA	154	20 (#12-32)	60.1	50	3	1	7.0
	β-tub Reverse	CTGGTTCCCACAT TGTCCTC		20 (#165-185)	60.4	55	5	0	7.9
(Hadfield et al., 2011)	LIB13 Forward	TCCTTGAAATGAA TATTTGTGACTCG		26 (#228-253)	58.3	35		2	
	Lib13 Reverse	TTAATGTGGTAGT TGCGGTTGAAC		24 (370-393)	60.02	42		5	

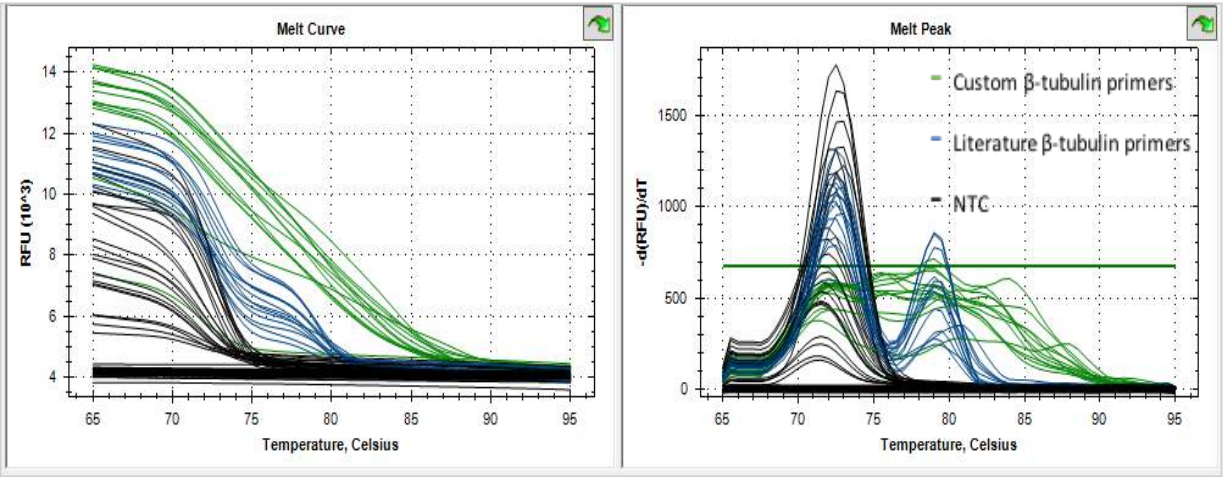


Figure 3-2: Melting curve analysis of custom designed and literature primers flanking a segment of the *C. parvum* β-tubulin gene.

were tested. The LIB13 primers were chosen based on literature with well-documented qPCR methods confirmed by additional analysis methods (Hadfield et al., 2011). With the new primers, successful amplification was obtained for samples containing as few as 3.2 opPCR per PCR in both fecal and PBS matrices (Figure 3-3, 3-4). Melting curve analysis of the PBS and fecal samples were similar to each other with maxima occurring at 75-76°C (Figure 3-4). No non-specific amplification was observed for the LIB13 primers and NTCs had C_q >42 with melting peaks far below the established threshold. Samples containing 320, 32, and 3.2 opPCR are consistent with a linear regression with slope and R^2 of -3.17 and 0.9630 for PBS and -3.59 and 0.9978 for fecal-derived samples (Figure 3-3). The slopes obtained correspond to acceptable qPCR efficiencies (E) of 107% and 90% respectively as calculated from the equation:

$$E = \left(10^{-\frac{1}{slope}} - 1\right) x\ 100\% \qquad \text{(Applied Biosystems, 2004).}$$

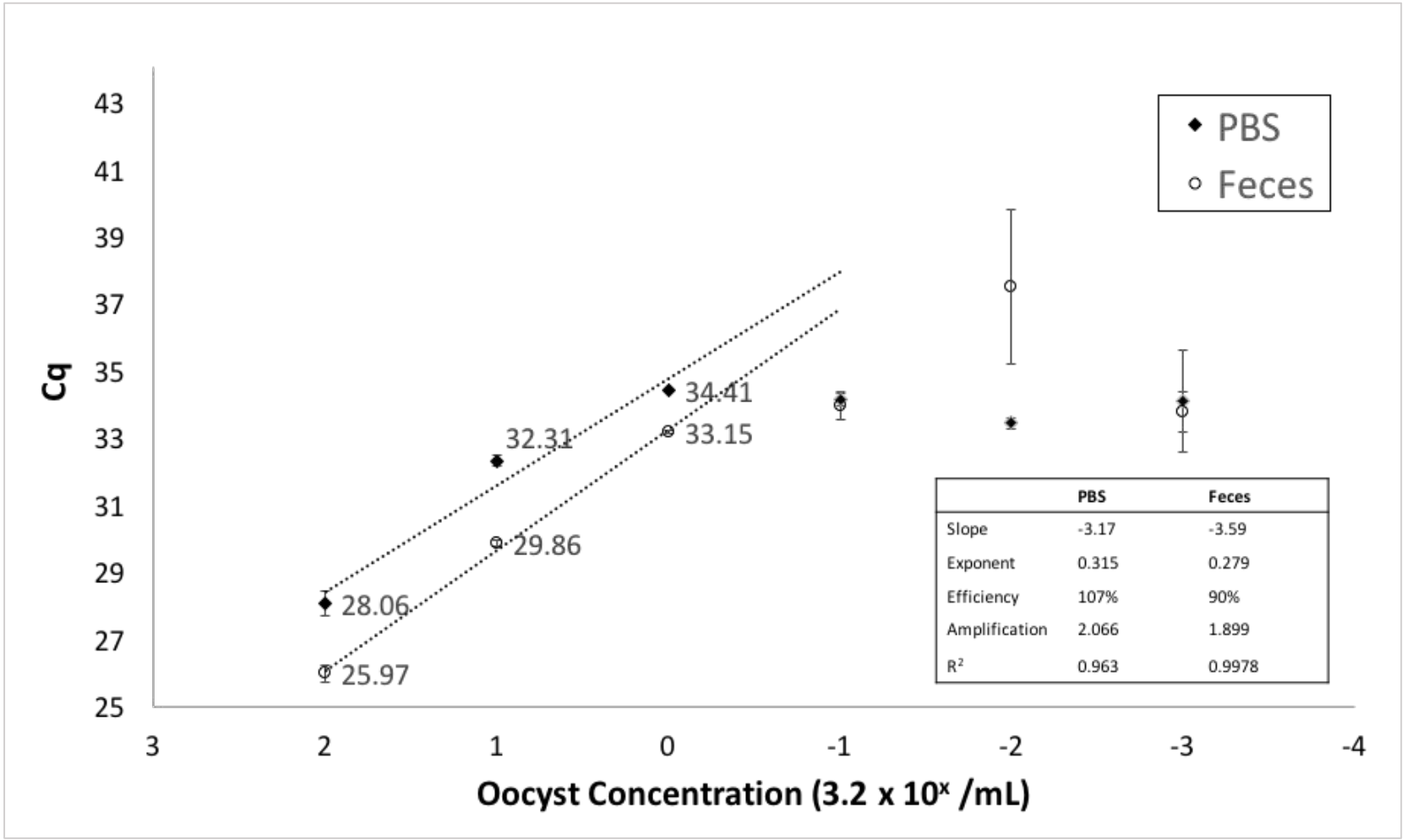


Figure 3-3: qPCR C_q values for dilution series performed on DNA extracted from spiked PBS or fecal material. Oocyst concentrations below 3.2 oocysts per qPCR reaction gave aberrant Cq values and were not included in the efficiency model. n = 3.

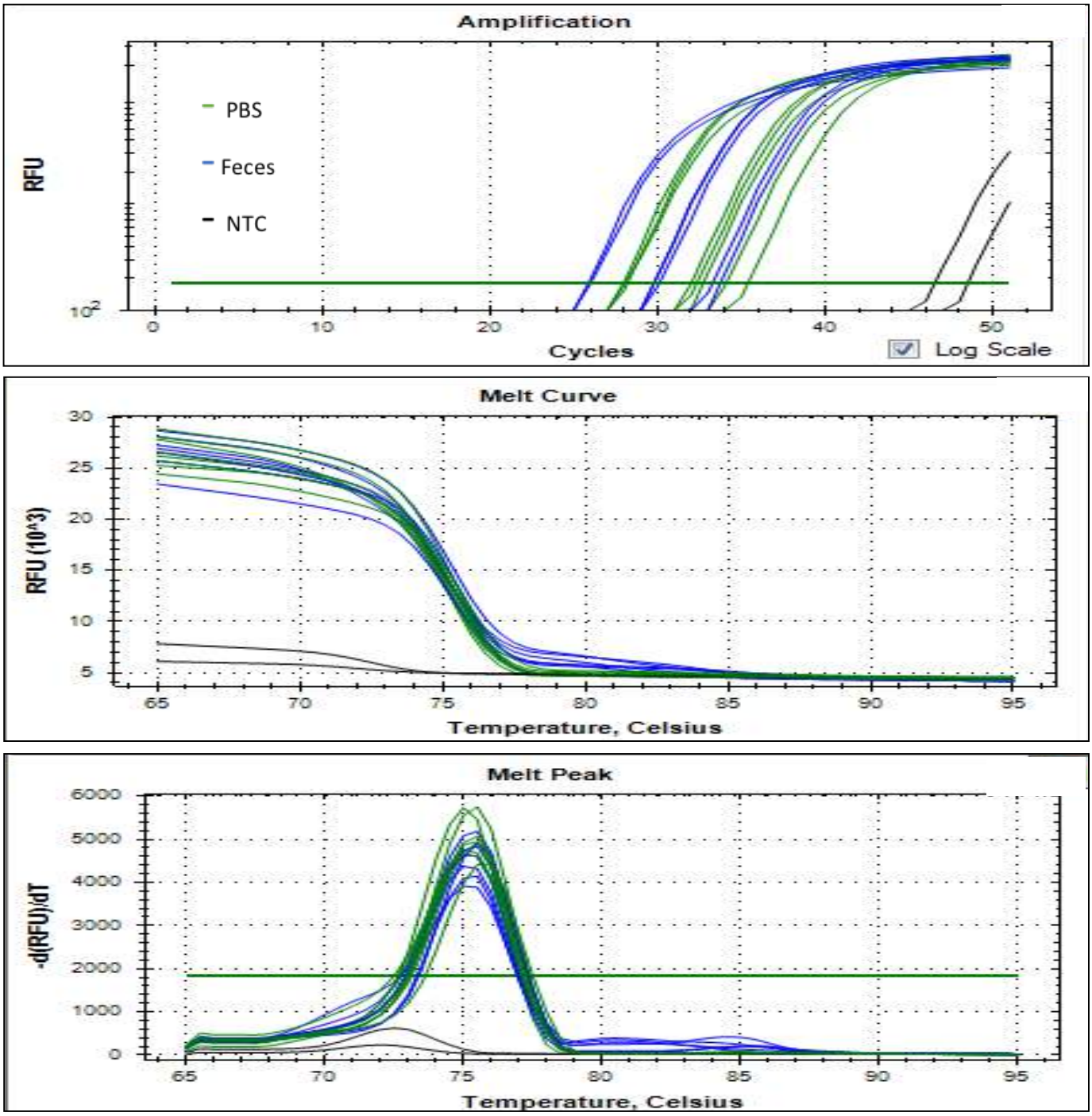


Figure 3-4: qPCR amplification, melt curve and melt peak plots for dilution series of ~320, 32, and 3.2 oocysts/mL in both PBS and fecal material.

An R^2 of 0.9653 suggests minor dilution error or inaccuracies obtained near the limit of detection, but this regression value is acceptable for the purpose of this test. DNA in the PBS suspension showed C_qs that were 1.94 ± 0.28 higher than DNA in fecal suspensions, indicating either a higher initial concentration of DNA in the fecal samples or better removal of PCR inhibitors in the fecal samples. The discrepancy is likely attributed to the DNAextraction process; the fecal sample contained debris that may have assisted in rupturing the oocyst cell wall during bead beating, granting more DNA in the final spin column effluent.

Dilutions containing 0.32, 0.032, and 0.0032 opPCR gave C_qs that were highly inconsistent both with the expected linear regression and within replicates and were therefore omitted in the model (Figure 3-3). It was further supported in several qPCR trials that amplification was unreliable beyond C_q 37 and NTCs often had C_q of 40-42 or had threshold breakthrough beyond tested amplification cycles.

3.3: Discussion

For qPCR, DNA derived from the fecal samples showed amplification efficiency and an R^2 coefficient comparable to DNA derived from PBS samples. PCR inhibition in the fecal samples was therefore minimal, which contrasts with the previously noted concerns raised in the literature. The low inhibition is likely an effect of the Mo Bio Powersoil™ DNA extraction kit's patented Inhibitor Removal Technology® (IRT) (Braid et al., 2003) or lower-than-expected concentrations of PCR inhibitors in the fecal samples. The ability of qPCR to efficiently amplify DNA in feces shows that with proper sample clean-up, the potential of interference by PCR inhibitors is not a concern. Quantification of humic acid substances was not performed on the feces and the actual concentration of inhibitors was unknown. It is

therefore possible that insufficient concentrations of PCR inhibitors were present in the feces. Regardless, qPCR was capable of efficient amplification in bovine feces and can be considered as a reasonable method of monitoring *C. parvum* found in the field.

The observed qPCR detection limit of ~3.2 opPCR, or 350 opg, is consistent with the 2 opg (200 opg) reported by (Hadfield et al., 2011). For direct comparison, the nPCR detection limit I obtained in the present study was 0.45 opPCR (5 opg). Of note, the low opPCR value obtained using nPCR is due to the difference in sample processing in which seeded fecal material was concentrated by pelleting prior to DNA extraction. Accordingly, the opg value should be viewed as a detection limit inclusive of sample processing and PCR whereas the opPCR values should be used to compare the efficacy of the two methods as it reflects only PCR detection capabilities. From the opPCR data, the limit of detection for nPCR was ~7-fold lower than for qPCR. Despite this difference, both methods proved capable of detecting sufficiently low numbers of oocysts and are therefore both legitimate methods for tracking persistence in environmental samples. It should be noted that since the nPCR and qPCR methods used in this study were performed with primers flanking different gene targets amplification efficiency cannot be directly compared. However, the LIB13 primers have been previously shown to have a greater sensitivity than primers flanking the 18S SSU rRNA gene (29/30 vs. 26/30 samples positive for *C. parvum* at less than 4 opPCR). The efficiencies between the LIB13 primers for qPCR and the 18S rRNA gene primers for nPCR are unlikely to be widely different given that both provided similar sensitivities by qPCR. Although direct comparison between nPCR and qPCR efficiencies and sensitivities were not performed in the current study, the ability of either method in detecting low oocyst

concentrations in feces was assessed and qPCR was found to give acceptable and comparable detection limits in both PBS and feces matrices.

Despite the acceptable performance of qPCR and its ability to provide relative quantification data, nPCR was pursued for the summer persistence study. This decision was heavily based on the already-purchased stock of nPCR reagents remaining from the winter persistence study; the high degree of optimization and confidence gained by regularly using nPCR for a year; the higher qPCR costs associated with performing all proposed 324 samples in triplicate--or even duplicate--in addition to standard curves; and an interest to generate data consistent with the nPCR-based winter persistence study. It was recognized that qPCR would have provided better resolution as to the differing persistence between each sample and the shading conditions. However, the priority in this study was determining maximum possible persistence time of *C. parvum*, which depends on the ability to reliably detect the lowest concentration of oocyst DNA.

For the winter and summer study, a spiking volume of 100µL of the *C. parvum* stock solution (yielding 12 opg feces or 1.13 ± 0.29 opPCR) was chosen for the persistence study with consideration of the nPCR limit of detection, costs of spiking 35 fecal pats, concentration of 20.5g fecal material during sample processing, and reduction of potential environmental contamination with biohazardous material.

CHAPTER 4
WINTER PERSISTENCE STUDY

4.1: Summary

The purpose of the winter persistence study was to examine the length of time *C. parvum* will persist in bovine feces under the environmental stressors found during the fall and winter seasons using nPCR as the detection method. In addition to evaluating the persistence time during the seasonal changeover, the study investigates whether different shading conditions affect persistence. Factors such as water content, relative humidity, and temperature were assessed in addition to *C. parvum* persistence to gain insight into which, if any, prominent environmental variables may influence persistence. UV light was not measured during this study because fecal pats were covered with snow for a majority of the study and UV light did not vary considerably between plots during the winter season.

Methodology Overview:

On September 27th, 2014, *C. parvum* spiked fecal pats were placed in fenced plots characterized as heavily, moderately, and lightly forested (section 2.1.1-2.1.4). Samples were withdrawn from each plot at daily, weekly, then monthly time points up to January 10, 2015, and analyzed for presence of *C. parvum* as determined by amplifiable *C. parvum* 18S rRNA gene (section 2.1.5-2.1.8 and 2.1.10). A total of three samples were taken from each plot per sampling day and were each split into three sub-triplicates. These samples were then assayed by nPCR and persistence was reported in terms of the number of fecal pats that contained at least one sub-triplicate positive for *C. parvum*. Temperatures were taken from local meteorological records from nearby facilities (section 2.1.11) and physical characteristics of the fecal pats and snow cover were observed throughout the winter season.

Morphology of freeze-thawed oocysts was evaluated by exposing oocysts to a successive number of freezing and thawing cycles and then monitoring oocyst wall integrity using fluorescence staining and fluorescence microscopy (section 2.1.12). Damaged oocysts and extracted DNA were tested for their potential to give positive results by nPCR (section 2.2.1, 2.1.8, 2.1.10).

Flushing of post-winter fecal pats was performed using the flume device shown in section 2.1.13 in order to test if residual oocysts remained in fecal pats despite no detection by nPCR.

Hypothesis:

Fecal samples in lightly forested plots will show longest *C. parvum* persistence time due to higher average temperatures and fewer freeze-thaw events. Conversely, samples in heavily forested plots will show shortest persistence due to lower temperatures and more frequent freezing and thawing. Due to winter freezing, feces will not harbor oocysts beyond the winter and will not pose a risk during spring freshet.

4.2: Results

4.2.1: Winter persistence of *C. parvum* in differentially exposed bovine feces

Persistence time was monitored until *C. parvum* was no longer detectable by nPCR (LOD at 5 oocysts/g for complete process). Initial sampling was performed at daily intervals to assess any immediate losses in oocysts in the fecal pats and then relaxed to weekly and monthly intervals to minimize expenditures. Because nPCR does not provide oocyst numeration, semi-quantification was performed by tracking the relative proportion of fecal pats positive for *C. parvum* in each plot. A fecal pat with as few as one positive sub-triplicate was reported as a positive fecal pat.

For each shading condition, the proportion of fecal pats positive for *C. parvum* were plotted with respect to exposure time (Figure 4-1). Heavily Forested (HF) and Moderately Forested (MF) plots contained detectable *C. parvum* (1/3 fecal pats) up to 41 days (Nov 6, 2014) of winter exposure but were non-detect at 67 days (Dec 3, 2014). The Lightly Forested (LF) plot condition showed longer persistence with detectable *C. parvum* (1/3 fecal pats) up to 67 days, but were non-detect in future sampling at 106 days. It was observed in the LF plot that fecal samples collected on Nov 6th and Dec 2nd were neither as frozen nor as difficult to collect as the feces from the MF and HF plots. Feces in control patties contained detectable oocysts in 3/3 samples beyond the final sampling time at 137 days.

Regression analysis of the data was not performed due to the wide confidence intervals associated with binomial proportions in data sets with small sample sizes (Figure 4-1). Although it can be visually observed that all three of the environmental conditions have similar persistence and decay of *C. parvum*, a predictive model cannot be ascertained. Statistical comparisons of the persistence observed among plot conditions using either analysis of covariance (ANCOVA) or a generalized linear mixed model (GLMM) is also uninformative due to the high variance. While interpolation of the data is impractical, the maximum persistence time of *C. parvum* during winter exposure can at least be deduced, which was the core objective of the study.

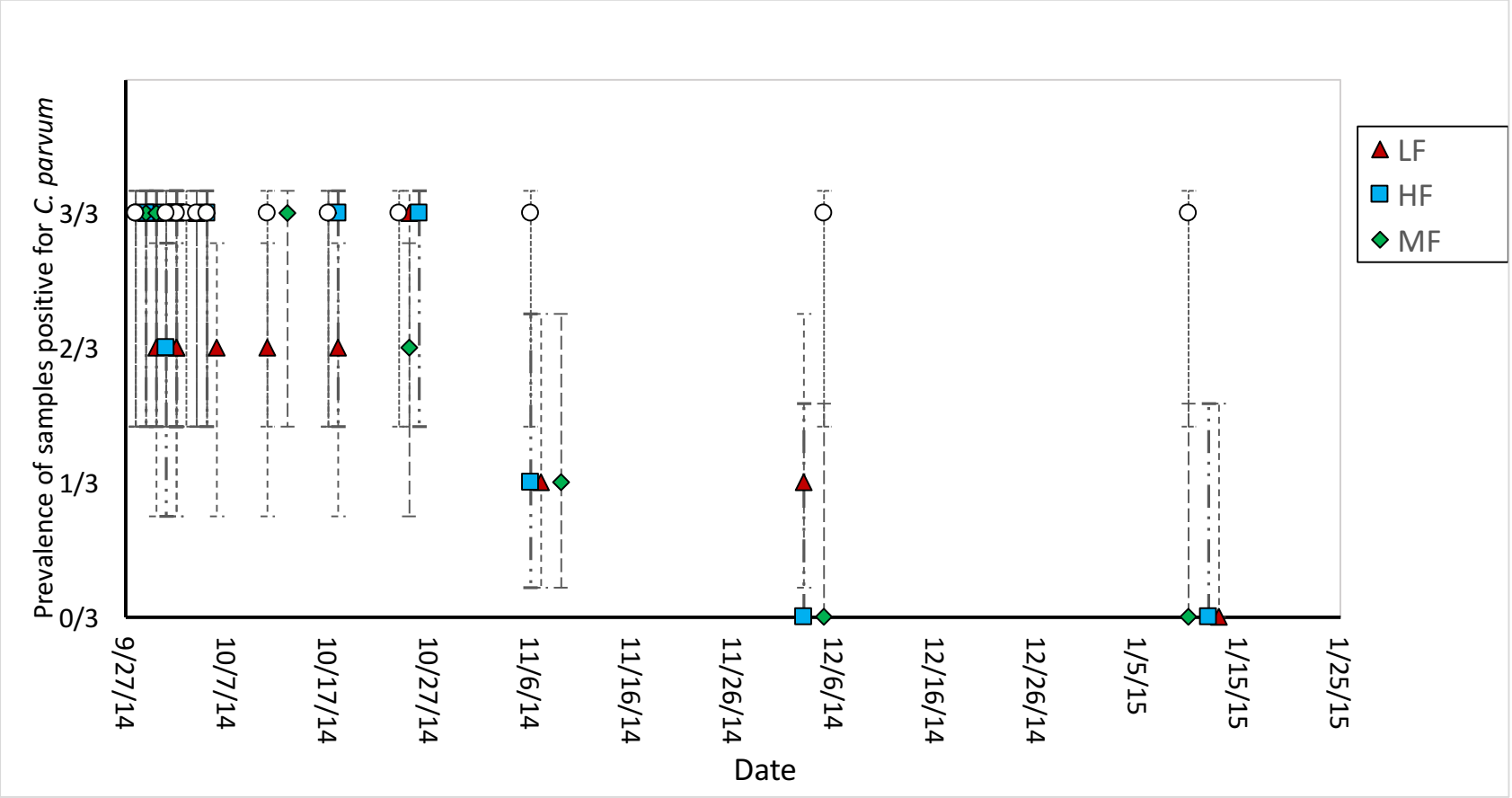


Figure 4-1: *C. parvum* persistence in winter exposed fecal pats under different shading conditions (n = 3). Error bars represent 90% binomial proportion confidence intervals using Agresti-Coull approximations.

4.2.2: Meteorological fluctuations during winter investigation

Temperature records from Kelowna International Airport (located 1.87 km from study plots) show first signs of freezing on November 2, 2014 (37 days of exposure to samples) (Figure 4-2). Temperatures manually taken at each plot at 1:00PM during the first two months of the persistence study showed that the average ambient temperature at the HF plot was 2-5°C lower than the LF plot. The average MF plot temperature was between these values, as expected. Four freeze-thaw events (in which temperatures fell below -3°C and above +3°C) were observed in November, which may have reduced the presence of *C. parvum.* Of interest is the reduction in oocyst persistence as observed between 28 and 41 days of exposure (October 24, 2014 to November 6, 2014) (Figure 4-1). Within this time, a single freeze-thaw event occurred which may be responsible for the rapid drop in samples positive for *C. parvum* (HF$\bar{x}$: 3/3 to 1/3, MF$\bar{x}$: 2/3 to 1/3, LF$\bar{x}$: 3/3 to 1/3). However, during this time span a snowfall event occurred and may have provided insulation and protection against freeze-thaw events. Snow may have dampened the thermal fluctuations that occur

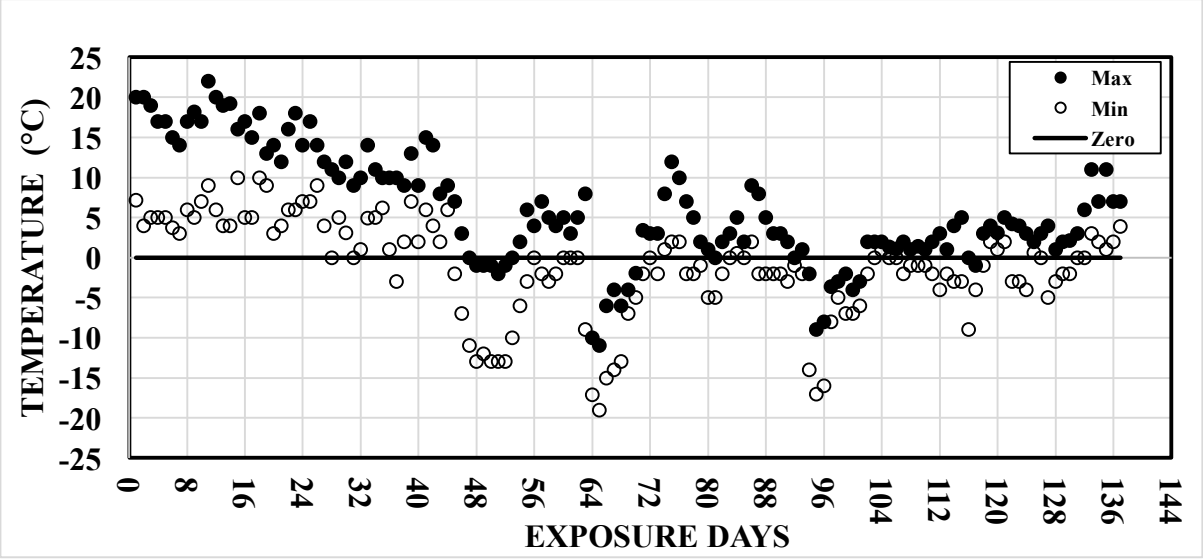


Figure 4-2: Local maximum and minimum temperature values at the persistence sites during the winter studies (day 1 = September 27, 2014).

throughout the winter and could be a factor in prolonging oocyst survival. Snow accumulation was greatest on the LF plot (maximum of 46 cm) due to lack of natural cover and was lowest in the HF plots (maximum of 22cm). This observation may explain the slightly longer detection of oocysts in the LF plot during the winter, although the difference in persistence time could be attributed to natural variance in the fecal pats.

It was observed during the day 42 and day 67 sample collection that fecal pats in the LF plot remained only moderately frozen and were easier to extract than pats in either HF or MF plots. The ease of collection may have been attributed to the slightly higher temperatures and greater coverage of snow in the LF plot that likely insulated the fecal pats from colder air temperatures. The higher local temperatures and insulation in the LF plot are noted as potential reasons for the potentially longer persistence time in this meadow-like environment and deserves further investigation.

Since snowfall was the predominant form of precipitation and the potential direct leaching of oocysts from the fecal pats was considered an unlikely event, supplementary research was directed towards the influence of freeze-thaw events. *C. parvum* leaching studies have been previously explored by (Tate et al., 2000), but these studies were performed using direct rainfall on soft, fresh fecal pats. In contrast, prior to the first recorded precipitation event in our study all fecal pats had formed light crusting of ~2-4mm and were then covered by snow and remained frozen for a majority of the study.

4.2.3: Oocyst morphology after freeze-thaw

It was postulated that the reduced persistence observed after the first freeze-thaw could be attributed to the integrity of the oocyst wall after freeze-thaw cycles. Immunofluorescence microscopy on FITC- and DAPI-stained *C. parvum* oocysts after

successive freeze-thaw cycles (15 minutes -80°C, 15 minutes 70°C) revealed visible deformation and rupturing of oocyst cell walls as indicated by excystation or empty oocyst shells (Figure 4-4). Oocysts were categorized as intact (DAPI contained within ovoid-shaped FITC-stained oocyst wall), deformed (discontinuous and/or jagged oocyst wall), or ruptured (no DAPI inclusion inside of oocyst and a severely deformed oocyst wall). Prior to freezing and thawing, the oocyst sample consisted of 67% intact oocysts, 18% damaged, and 15% ruptured (Figure 4-4). The proportion of damaged and ruptured oocysts increased after successive thermal cycling and after exposure to three thermal cycles, 41% of the oocyst population was lost. Of the remaining oocysts, only 19 ± 3% contained fully intact cell wall and visible nuclei. Based on the progressive decrease in intact oocysts with respect to thermal cycles, it is expected that additional thermal cycles would result in even fewer intact oocysts. The complementary increase in deformed oocysts shows that the effect of freeze-thaw does not immediately lyse the oocyst but may instead weaken the oocyst wall. Irregular values for ruptured oocysts were due to the difficulty in differentiating completely ruptured oocysts from fluorescent debris.

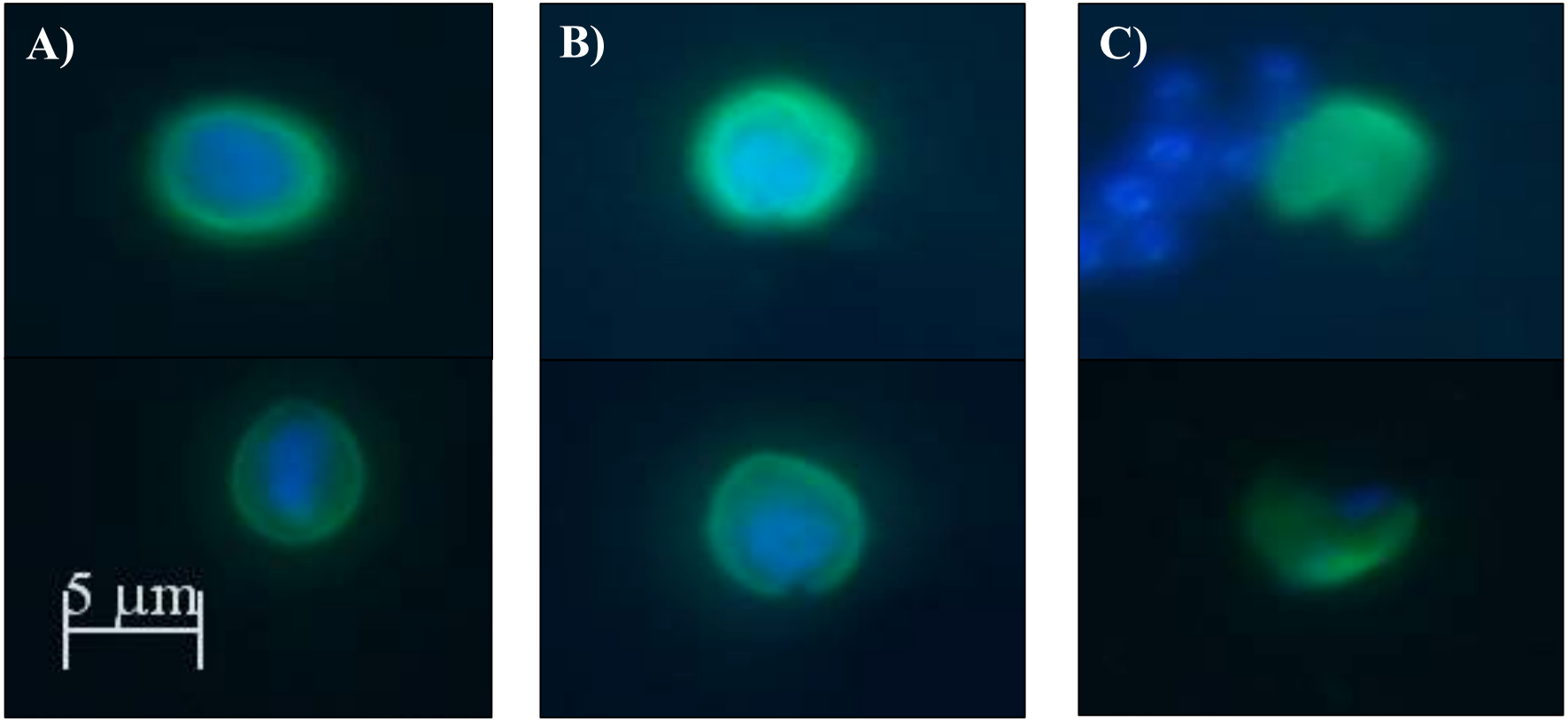


Figure 4-3: Epifluorescent images of FITC-mAb immunofluorescence and DAPI stained *C. parvum* oocysts at 100x magnification. Oocysts characterized as A) intact B) deformed C) ruptured.

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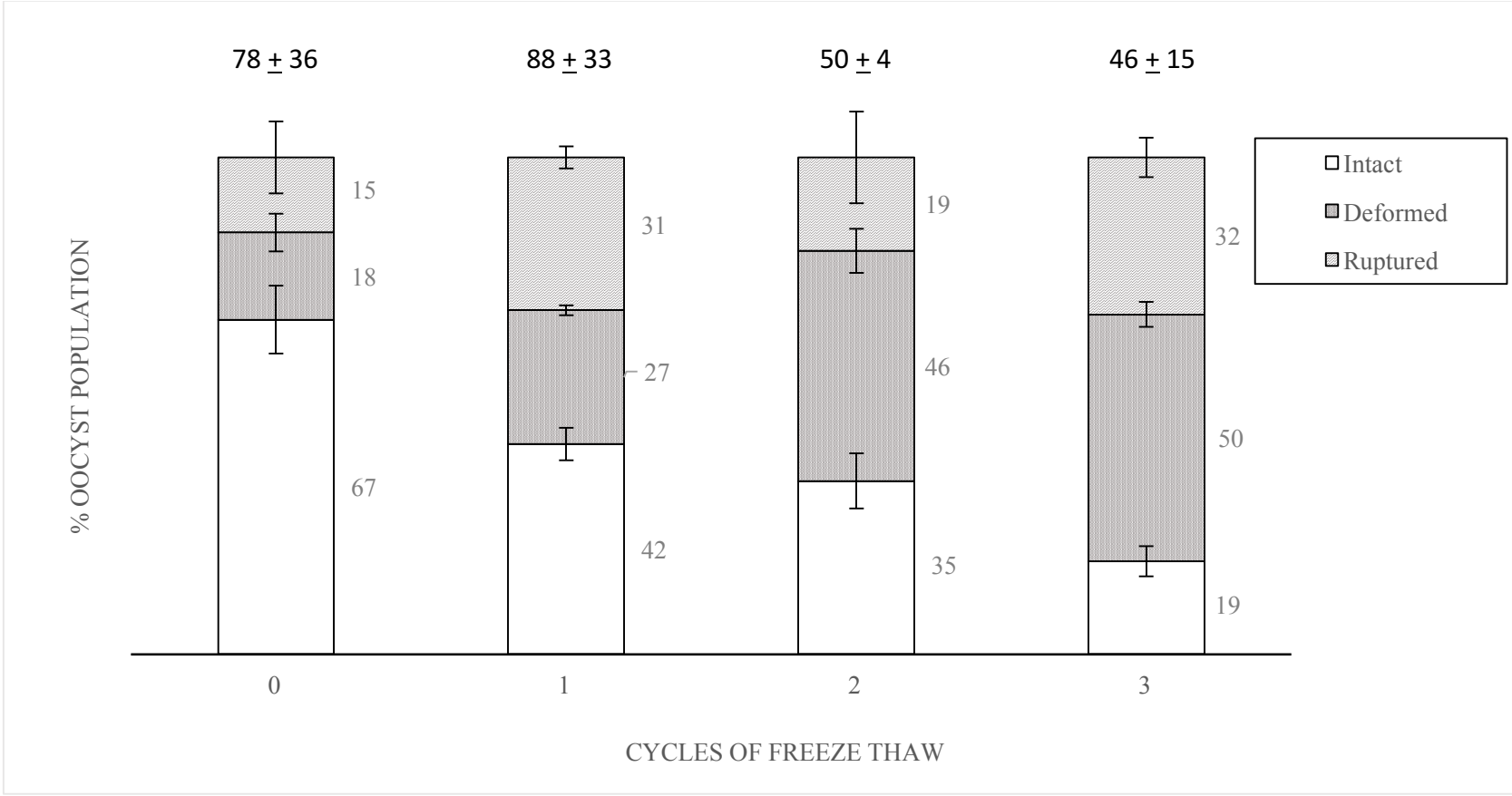

Figure 4-4: *C. parvum* oocyst morphology after 0, 1, 2, or 3 freeze-thaw events. Oocysts were classified as intact, deformed, or ruptured and represented as a percentage of the total number of oocysts counted. Average oocyst count and standard deviation (n = 3) reported above columns.

4.2.4: Detection of DNA from ruptured oocysts

The possibility of lysed oocysts or naked *C. parvum* DNA yielding positive results by nPCR was investigated by testing the persistence of thermally ruptured oocysts and *C. parvum* DNA spiked into autoclaved feces compared to the persistence of intact oocysts (see controls section 2.2.1). Fecal pats containing either freeze-thaw damaged *C. parvum* oocysts or DNA extracted from *C. parvum* showed remarkably shorter persistence compared to feces containing intact oocysts (Table 4-2). Freeze-thaw ruptured oocysts were rendered non-detectable within 48 hours of spiking whereas DNA spiked samples were non-detectable within one hour. Interestingly, 1/6 of the DNA-spiked fecal samples were positive at 168 hours despite previous absence. The single positive result may have been a result of contamination generated during the nPCR amplification process or a single stray oocyst in the feces which may have resisted oocyst removal procedures.

Table 4-1: Persistence of differentially treated *C. parvum* (intact, ruptured via freeze-thaw cycling, or extracted DNA) spiked into autoclaved bovine feces at 4°C.

	EXPOSURE (HOURS)			
	1	**48**	**96**	**169**
FECAL SPIKE	**Samples positive for *C. parvum*/total samples**			
FREEZE-THAW OOCYSTS	2/6	0/6	0/6	0/6
OOCYST DNA	0/6	0/6	0/6	1/6
UNDISTURBED OOCYSTS	9/9	9/9	9/9	9/9

4.2.5: Flushing of exposed fecal pats

Cow feces deposited near streams have the possibility of becoming submerged or flushed with snowmelt, potentially increasing the risk of oocyst transport from feces into

drinking water sources. The risk of such hydrological events on *C. parvum* transport and the characteristics of the feces affecting this risk was tested on the remaining fecal samples from the winter persistence study in an effort to emulate the effects of spring freshet on fecal pats deposited at the end of fall grazing period.

Although the fecal samples that were subjected to winter exposure were not detectable by nPCR after 106 days, the fecal pats were collected from the sampling site on May 14th for leaching potential. Neither nested PCR or EPA Method 1623 revealed any presence of *C. parvum* in any of the flushed fecal pats. A higher flushing rate was not tested since preliminary attempts resulted in movement of the feces down the flume or breaking of the fecal pats.

4.3: Discussion

The core finding in the winter study is that in all of the environmentally exposed plots, oocysts were non-detectable prior to 106 days of exposure. Superimposing these findings on an annual water level plot (Figure 4-5) provides context to winter oocyst persistence near British Columbia watersheds. Oocysts deposited as late as September 27th do not remain in the fecal pats into spring freshet (equivalent to approximately 186 days of exposure), suggesting that fall-deposited feces near riparian zones are of minimal risk during high spring riparian flow events. This finding was further supported by the lack of detectable oocysts in effluent after five hours of water flushing on the fecal pats collected after winter exposure.

Control studies in which spiked fecal pats were stored at 4°C in a refrigerator contained detectable *C. parvum* in 3/3 samples beyond 137 days, indicating that control

samples are far less susceptible to oocyst loss, either by physical transportation or by oocyst death, than environmental samples. Persistence observed for the control sample is

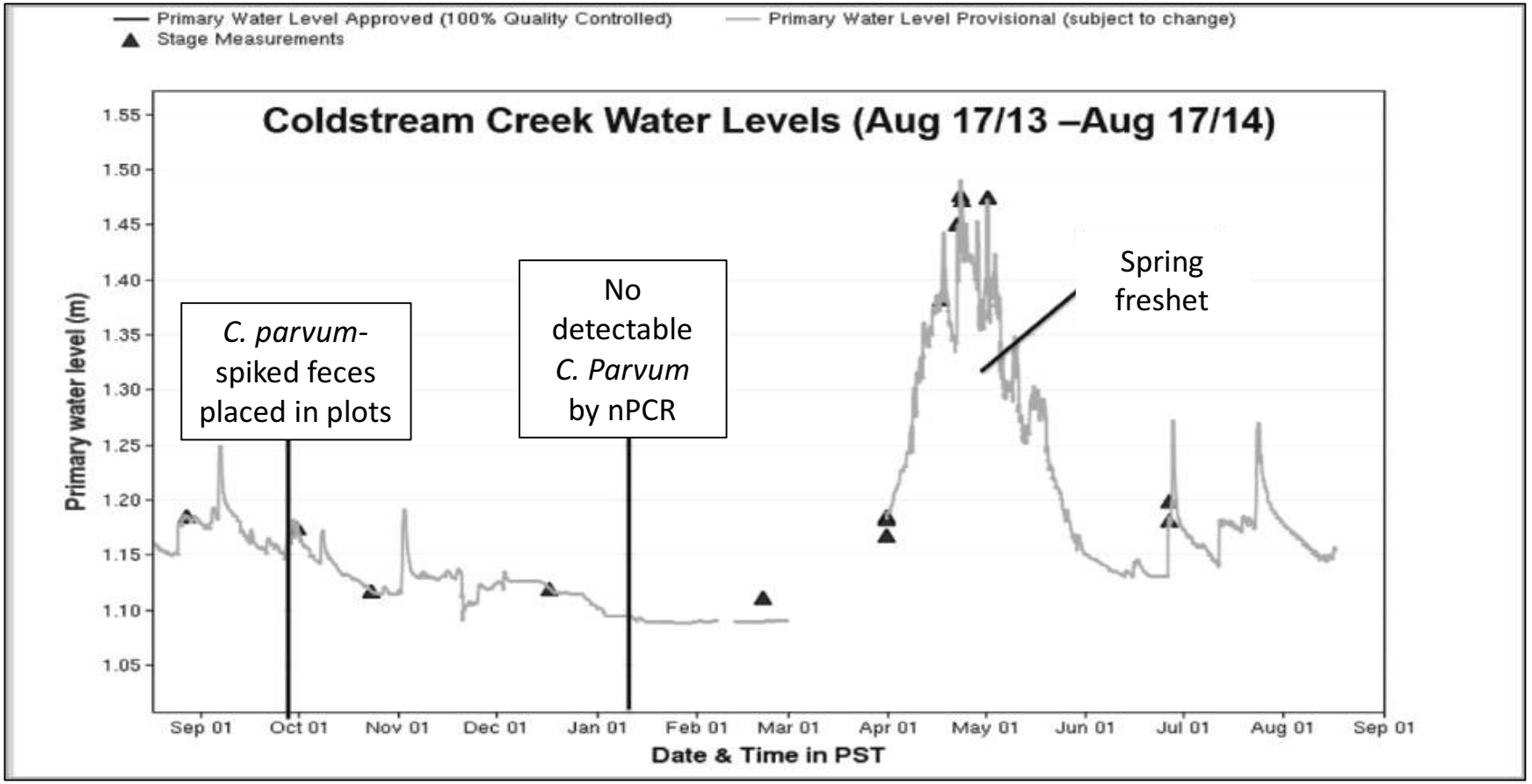

Figure 4-5: 2014 Real-time hydrometric data obtained from Environment Canada Water Office for Coldstream Creek, BC (intake 08NM142). Annotations were added to indicate date of inoculation and loss of *C. parvum* detection.

comparable to values observed in the literature for oocysts in fecal material at 4°C (Peng et al., 2008). Control samples were free of UV light, temperature swings (e.g. freeze-thaw events), water-facilitated transport, and other environmental exposures that may directly impair oocyst persistence.

From the winter persistence study only limited environmental parameters were collected, including overall temperature (not plot specific), precipitation, plot snowfall depth, and fecal consistency. From the limited meteorological data, it cannot be ascertained as to which environmental factor(s) most affected *C. parvum* persistence. It can only be concluded that *C. parvum* in feces exposed to the collective meteorological factors of the Okanagan winter were rendered non-detectable by nPCR by 106 days in all shading conditions. The snow coverage observed at all of the environmental plots likely affected the fecal pats in all plots similarly, potentially shielding the oocysts from direct exposure of UV light and wind chill, therefore buffering the factors that may differ among plots.

From the literature, freeze-thaw cycles are well cited as a method of lysing *Cryptosporidium* oocysts and were proposed as a factor on oocyst decay in the winter-exposed feces due to the observed temperature fluctuations. The supplementary investigation of the influence of freeze-thaw cycles on oocyst morphology (Figure 4-3, 4-4) revealed that as many as three freeze-thaw events did not sufficiently rupture the cell walls of 19% of the observed oocyst population. Although these remaining oocysts appeared intact and incorporated both FITC and DAPI stains, their viability could not be confirmed by microscopy. Oocysts subjected to freeze-thaw cycles were detectable by nPCR in two of the six tested samples after 1 hour of fecal exposure but were not detectable by 48 hours. The weakened oocyst wall may act as a temporary protective carrier for DNA during sample

processing, but prolonged exposure liberates DNA to the fecal environment and genomic DNA in thawed feces has been shown to fragment after as few as 24 hours at room temperature (Cardona et al., 2012). DNA spiked into feces was not detectable at room temperature after 1 hour, which was expected due to the nature of the sample processing method in which fecal material in PBS was centrifuged at 3000 × g, resulting in discard of free DNA within the supernatant. The likelihood of ruptured oocysts or residual oocyst DNA yielding long-term positive results in the field samples was therefore low due to the digestion or denaturation of free DNA by the fecal environment (Wiedenmann et al., 1998). Accordingly, the persistence data from field samples subjected to extended environmental exposure predominantly reflected intact oocysts capable of protecting cellular DNA. The detection of *C. parvum* via a simplified sample preparation and nPCR is dependent on oocyst wall integrity; weakened oocysts may provide positive results by nPCR if they are processed immediately, but exposing weakened oocysts to feces at or beyond 48 hours appears to exacerbate the decay of DNA from the oocyst and render the damaged oocysts non-detectable.

The winter persistence of *C. parvum* in feces and in soil has been investigated prior to this study using a vital dye permeability assay and sentinel chambers in New York and Norway (Jenkins et al., 1999; Robertson and Gjerde, 2004). However, these studies differ from the current study since they investigated the survivability of oocysts in piled manure or in soil, not in fecal pats. Additionally, neither study investigated the influence of different shading environments on persistence. Regardless, these studies serve as a reference for persistence time in similar conditions. Jenkins et al. (1999) detected viable oocysts up to 140 days in winter manure piles, which provided an insulated environment ranging from 5°C to

30°C from November 1996 to May 1997 (Jenkins et al., 1999). Significantly shorter persistence times were expected in the present Okanagan study due to colder temperatures (as low as -19°C) and a greater number of freeze-thaws affecting the exposed 1kg fecal pats. In the study by Robertson and Gjerde (2004), oocyst persistence was assessed during the Norwegian winter with sentinel chambers containing only 0.8g of *C. parvum*-inoculated feces, which were then covered with soil. Neither study assessed persistence in a naturally-sized fecal pat nor with oocysts in direct contact with the environment. Additionally, both Jenkins et al. (1999) and Robertson and Gjerde (2004), as well as my own preliminary work, have recognized that the recovery and detection of trace oocysts in environmental samples can be difficult via vital dye and immunofluorescence assays (IFAs) due to opaque detritus and cross-reactivity (Robertson et al., 1998). Direct comparisons between IFA approaches and nPCR have confirmed that nPCR is less likely to misreport *Cryptosporidium* oocysts due to cross-reactivity (Mayer and Palmer, 1996). Both the aforementioned vital dye studies show that using the vital dye assay, oocyst viability was lost between 75 and 140 days of winter exposure. Despite the differences in methodology, the observed oocyst persistence time of 67 to 106 days determined by nPCR is comparable to previously obtained vital dye results.

CHAPTER 5
SUMMER PERSISTENCE STUDY

5.1: Summary

Spring and summer environmental conditions differ greatly from fall and winter conditions in the Okanagan, particularly in terms of average temperature, UV exposure, and precipitation. In sunny, dry areas, summer ground temperatures can reach pasteurization temperature (~72°C), potentially rendering *C. parvum* noninfectious within five seconds (Harp et al., 1996). Additionally, summer UVAB light exposure often exceeds over 10mJ cm^{-2}, which is sufficient to permanently inactivate *C. parvum* oocysts (Mofidi et al., 2001). Shading factors and fecal shielding are therefore expected to play a grander role in *C. parvum* persistence during the summer due to the greater sensitivity of the oocysts to UV light and high temperatures observed in open, meadow-like areas on grazing land. Information as to the impact of shading and the associated environmental factors on oocyst persistence in bovine feces can shape current grazing practices by identifying high risk sites on crown land. Understanding these sensitive areas is essential for the development of accurate BMPs that best mitigate pathogen transport risk from livestock to drinking water.

The purpose of the summer study was to evaluate the persistence time of *C. parvum* under the harsher sun exposure and warmer, drier climate observed during the summer. Humidity, moisture content, UV exposure, dew point, and temperature were recorded in addition to *C. parvum* persistence to gain insight into which, if any, environmental variables significantly correlate with a decline in *C. parvum* persistence. This study also provides information on the rate of loss of extractable DNA during summer months and the environmental conditions that favor/disfavor oocyst survival. Three different shading

conditions (identical to those used during the winter persistence study) were observed to evaluate the influence of common environmental scenarios observed in Okanagan Crown Land on *C. parvum* persistence in feces.

Method Overview:

Similar to the winter persistence study (Chapter 4), *C. parvum* spiked fecal pats were placed in different outdoor plots characterized as heavily, moderately, and lightly shaded (methods 2.1.1-2.1.4). Fecal pats were deposited on June 23rd, 2015 and collected on daily, weekly, monthly, and then intermittently until March 19th, 2016, which extends through both summer and winter exposures. Samples were analyzed for presence of *C. parvum* as determined by amplifiable *C. parvum* 18S rRNA gene (section 2.1.5-2.1.8 and 2.1.10). A total of 9 replicates from each plot per sampling day were tested and persistence was reported in terms of the proportion of the samples positive for *C. parvum.* Temperatures were taken both from local meteorological records from nearby facilities and using plot-specific data-loggers that also collected relative humidity and dew point (section 2.1.11). UVAB light was measured during sampling visits to evaluate relative UV intensities among plots (section 2.1.11). Gravimetric weights were also obtained for every fecal pat sampled in order to assess relative desiccation of fecal pats throughout the summer. Physical characteristics of the fecal pats and snow cover were observed throughout the summer and winter seasons.

Hypothesis:

Fecal samples under heavily forested conditions will show longest *C. parvum* persistence times due to relatively less UV exposure and lower average temperatures. All samples will show shorter persistence times than those observed during the winter persistence study due to higher UV exposure and average temperatures.

5.2: Results

5.2.1: Summer persistence of *C. parvum* in differentially exposed bovine feces

From fecal pats deposited on June 23rd, 2015, oocysts were no longer detectable in the LF plot by the end of the summer, on September 1st, 2015 (71 days total persistence time) (Figure 5-1). However, on this date 3/3 of the fecal pats in the HF condition and 2/3 of the fecal pats in the MF condition still contained *C. parvum*. An additional sample collection at 227 days (February 4, 2016) was performed to verify loss of the oocysts over the winter as suggested by the winter persistence data. Interestingly, on day 227 both the HF and MF plots contained *C. parvum* despite winter exposure (2/3 and 1/3 positive fecal pats, respectively). During collection of these samples it was observed that fecal pats in the heavily and moderately forested plots were covered by 4-10 cm of snow whereas winter samples collected the previous year were free of snow cover. Another sampling of fecal pats was performed at 271 days (March 19, 2016) to understand whether the final snow melt and potential thawing of oocysts reduced the presence of oocysts from the fecal pats. At this point, no oocysts were detected in the fecal pats from the MF plot, but 1/3 fecal samples from the HF plot remained positive.

As noted in the winter persistence study results, regression analysis of the data was not performed due to the wide confidence intervals associated with binomial proportions in data sets with small sample sizes. A visual inspection of the scatterplot (Figure 5-1) shows that the LF plot instigated decay much sooner than the other two plots and the control.

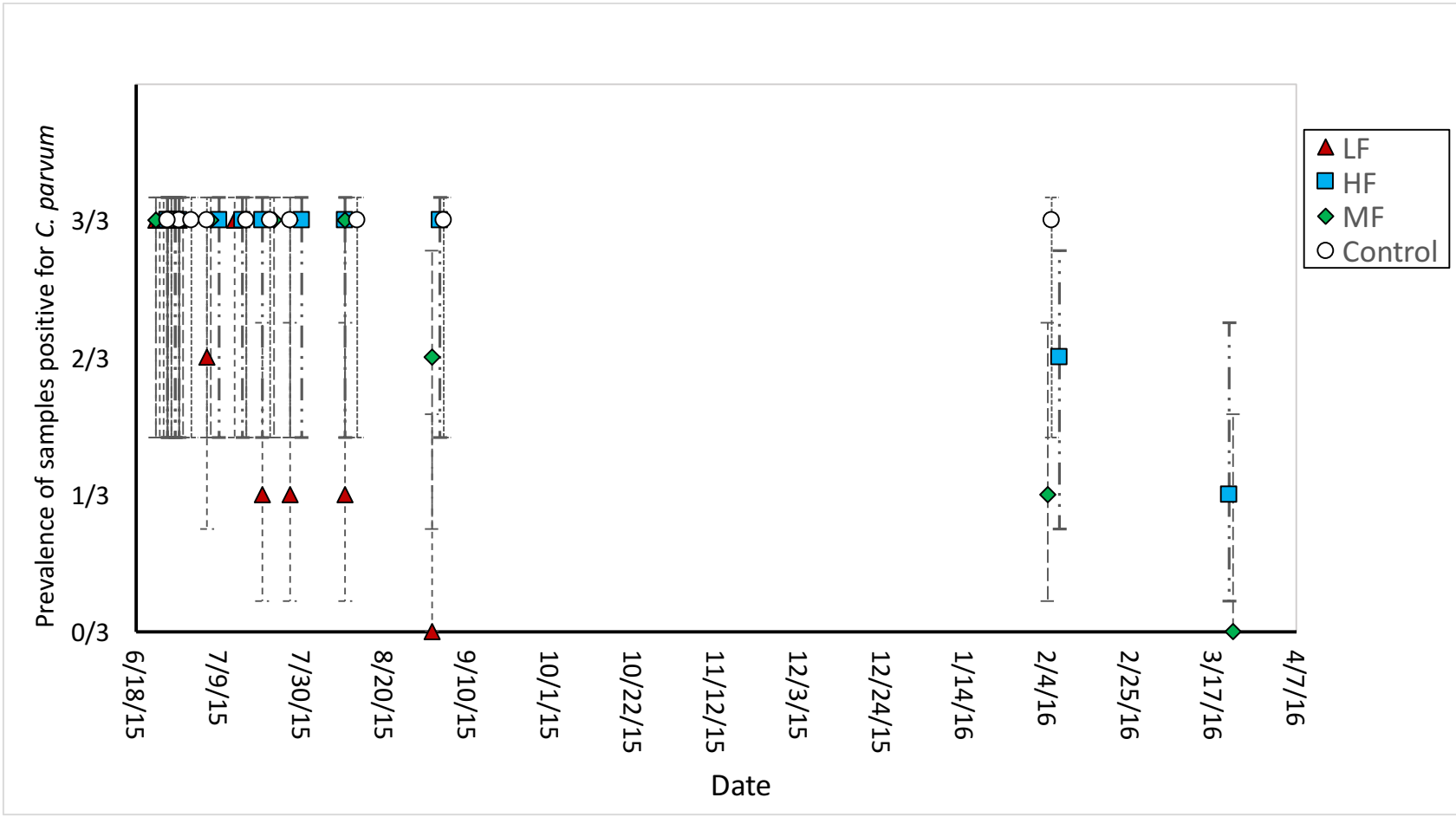

Figure 5-1: *C. parvum* persistence in winter exposed fecal pats under different shading conditions (n = 3). Error bars represent 90% binomial proportion confidence intervals using Agresti-Coull approximations.

5.2.2: Meteorological fluctuations during summer investigation

Fecal pats subjected to higher temperatures and greater UV exposure showed a more rapid decline in detectable oocysts than fecal pats in the shaded conditions. The LF meadow-like plot experienced highly unfavorable conditions for *C. parvum*, including ground temperatures up to 65°C, frequent UV exposure above 10,000 $\mu J/cm^2$, and a decrease in water content to <4% (w/w) within 14 days (Figure 5-2, 5-3). Conversely, the HF plot had consistently lower temperatures and lowest UV exposure (average intensity <1,000 $\mu J/cm^2$). Water content in these fecal pats fell as low as 5% (w/w) by 14 days and remained near 8% for the remainder of the study, which was attributed to the shielding of rain by the shading tarp. As expected, the MF condition consistently showed average UV intensities of 3,000 $\mu J/cm^2$, which falls between the UV intensities observed in the HF and LF plots. Feces in the MF plot had higher water content for a majority (days 20 to 71) of the study due partially to its milder sun exposure compared to the LF plot as well as the absence of a shading tarp. Other meteorological factors such as dew point and humidity were similar among the conditions. The HF plot had consistently narrower and lower range of relative humidity and dew point (Figure 5-3), which coincides with the low fecal moisture content observed during gravimetric weighing (Figure 5-2).

Throughout the course of the study, very few precipitation events occurred and the potential of water transport was considered low. Considerable rainfall (~13.8mm) occurred between 32-34 days of exposure and the potential influence of this rainfall event on oocyst leaching was addressed by sampling the following day (day 35 data point). No drastic decrease in samples positive for *C. parvum* occurred compared to the previous week suggesting that the rainfall did not transport considerable amounts of oocysts from the feces.

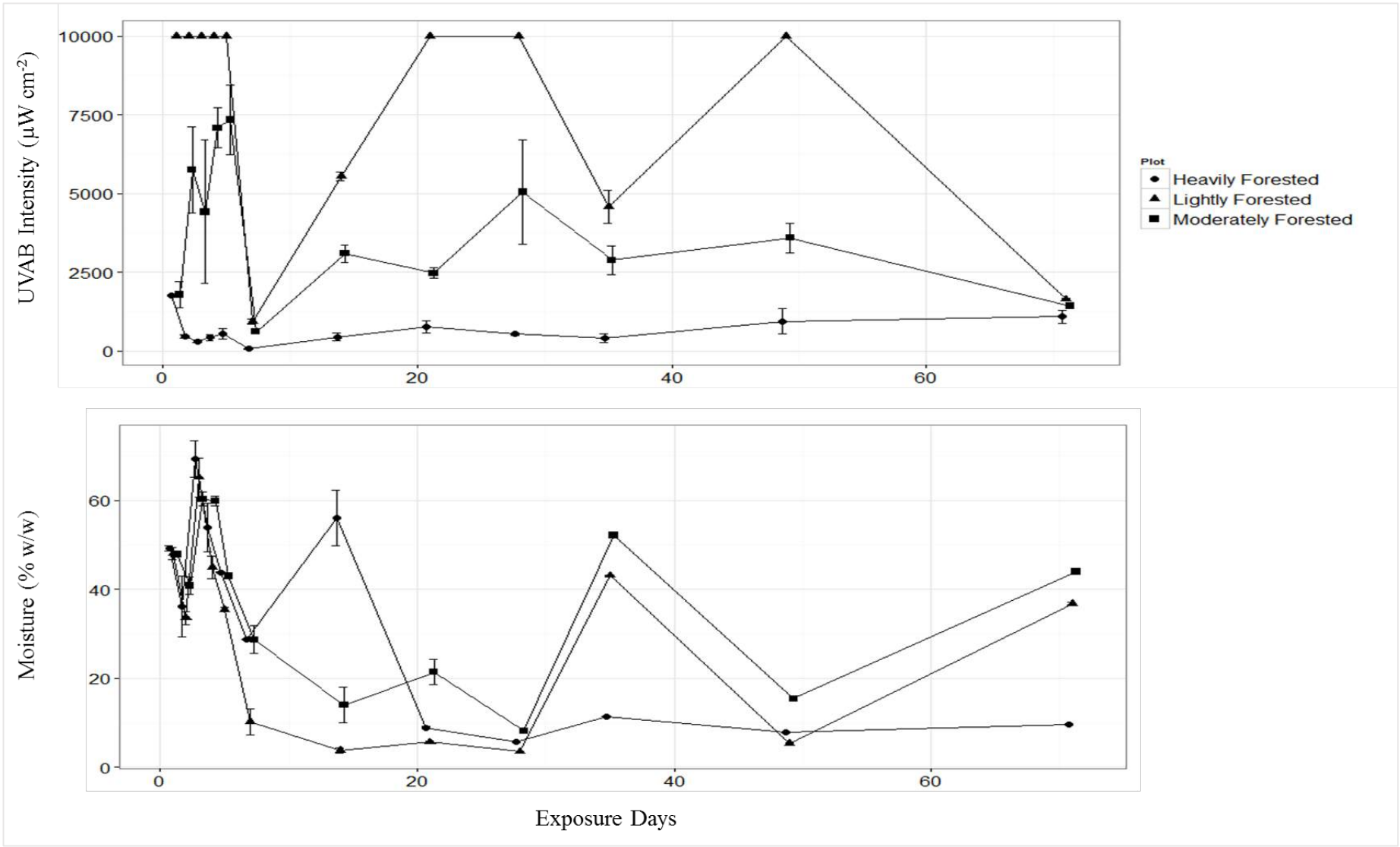

Figure 5-2: UVAB intensity and gravimetric fecal moisture (%w/w) as observed at each plot during sampling days from June 26, 2015 (day 1) to September 1, 2015 (day 71).

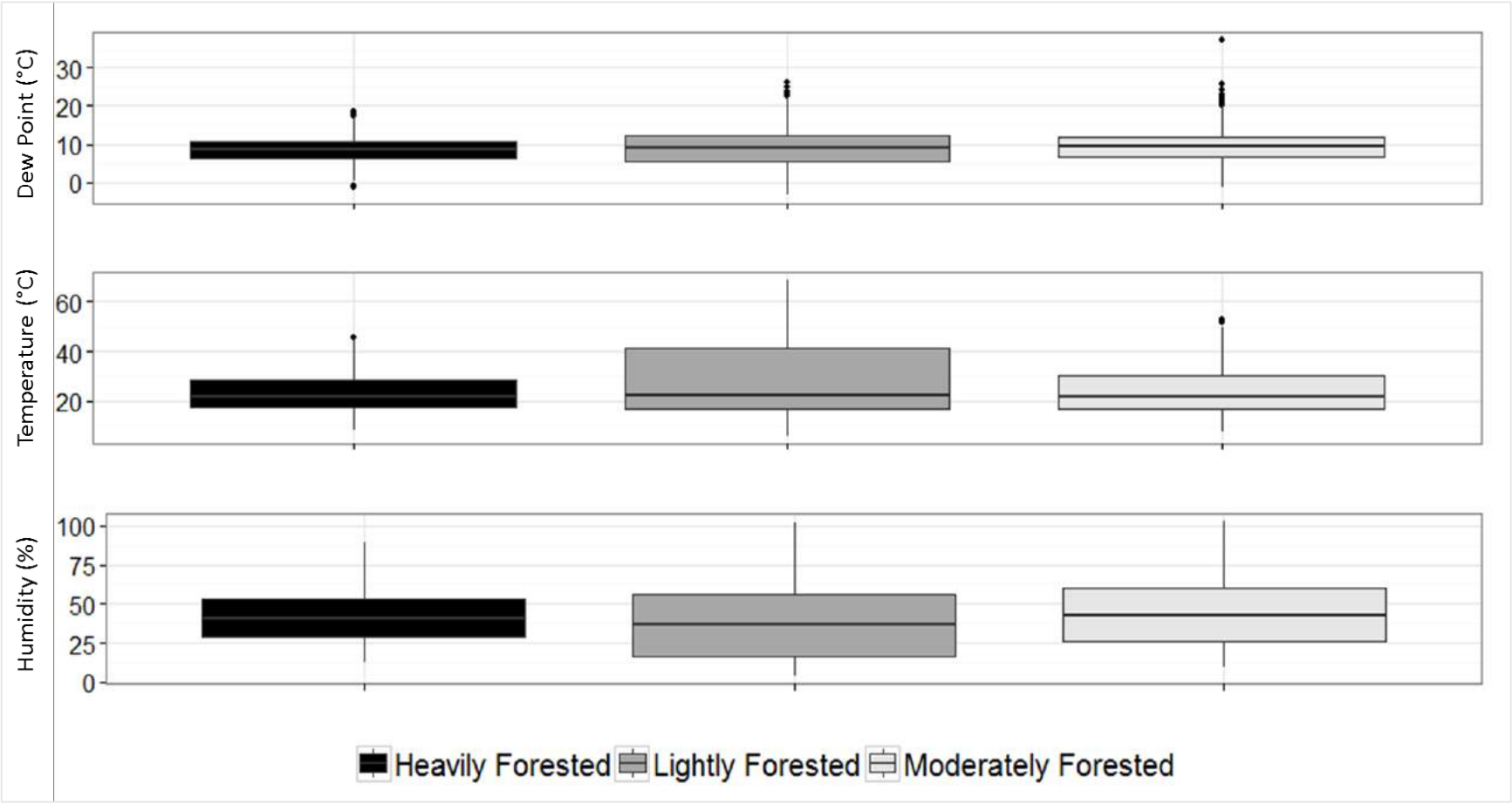

Figure 5-3: Boxplots of data logger recordings for dewpoint, temperature, and humidity at each of the three shading conditions. n = 1659 from June 26, 2015 to September 2, 2015.

This discovery is supported by previously performed rainfall studies on comparably vegetated plots (Davies et al., 2004). The HF plot received lowest amount of precipitation, either in form of rain or snow, due to the shading tarp.

5.3: Discussion

It was evident from the LF summer persistence data that excessive solar exposure in poorly shaded plots impairs oocyst longevity, implicating UV light as an antagonist of persistence. In plots shaded from UV light, it is possible that mild desiccation and fecal crusting may be effective in cryopreserving *C. parvum* oocysts and this phenomenon requires further investigation.

It was surprising to observe that seeded feces deposited in shaded plots during the summer show persistence beyond both the summer and the winter seasons. Annotating the summer findings on an annual water level plot (Figure 5-4) shows that *C. parvum* persistence in the LF plot does not extend into spring freshet. In contrast, the MF conditions may foster *C. parvum* up to early spring freshet and could pose a risk to nearby water resources if BMPs and grazing practices are not adequately implemented. The HF conditions fostered *C. parvum* oocysts in feces beyond the start of early spring freshet and may require more thorough monitoring than *C. parvum* infected feces in the MF and LF environments. From the summer study, it was determined that lightly forested conditions such as a those found in meadows discourage oocyst persistence due to the extremely high ground temperatures and high ultraviolet light exposure. Shaded conditions better foster oocysts during the summer and may even prolong persistence by creating a dry fecal crust that shields oocysts from UV and/or ice crystals that form during winter freeze-thaw.

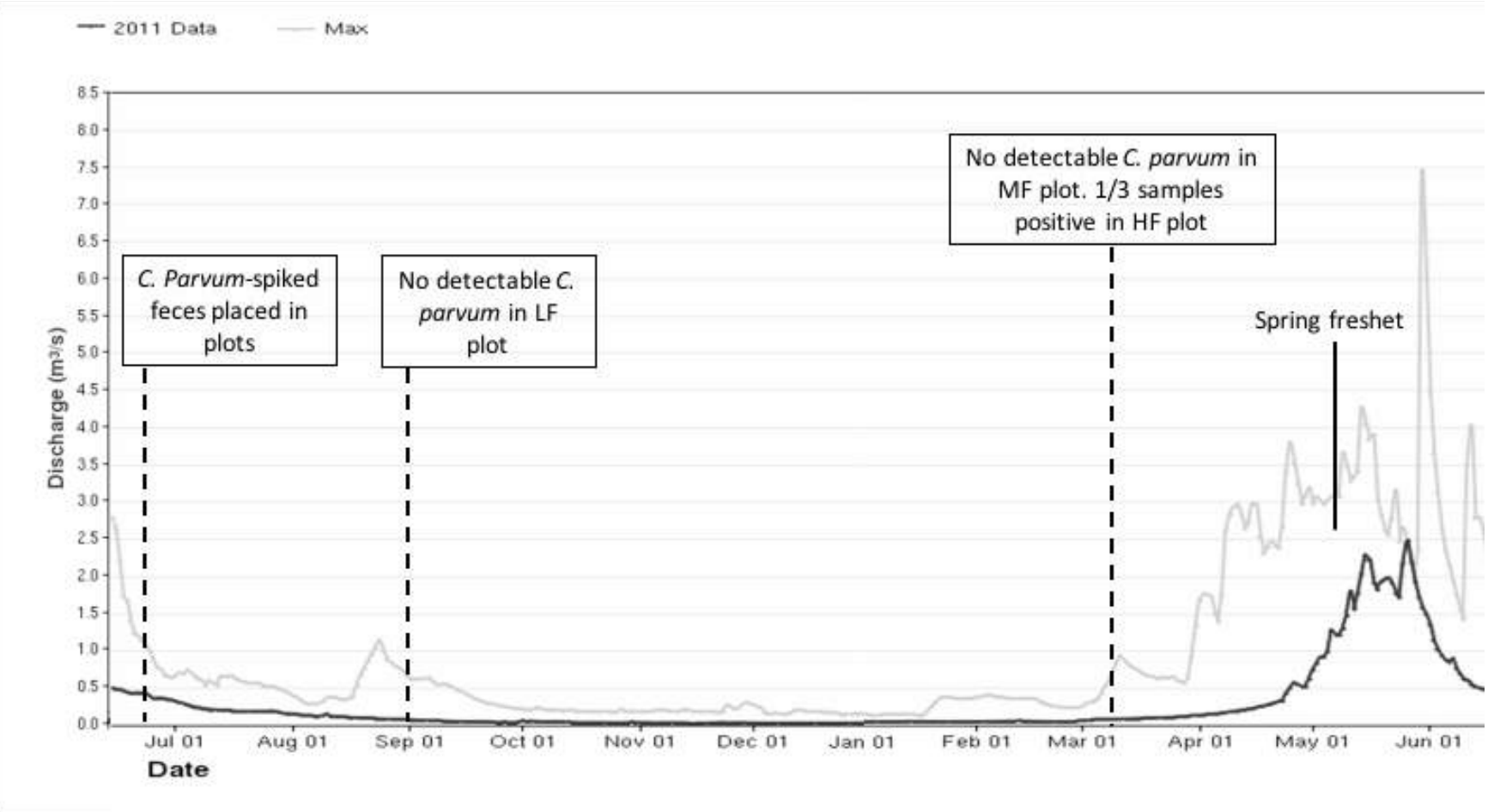

Figure 5-4: 2015 Real-time hydrometric data obtained from Environment Canada Water Office for Coldstream Creek, BC (intake 08NM142). Annotation indicate date of inoculation and loss of *C. parvum* detection for each of the plots.

In controlled laboratory studies, short pulses of 7.5-11 mJ cm^{-2} UV light have been shown to be sufficient in killing oocysts (Mofidi et al., 2001), yet in the environment, *C. parvum* persisted nearly three months in bovine feces subjected to comparable UV exposure (10 mJ cm^{-2}). The longer persistence in bovine feces compared to buffered solutions is partly attributed to the protective environment of the fecal pat itself, which can shield UV light from internally residing oocysts. Details as to the influence of the fecal matrix composition on oocyst persistence is limited and requires future investigation.

The persistence time disparity between the summer-deposited fecal pats and the winter-deposited fecal pats was likely due to meteorological differences between the two studies. It was first hypothesized that the 2015 winter season temperatures may have been insufficient to kill the oocysts that persisted throughout the summer. However, during both the summer and winter persistence trials, the minimum, maximum, and average temperatures observed during the winter season were similar (Figure 5-5, Table 5-2). In contrast to expectations, the 2015-2016 winter season had four more freeze-thaw events and longer consecutive freezing days compared to the 2014-2015 winter season. It was therefore unlikely that winter temperatures alone could be responsible for the slower oocyst decay observed for the summer-deposited fecal pats. However, snow temperatures and fecal pat temperatures were not taken and these temperatures may have differed between the two studies depending on snow cover depth and insulation. Alternatively, summer exposure may have influenced the sensitivity of the fecal oocysts to winter effects in the form of cryotolerance. Of particular note is the significantly lower water content in the HF feces. These samples were on average 10-15% drier than feces in the MF and LF plots (Figure 5-2) and required considerably more force to withdraw samples due to cement-like consistency.

Table 5-1: Temperature data for both the 2014-2015 and 2015-2016 winter season.

	SEPTEMBER 27, 2014-FEBRUARY 10, 2015	SEPTEMBER 27, 2015-FEBRUARY 10, 2016
MINIMUM TEMPERATURE	-19°C	-18°C
MAXIMUM TEMPERATURE	22°C	22°C
AVERAGE TEMPERATURE	3°C	2°C
# OF FREEZE-THAW EVENTS	5	9
LONGEST CONSECUTIVE FREEZING DAYS	8	12

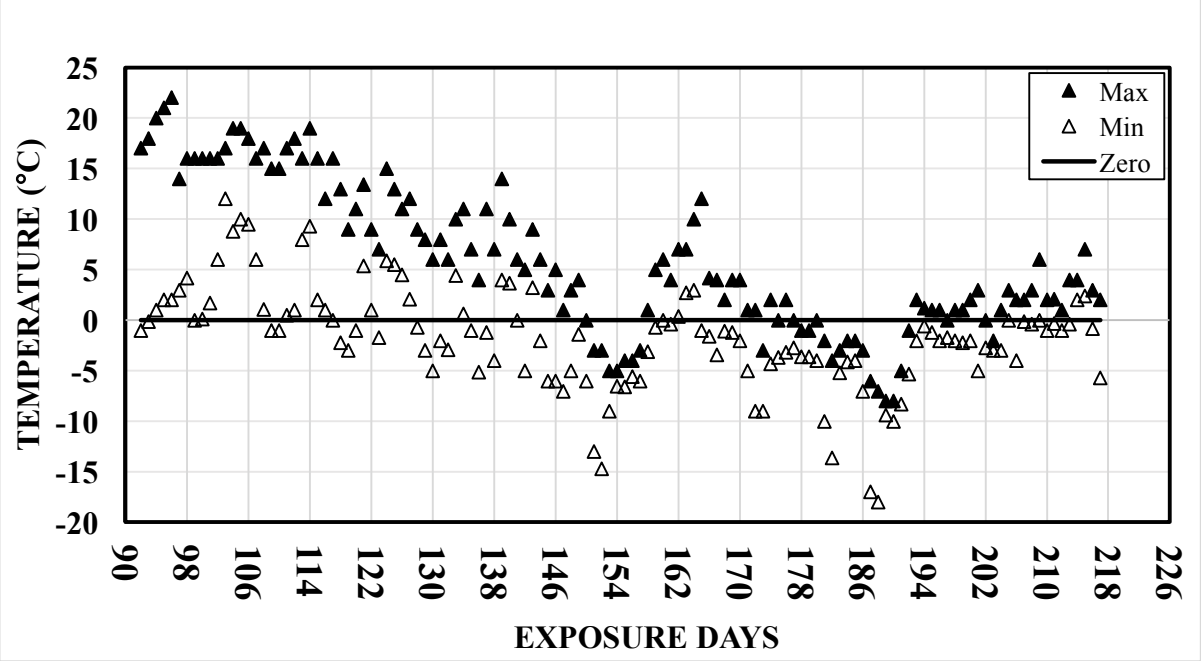

Figure 5-5: Local maximum and minimum temperature values at the persistence sites during the winter season of the summer deposited fecal pats (day 90 = September 27, 2015).

While complete desiccation has been shown to kill oocysts (Robertson et al., 1992), mild desiccation may play an important role against freeze-thaw lysis of oocysts by reducing the amount of water in the fecal pat and the according ice crystal formation at sub-zero temperatures (Walker et al., 2006). The desiccation of the fecal pats was therefore proposed as a contributor to winter cryopreservation. While freeze-thaw cycles do indeed rupture *C. parvum* oocysts, this effect may be suppressed in feces with very little water content.

CHAPTER 6
CONCLUSION

6.1: Response to research objectives

The first research objective was to evaluate nested PCR as a method for measuring oocyst persistence in bovine fecal samples and compare nested PCR with alternative molecular detection techniques such as qPCR. Although nested PCR was unable to provide a direct indication of oocyst viability, it did serve as a semi-quantitative method for determining the maximum duration that the oocysts may remain present in the fecal pats. An oocyst is not considered viable if the DNA is not present inside an intact oocyst, and thus the absence of 18S rRNA gene in *C. parvum* from fecal pats is a measure of non-viability, which is an important datum in forest and watershed monitoring. In supplementary studies it was observed that after 48 hours of exposure to feces, the sample preparation and nested PCR protocol did not detect leaked oocyst DNA from freeze-thaw ruptured oocysts, making this assay useful for selective detection of intact oocysts. Nested PCR was found to provide a comparable limit of detection compared to qPCR and showed few complications during either of the seasonal persistence studies.

The second and third research objectives were to provide information as to the maximum time that *C. parvum* remains detectable on Okanagan grazing land during winter and summer exposure conditions and to identify influence(s) of shading conditions and key environmental factors on *C. parvum* persistence during winter and summer exposures. For the winter season, *C. parvum* DNA was not detectable in bovine feces beyond 106 days under the heavily, moderately, and lightly forested shading conditions. The influence of winter shading conditions could not be statistically evaluated due to the high variance

associated with binomial proportion data and a small population size. This study has provided supporting evidence that *C. parvum* in fecal pats deposited in the fall does not persist within the feces throughout the winter and into late May, when spring freshet is common. Freeze-thaw events were shown to accelerate the loss of *C. parvum* from feces in natural environments, causing considerable deformation of oocyst cell walls. Oocysts subjected to as few as three freezing and thawing cycles showed a 41% reduction in the original population with only 19% of the remaining detectable oocysts having an intact oocyst wall. The effect of snow cover on the fecal pats may protect the oocysts from rapid temperature swings and extreme low temperatures. From the winter persistence data, it is recommended that ranchers discourage cattle access near community watersheds at least 106 days prior to spring freshet. This scheduling restriction is unlikely to be problematic as grazing cattle are sheltered during the winter months. However, changes in winter temperatures, the frequency of freeze-thaw events, and the timing of spring freshet will influence the risk of *C. parvum* transmission from bovine feces to community watersheds.

Whereas shading had little effect on oocyst longevity during the winter, shading appeared to influence summer-exposed oocysts. High UV light and elevated ground temperatures rendered oocysts non-detectable (0/3 positive fecal pats) by 71 days in lightly forested plots while heavily forested plots retained oocysts (3/3 positive fecal pats) beyond 71 days. The moderately forested plot showed a decrease in oocyst presence after 35 days of exposure and 2/3 tested fecal pats were positive at 71 days. Feces in moderately forested conditions were positive for *C. parvum* until 271 days of exposure and the highly forested condition still contained 1/3 positive fecal pats at this time. Persistence beyond both the summer and winter exposures was unexpected in light of the previous winter persistence

data. *C. parvum* in fecal pats deposited during the summer may persist into spring freshet, and BMPs are encouraged to minimize cattle presence in sensitive riparian zones. The persistence of June-deposited seeded feces into March raises questions as to how the winter exposures may have differed or how the summer environment may have shielded the oocysts from freezing and thawing during the winter. Further studies are required to evaluate if mild desiccation or fecal crusting acts as a means of preservation for *C. parvum*.

6.2: Research impact

This study is the first to implement nested PCR as a method of evaluating *C. parvum* persistence in bovine feces in natural environments. It is also the first to monitor *C. parvum* persistence throughout both a summer and winter season and under different shading conditions. Nested PCR was determined as a valid approach for assessing maximum persistence time and results were comparable to literature findings that used the DAPI/PI vital dye method. The results obtained during this research provide detail as to the maximum duration that *C. parvum* can remain as an intact oocyst in bovine feces on Crown Land. Key factors that affect *C. parvum* persistence such as UV and freezing/thawing, as well as different shading environments, will be of use in the development of best management practices that seek to reduce *C. parvum* persistence both for Okanagan range operations and range operations in other climates. From the study, it can be concluded that grazing cattle should be discouraged from prolonged presence in heavily shaded riparian regions during the summer. During the winter, it is recommended to monitor freezing temperatures on grazing land to assess whether *C. parvum* may undergo lysis by natural freeze-thaw cycling.

6.3: Future research

Several questions raised while conducting this study could not be investigated due to restrictions in project time, bioethical limitations, or resources. The following areas should be examined during future studies in order to better assess the efficacy of nested PCR as a detection method and the influence of natural environmental exposure on *C. parvum* in feces:

- The relationship between the nPCR method used and *C. parvum* viability or infectivity.
- The influence of different fecal compositions on *C. parvum* persistence.
- Whether UV light or high temperature was the most influential factor on *C. parvum* decay in the summer in the LF meadow-like plot.
- Whether mild desiccation and fecal crusting prolong *C. parvum* persistence when exposed to winter factors.
- How *C. parvum* persistence will be effected by higher or lower initial oocyst concentration.

6.4: Alternative statistical approaches and experiment design

It was determined after completing the two seasonal studies that flaws existed in the experiment setup. Attempts were made to ensure that the data reported in the body of the thesis are as accurate as possible given the acknowledged flaws. The purpose of this section is to address these mistakes and provide insight into alternative methods to further improve the integrity of future experiments regarding *C. parvum* persistence.

Specifically, complications arose in comparing the four plot conditions (HF, MF, LF, and Control) due to inadequate sample size and the difficulty in processing binary data. Four perspectives are presented below as possible interpretations of the data sample size. A criticism of the ANCOVA method is also presented, followed by a discussion of an improved

experiment design. A schematic of the Perspective I, II, and III approaches is provided at the end of the section (Figure 6-1).

Perspective I: n = 9

A sample size of nine suggests that each of the sub-triplicates from the three fecal pats per plot are independent samples. It was originally proposed that the nine subsamples reflected the variability within a plot and served as a descriptor of *C. parvum* presence in the form of a proportion of positive results. While the increased sample size provided greater resolution, this effect was artificially generated. Since the sub-triplicates were derived from a fecal pellet collected mid-processing and not directly from different fecal pats, they cannot be viewed as true independent replicates and therefore $n \neq 9$. If, however, the samples were taken from nine different plots of the same condition, then the sample size would be nine for that condition.

From the n = 9 approach, the means of the binary data at each sampling time were logarithmically transformed to yield first-order kinetic models of the data. Given that the un-transformed data violated the assumptions of normality and equal variances, any subsequent transformations would require dubious statistical manipulation and would likely be flawed.

Plots illustrating the proportion of fecal pats positive for *C. parvum* (n = 9) using both zero- and first-order kinetics for both the winter and summer trials are provided in Appendix D, (Figure D-1 and D-2). The error bars in these plots reflect standard error of the mean on the binary data. This error calculation is fraudulent in that it both assumes nine independent samples and that the data are normally distributed. The latter assumption is most glaringly violated in instances of binary data, especially in small sample sizes. A more accurate

method of calculating error was discussed in section 2.1.14 and was used in the plots presented in both Chapter 4 and Chapter 5.

Perspective II: n = 3 with proportional positives

Instead of viewing each sub-triplicate as an independent sampling, an alternative perspective is to only view the three parent fecal samples from each plot as independent samples. In this scenario, the sub-triplicates are representatives of variability within each of the fecal pellets. Data for the samples taken from a plot at a given time could be grouped as nested sets such as [(+, -, -), (+, -, -), (+, +, -)] and reported as respective proportions of positive samples, e.g. [(1/3), (1/3), (2/3)]. This approach has a sample size of three (n = 3) and uses the sub-triplicates as descriptors of *C. parvum* within the fecal pellet. However, the assumption is that a fecal pellet with a greater number of *C. parvum* oocysts will have greater dispersion throughout the pellet. For example, a fecal pat characterized by 3/3 positive sub-triplicates would be assumed to have more oocysts than a fecal pat characterized by 1/3 positive sub-triplicates. This assumption should be treated with caution. Since the results are not reported quantitatively, it is unknown whether a poorly homogenized fecal pellet described as '1/3 positive sub-triplicates' does indeed contain fewer oocysts than a well homogenized fecal pat described as '3/3 positive sub-triplicates'.

It is important to note that the persistence means calculated from this approach are identical to the means calculated using the n=9 approach. As a result, both zero-order and first-order plots of the means gave linear models with identical slopes. However, the standard errors calculated for both the n=9 and n=3 approaches do differ because of the way the data are aggregated into sets (i.e. ordering of the data matter). For example, the set of [(-,+,+),(-,+,+),(-,+,+)] for n=9 and the equivalent set of [(2/3),(2/3),(2/3)] for n=3 would yield $\bar{x}$=0.67

by both methods, but would yield S=0.5 and S=0, respectively. Again, the conventional calculation of standard deviation or standard error is inaccurate when using binomial data.

Perspective III: n = 3, with absolute positives

A more precautious approach is to treat any positive value observed in the sub-triplicate set as a positive result for the entire fecal pat. For example, a fecal pat described by a sub-triplicate set of (+, +, +) and another fecal pat described by (+, -, -) would both be simply denoted as positive fecal pats. Using the previous example set of [(-,+,+),(-,+,+),(-,+,+)], the conclusion would be that 3/3 fecal pats in the plot were positive for *C. parvum*. Only samples with a sub-triplicate set of (-, -, -) would be considered negative for *C. parvum*. Using this criterion, the sub-triplicates serve only to assess if any *C. parvum* exist in the feces, not whether heterogeneity occurs in the fecal pats. The consequence of interpreting a single positive sub-triplicate as a positive fecal pat is a lower degree of resolution in the data and a disregard for variation within fecal pats. However, this loss in resolution is compensated by the higher integrity in the results and the lower risk of committing detection error (i.e. the possibility that observed absence is actually due to an inadequate detection by the instrument). The 'n = 3 with absolute positives' interpretation was used for the construction of the persistence plots for both the winter and summer studies (Figure 4-1, 5-1).

Perspective IV: n = 1

In both of the previous scenarios in which n = 3 it is presumed that the three fecal pats sampled in each plot are independent samplings. However, the three fecal pats are withdrawn from a plot that is defined with a singular shading characteristic (i.e. either heavily, moderately, or lightly forested). If the only variable being tested is shading condition, and only one shading plot of a given type is observed, the sample size is therefore one. By this

logic, all of the fecal pats and sub-triplicates in each plot are therefore sub-samples that describe sampling variation and no true replication of the plot condition was performed. It was argued that subdividing the original plot into smaller enclosed plots would nullify the n = 1 perspective. However, doing so commits pseudo-replication since it does not make an effort to preemptively maximize dispersion (Hurlbert, 1984).

If indeed none of the fecal pats or sub-samples were independent samplings, the observed effect of a particular shading type on *C. parvum* was reliant on observations from only one plot, which would be inadequate for reasonable extrapolation of the results. In response to this claim, it should be remembered that this experiment did not seek to specifically define the influence of a single shading condition on *C. parvum* persistence, but rather investigated whether different shading conditions showed significant differences in the rate at which *C. parvum* decayed. It is agreed that if a detailed model were required for the influence of meadow-like environments on oocyst decay, then an experiment should be performed with that goal as a priority (e.g. testing numerous meadow-like plots in different climates). Such an experiment would require significantly more plots and sampling. Even under the perspective that n = 1, it is visually evident from summer persistence plots (Figure 5-1) that there are significant differences in oocyst decay times between lightly forested and other exposure conditions.

The perspective of n = 1 has validity. But the data from several samplings and sub-samplings serve as descriptive data that should not be ignored solely because of partition semantics. The binomial data obtained from performing multiple samplings at a given time in the plot should be at least reported, even if it must be accompanied with a disclaimer.

Criticisms of the performed statistical method

The use of ANOVA or ANCOVA to evaluate binary or binomial data is controversial because such data often violate assumptions of normality and equal variance. This was observed in the control and MF winter study groups and in all of the summer study groups, as assessed by the Shapiro-Wilk's test ($p > 0.05$). Whereas the groups in the winter study did show homogeneity of variances by Levene's test of equal variances, the groups in the summer study violated this assumption ($p < 0.05$). Even if normality was observed, the limited sample size used in this study makes it difficult to perform ANCOVA confidently.

It is interesting, however, that the ANCOVA results did reflect the visually apparent differences among the exposure conditions (Appendix C). Especially in the case of the summer persistence data, ANCOVA-LSD reveals significant differences between the LF condition and all other plot types. In the winter condition, no differences are observed among the three environmental exposures, which are all statistically different from the control.

A more reasonable alternative to ANCOVA is a Generalized Linear Mixed Model (GLMM). A GLMM is often used when working with non-normal data that cannot be easily transformed into workable, normalized sets. A GLMM is a more complex variation of a generalized linear model (GLiM), which uses the framework of a linear regression model with the added component of an embedded link function. These link functions vary depending on the data to be analyzed, but their general role is to account for non-normal distribution of data. For instance, a linear model can be generated from binary data by employing a 'logit' function that yields a predicted probability of the binary outcome occurring. One of the assumptions of GLiMs is that all of the samples are completely independent, which is not always possible when performing longitudinal studies on the same

sample(s). GLMMs are useful in these instances because both fixed and random effects can be implemented in the model to best account for potential non-independencies. For the persistence study, the fixed effects would be the observed presence/absence of *C. parvum* and the plot condition and random effects would include the variation observed among replicates and the variation among plot conditions. Using a Likelihood Ratio Test, the intercepts and slopes of the GLMM for each condition as well as a null model (a model that does not include the variable of interest, e.g. plot condition or time) can be compared and a p-value is generated for assessing statistical significance.

Unfortunately, the reliability of a GLMM is often dependent on robust sample sizes, especially when working with binary or binomial data. Furthermore, GLMMs are more complex than the commonly implemented statistical tests such as t-tests, chi-square tests, ANOVA, and ANCOVA. Indeed, a majority (58%) of ecological and evolutionary publications from 2005 to 2008 were found to use GLMM incorrectly (Bolker et al., 2008). This misinterpretation of GLMM is most commonly due to the slew of assumption tests, nested algorithms, and link functions available to ecologists as well as the tempting choice to easily automate GLMMs using statistical software without understanding the requisite assumptions and parameters (Bolker et al., 2008).

After addressing the aforementioned limitations of the sample size and understanding the limitations and prerequisites of GLMM, this statistical tool would be the ideal method of testing the null hypotheses in the winter and summer studies.

Improved experiment design

It is recognized that inadequate experiment design was performed for the winter and summer persistence studies. The first major flaw was in not replicating the plot condition

(resulting in pseudo-replication as already discussed) and the second flaw was in performing sub-sampling at random. The random sub-sampling made it impossible to track persistence in a single fecal pat and resulted in a higher potential of random effects interfering with the data due to unknown variance in exposure even within the same plot. By testing the same fecal pats at each time point, stronger inferences can be made from the longitudinal data.

For future work, it is recommended that at least three to five plots are constructed for each of the tested shading conditions, with each of the plots containing at least three fecal pats. In this approach, the different plots serve as independent sampling sites and dispersion is maximized to prevent pseudo-replication. If three plots are used for each of the three condition, and each of the plots contain three fecal pats, then a total of $3^3 = 27$ samples are withdrawn and analyzed at each time point. This is the same number of samples tested in the original design, but it provides a more reliable description of the persistence of *C. parvum* in each of the plot conditions.

In addition to altering the experiment design, the use of qPCR instead of nPCR would improve the statistical interpretation of the results by providing continuous rather than binary data. The use of qPCR was not permitted in this study due to the request from funding partners that only EPA-approved methodologies for *C. parvum* detection should be used. The results reported in Chapter 3 show that qPCR is, at the very least, a viable alternative and would have been worth pursuing.

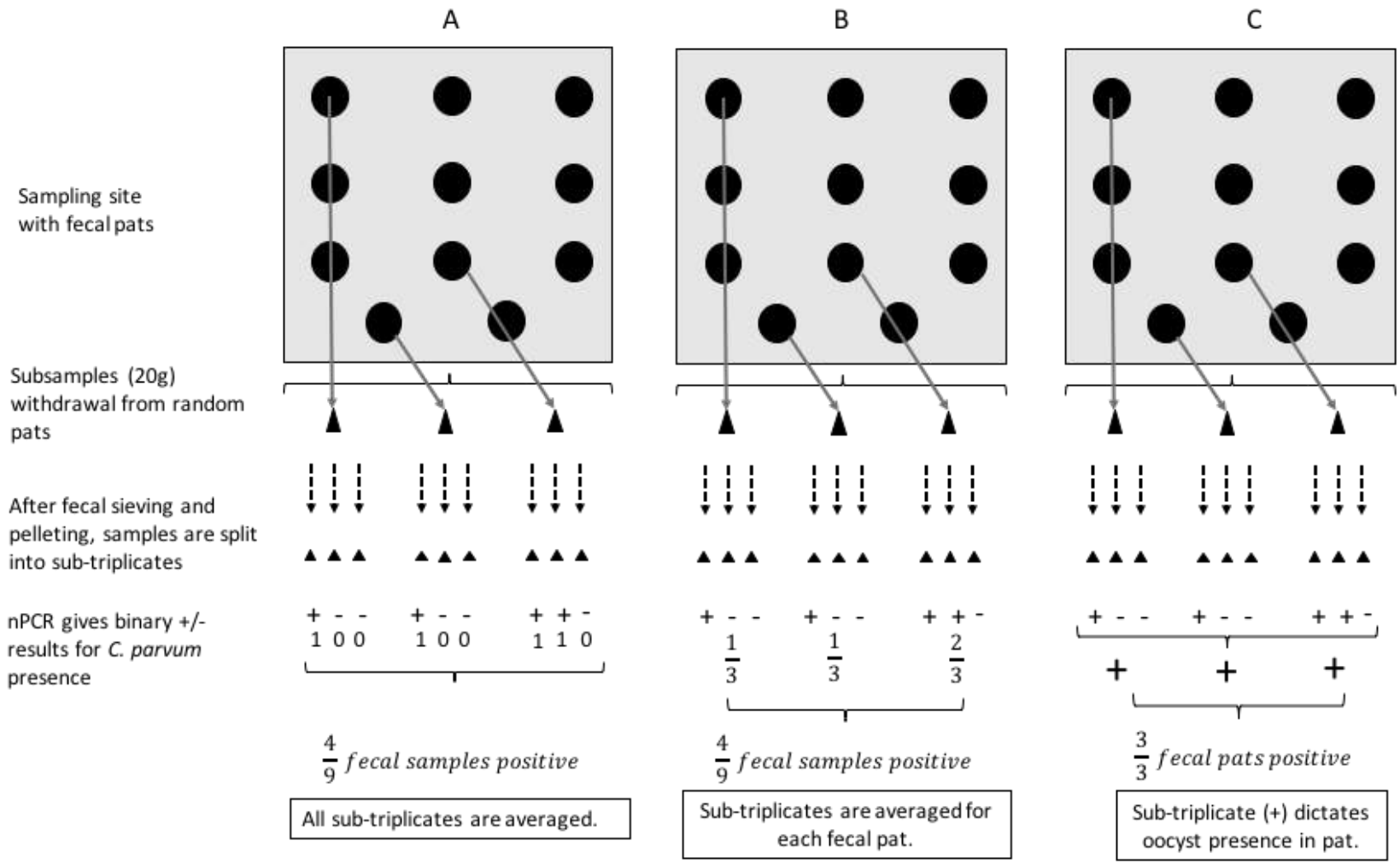

Figure 6-1: Schematic of the different approaches in aggregating the field sample data. A) n=9 B) n=3 with proportional positives C) n=3 with absolute positives.

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APPENDICES

Appendix A: Chemical recipes

1L 1x PBS:

- 8g of NaCl
- 0.2g of KCl
- 1.44g of Na_2HPO_4
- 0.24g of KH_2PO_4
- pH to 7.4 with HCl.
- Up to 1L in RO H_2O.
- Autoclaved

1L 1x TBE:

- 10.8 g Tris
- 5.5 g Boric acid
- 4 ml 0.5 M Na_2EDTA (pH 8.0)
- Up to 1L in Type 1 water

1 L Elution buffer:

- 1% v/v Laureth 12
- 0.01% v/v antifoam A
- 0.01% v/v EDTA
- Up to 1L in RO water

Appendix B: Calculations

Nested PCR Calculation of oocysts/g feces:

$$\frac{(1.23\ x\ 10^5 oocysts)}{(1.00\text{mL stock solution})} x\ (0.040\text{mL stock used}) x\ \frac{1}{(1000\text{g feces})} = \mathbf{\frac{4.9\ oocysts}{g\ feces}}$$

Nested PCR Calculation of oocysts/PCR:

$$\frac{(4.9\ \text{oocysts})}{(\text{g feces})} x\ (20.5\text{g feces for pelleting})\ x \frac{(0.600\text{mL subsample for DNA extraction})}{(5\ \text{mL total pellet volume})}\ x$$

$$(75\%\ \text{DNA extraction efficiency}*)\ x\ \frac{(5\text{uL DNA used in nPCR})}{(100\text{uL total DNA extract volume})}$$

$$= \mathbf{\frac{0.45\ oocyst\ equivalents\ of\ DNA}{nPCR\ reaction}}$$

qPCR Calculation of oocysts/g feces:

$$\frac{(1.23\ x\ 10^5 oocysts)}{(1.00\text{mL stock solution})} x\ (0.200\text{mL stock used})\ x\ \frac{1}{(0.700\text{g feces})}\ \text{x} \frac{1}{(100\text{X dilution factor})}$$

$$= \mathbf{\frac{351\ oocysts}{g\ feces}}$$

qPCR Calculation of oocysts/PCR:

$$\frac{(351\ \text{oocysts})}{(\text{g feces})} \text{x}\ \frac{(1\ \text{g feces})}{(0.700\text{mL resuspension})}\ \text{x}\ (0.600\text{mL subsample for extraction})\ \text{x}$$

$$(75\%\ \text{DNA extraction efficiency})\ \text{x}\ \frac{(2.0\text{uL DNA used in qPCR})}{(100\text{uL total DNA extract volume})}$$

$$= \mathbf{\frac{3.2\ oocyst\ equivalents\ of\ DNA}{qPCR\ reaction}}$$

*75% Extraction efficiency based on the required use of only 600uL of the 800uL supernatant during one of the DNA extraction processing stage

Appendix C: SPSS statistical outputs

The following SPSS ANCOVA and LSD outputs were obtained using the logarithmically transformed (ln) persistence means at each plot during each sampling time. Because the means and the slopes of the plotted data are identical for both the n=9 and n=3 (with proportional positives) approaches (section 6.4), the statistical outputs for the two approaches are identical as well. These outputs are provided solely as a reference for the originally performed statistical approach and are considered inaccurate.

Winter

ANCOVA significance report

Tests of Between-Subjects Effects

Dependent Variable: Log-transform of Winter Persistence data

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	16.481[a]	4	4.120	18.146	.000	.628
Intercept	117.381	1	117.381	516.959	.000	.923
time	11.887	1	11.887	52.352	.000	.549
group	4.594	3	1.531	6.744	.001	.320
Error	9.764	43	.227			
Total	154.739	48				
Corrected Total	26.245	47				

a. R Squared = .628 (Adjusted R Squared = .593)

Estimated Marginal Means

Dependent Variable: Log-transform of Winter Persistence data

group	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Control	2.112[a]	.138	1.834	2.389
Heavily Forested	1.312[a]	.138	1.034	1.589
Moderately Forested	1.702[a]	.138	1.425	1.979
Lightly Forested	1.419[a]	.138	1.142	1.697

a. Covariates appearing in the model are evaluated at the following values: time = 16.5833.

Pairwise Comparisons

Dependent Log-transform of Winter Persistence data

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.[a]
Control	Heavily Forested	.800*	.195	.000
	Moderately Forested	.410*	.195	.041
	Lightly Forested	.693*	.195	.001
Heavily Forested	Control	-.800*	.195	.000
	Moderately Forested	-.390	.195	.051
	Lightly Forested	-.107	.195	.584
Moderately Forested	Control	-.410*	.195	.041
	Heavily Forested	.390	.195	.051
	Lightly Forested	.283	.195	.153
Lightly Forested	Control	-.693*	.195	.001
	Heavily Forested	.107	.195	.584
	Moderately Forested	-.283	.195	.153

Based on estimated marginal means
*. The mean difference is significant at the .05 level.
a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Summer

ANCOVA significance report

Dependent Variable: Log-transform of Summer Persistence data

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	14.075[a]	4	3.519	17.477	.000	.630
Intercept	151.634	1	151.634	753.139	.000	.948
time	11.104	1	11.104	55.151	.000	.574
group	4.714	3	1.571	7.804	.000	.363
Error	8.255	41	.201			
Total	180.860	46				
Corrected Total	22.330	45				

a. R Squared = .630 (Adjusted R Squared = .594)

Estimated Marginal Means

Dependent Log-transform of Summer Persistence data

group	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Control	1.964[a]	.131	1.700	2.229
Heavily Forested	2.145[a]	.127	1.888	2.402
Moderately Forested	1.912[a]	.130	1.651	2.174
Lightly Forested	1.221[a]	.151	.916	1.526

a. Covariates appearing in the model are evaluated at the following values: time = 33.4130.

Pairwise Comparisons

Dependent Variable: Log-transform of Summer Persistence data

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.[a]
Control	Heavily Forested	-.181	.186	.336
	Moderately Forested	.052	.185	.781
	Lightly Forested	.743*	.198	.001
Heavily Forested	Control	.181	.186	.336
	Moderately Forested	.233	.181	.206
	Lightly Forested	.924*	.200	.000
Moderately Forested	Control	-.052	.185	.781
	Heavily Forested	-.233	.181	.206
	Lightly Forested	.691*	.200	.001
Lightly Forested	Control	-.743*	.198	.001
	Heavily Forested	-.924*	.200	.000
	Moderately Forested	-.691*	.200	.001

Based on estimated marginal means
*. The mean difference is significant at the .05 level.
a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Appendix D: Plots developed using alternative statistical methods.

n=9 plots

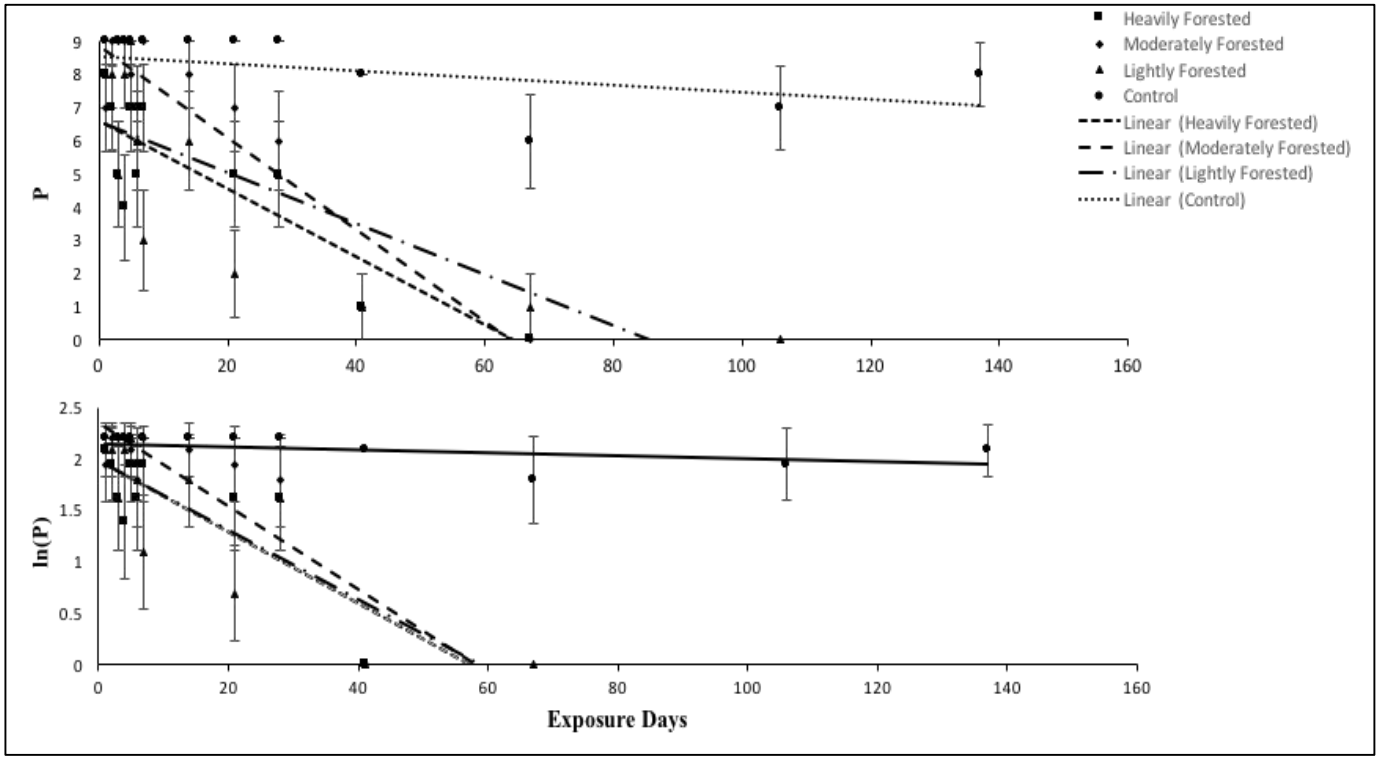

Figure D-1: Zero order (top) and first order (bottom) kinetic models of *C. parvum* persistence in winter exposed fecal pats under different shading conditions for n = 9. P is the proportion of samples positive for *C. parvum*. Exposure day 1 corresponds to September 27th, 2014

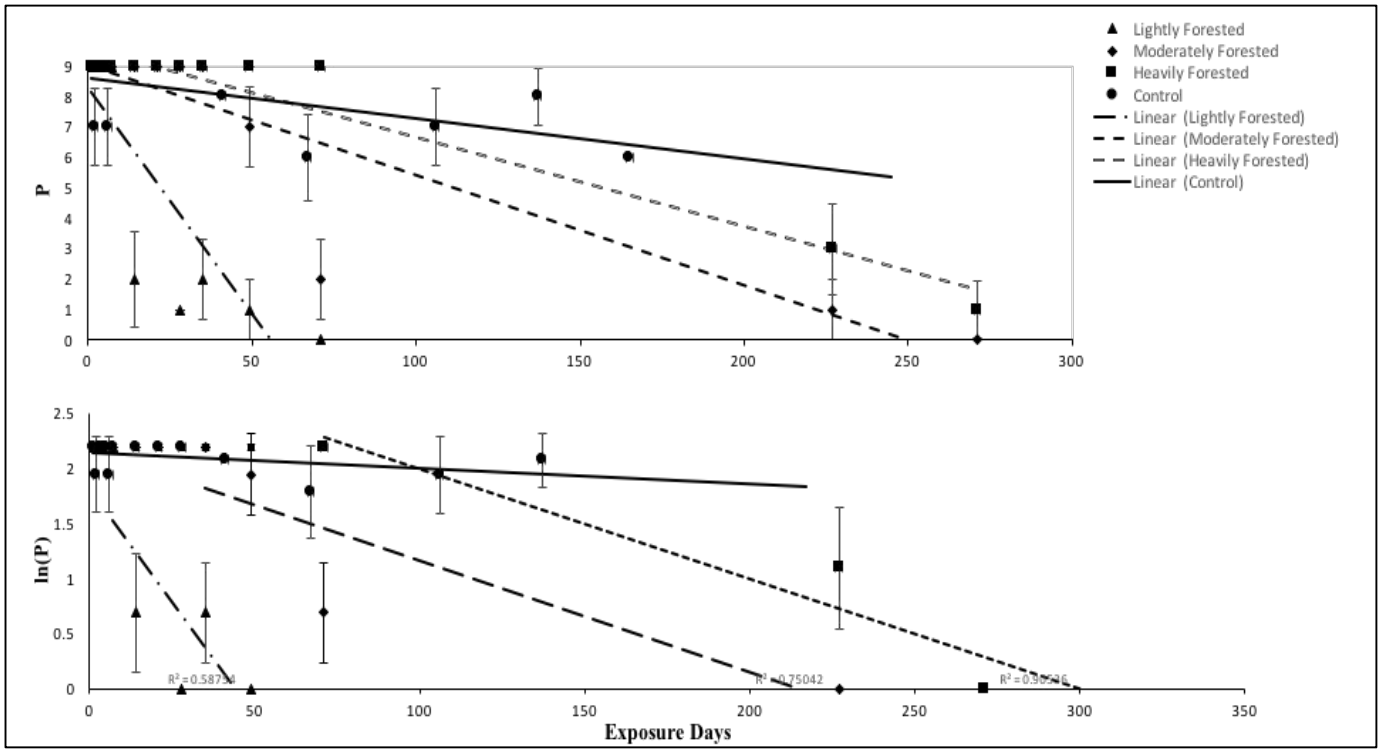

Figure D-2: Zero order (top) and first order (bottom) kinetic models of *C. parvum* persistence in summer exposed fecal pats under different shading conditions for n = 9. P is the proportion of samples positive for *C. parvum*. Exposure day 1 corresponds to June 23, 2015.

n=3 plots

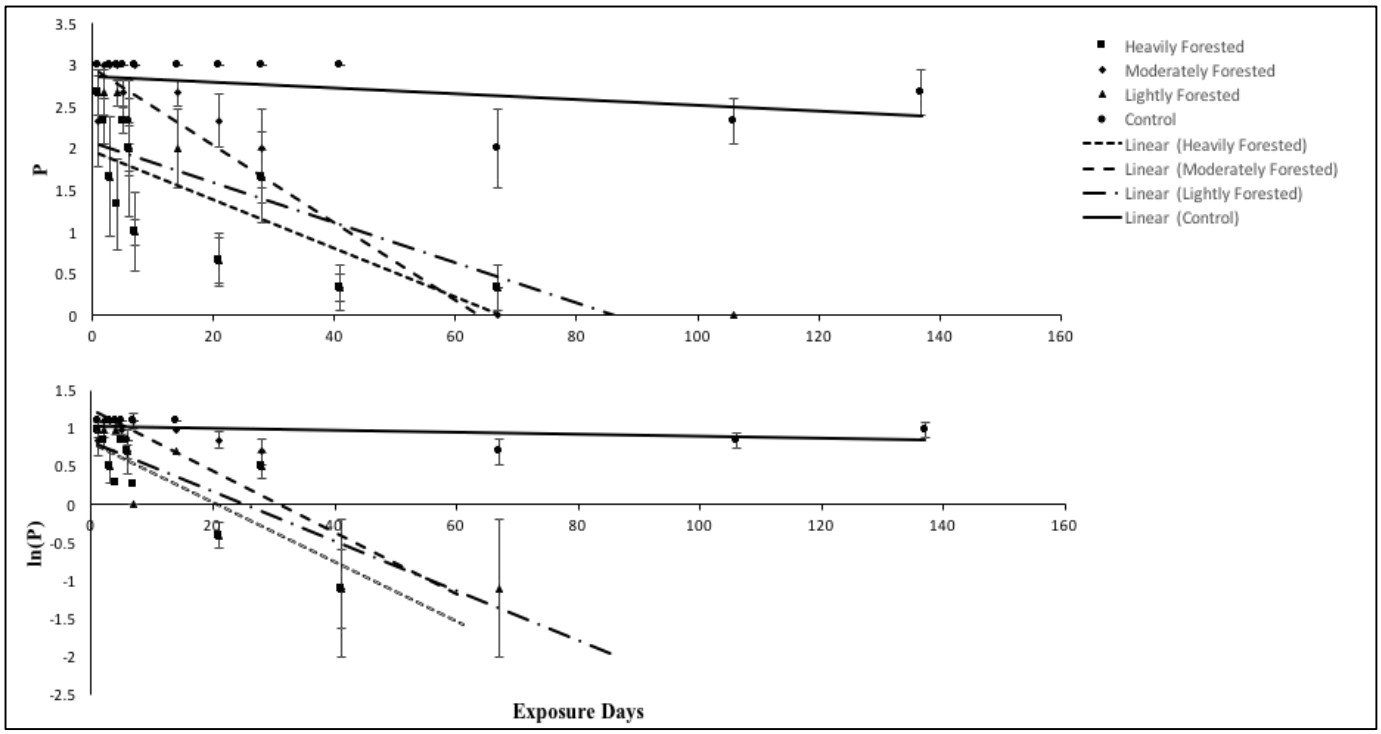

Figure D-3: Zero order (top) and first order (bottom) kinetic models of *C. parvum* persistence in winter exposed fecal pats under different shading conditions for n = 3. P is the proportion of samples positive for *C. parvum*. Exposure day 1 corresponds to September 27th, 2014

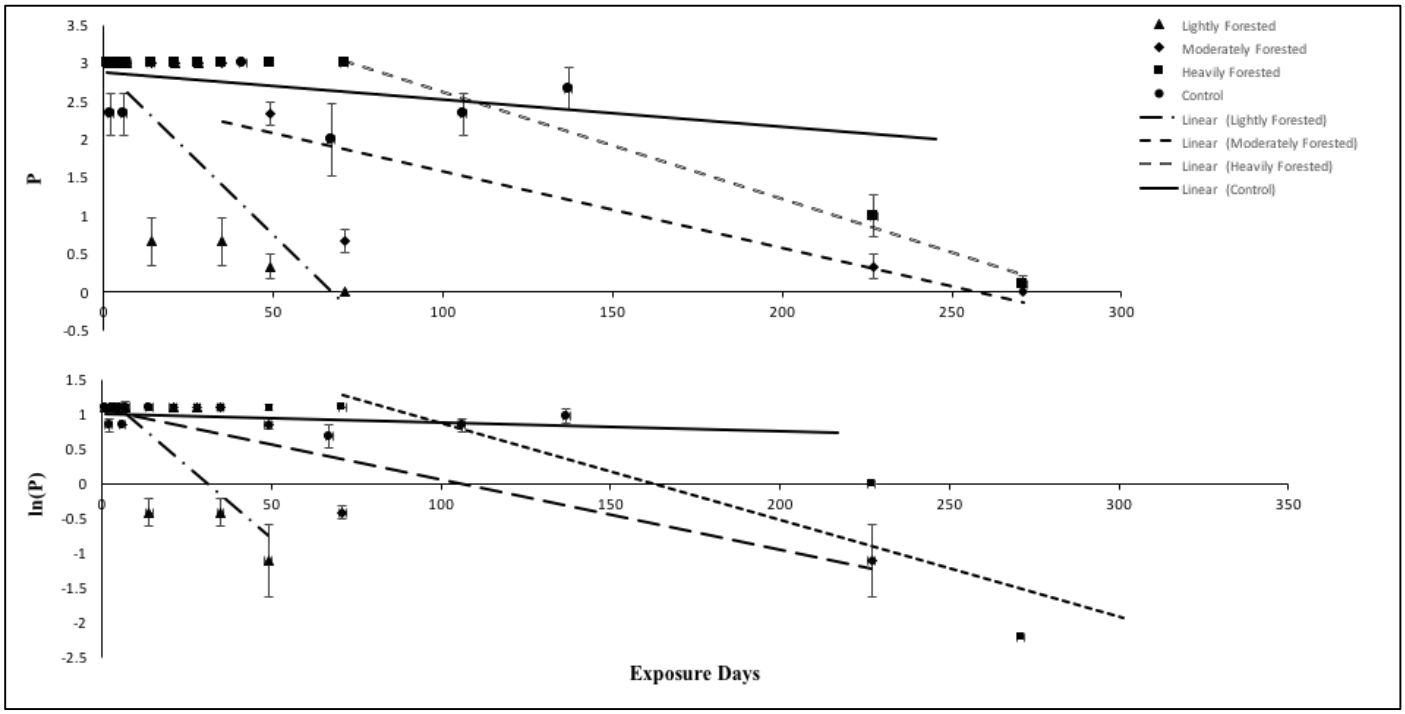

Figure D-4: Zero order (top) and first order (bottom) kinetic models of *C. parvum* persistence in summer exposed fecal pats under different shading conditions for n = 3. P is the proportion of samples positive for *C. parvum*. Exposure day 1 corresponds to June 23, 2015.