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The temporal distribution of planktonic protists in southwestern Alberta and their role in the persistence of the human pathogen *Campylobacter jejuni*

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Bachelor of Science, Acadia University, 2008

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MASTER OF SCIENCE
(Microbial Ecology/Molecular Biology)

Department of Biological Sciences
University of Lethbridge
LETHBRIDGE, ALBERTA, CANADA

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Dedication

I dedicate this thesis to my wife Melissa Thomas, to my parents Catherine and William Thomas, and my brother Ben Thomas.

ABSTRACT

The temporal dynamics of *Campylobacter jejuni* and protistan community structure in southwestern Alberta rivers were studied over a 1-year period. Culture-based isolations of *C. jejuni* were predominantly from the winter while real-time quantitative PCR indicated fluctuating densities of *C. jejuni* with no seasonal trend and no correlation with physicochemical properties of water. Protistan communities clustered according to season rather than location. Clinical *C. jejuni* isolates from southwestern Alberta survived longer when co-cultured with the model protozoan *Acanthamoeba polyphaga* than in the presence of by-products or growth medium. The use of wild type and mutant *C. jejuni* 81-176 strains revealed a cytotoxic effect toward *A. polyphaga,* and that invasion requires a functional flagellar apparatus but not quorum sensing nor cytolethal distending toxin. Combined, findings illuminate seasonal patterns of *C. jejuni* and protists and support the hypothesis that *C. jejuni* exploit phagotrophic protists as a survival mechanism in water.

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LIST OF ABBREVIATIONS

ACE	Abundance coverage estimator
AGI	Acute gastrointestinal illness
ASBE	*Arcobacter* selective isolation broth enrichment
ATCC	American Type Culture Collection
BE	Bolton enrichment
BSA	Bovine serum albumin
CC	Correlation coefficient
CCAP	Culture Collection of Algae and Protozoa
CDT	Cytolethal distending toxin
CFU	Colony Forming Unit
CGF	Comparative genomic fingerprinting
CHR	Chinook Health Region
CSB	Columbia agar supplemented with 5% sheep blood
DPG	2,3-diphosphoglycerate
DPO	Dual priming oligonucleotide
dsDNA	Double stranded deoxyribonucleic acid
EMA	Ethidium monoazide
ESRD	Alberta Environment and Sustainable Resource Development
FAM	6-carboxyfluorescein
GBS	Guillain-Barré Syndrome
gDNA	Genomic deoxyribonucleic acid
IAC	Internal amplification control
IBD	Inflammatory Bowel Disease
IBS	Inflammatory Bowel Syndrome
JM	Johnson and Murano
KM	Karmali agar
KS	Karmali agar + supplement
MF	Membrane filtration
MLST	Multilocus sequence typing
MOI	Multiplicity of infection

NCBI	National Center for Biotechnology Information
NMS	Non-metric multidimensional scaling
NTU	Nominal turbidity unit
OD	Optical density
OTU	Operational taxonomic unit
PAS	Page's amoeba saline
PCR	Polymerase chain reaction
PHAC	Public Health Agency of Canada
PFGE	Pulsed field gel electrophoresis
PYG	Peptone-yeast-glucose
qPCR	Quantitative polymerase chain reaction
ReA	Reactive arthritis
RFLP	Restriction fragment length polymorphism
RDP	Ribosomal database project
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SSR	Simple sequence repeats
SVR	Short variable region
T3SS	Type III secretion system
T3SSE	Type III secretion system effector
TCA	Tricarboxylic Acid Cycle
T-RF	Terminal restriction fragment
T-RFLP	Terminal restriction fragment length polymorphism
UV	Ultraviolet
VAP	Variably absent or present
VBNC	Viable but non-culturable
WHO	World Health Organization

CHAPTER ONE

Literature Review

1. Campylobacteria and *Campylobacter jejuni*

The *Campylobacter* genus was proposed by Sebald and Véron in 1963 (Millson *et al.*, 1991). At the time, the genus comprised two species: *Campylobacter fetus*, and *Campylobacter bubulus*, which has since been reclassified as *Campylobacter sputorum* (Forbes *et al.*, 2009). Members of the genus *Campylobacter* are gram negative, non-spore forming, and are mostly spirally curved rods measuring 0.2-0.8 by 0.5-5.0 µm in size (Vandamme *et al.*, 2005). *Campylobacter* species are microaerophiles, requiring oxygen concentrations ranging from 3 % to 15 % for growth. Furthermore, members of this genus are chemoorganotrophic, obtaining energy from amino acids or tricarboxylic acid (TCA) cycle intermediates (Vandamme *et al.*, 2005).

Currently, the *Campylobacter* genus consists of 25 recognized species (i.e. campylobacteria) (Debruyne *et al.*, 2009), many of which are not considered to be pathogenic in humans. Campylobacteria are fastidious to culture, but using advanced isolation techniques many have been recovered from human feces (Engberg *et al.*, 2000; Kulkarni *et al.*, 2002; Lastovica and le Roux, 2000, 2001; Lawson *et al.*, 1999; Maher *et al.*, 2003) as well as from the human oral cavity (Baker *et al.*, 2006). Among recognized pathogenic species, *C. jejuni* and *C. coli* are most commonly associated with disease in humans (Public Health Agency of Canada; http://dsol-smed.phac-aspc.gc.ca/dsol-smed/ndis/diseases/camp-eng.php). Campylobacteriosis (enteritis incited by campylobacteria) resulting from *C. jejuni* and *C. coli* infection is the leading cause of bacterial enteritis in the developed world (Wilson *et al.*, 2008). In Canada, the number of confirmed cases of enteritis due to campylobacteria is greater than that of all other

bacterial enteric pathogens combined (PHAC; http://dsol-smed.phac-aspc.gc.ca/dsol-smed/ndis/index-eng.php).

1.1. Evolutionary origin and current state of the *C. jejuni* species

C. jejuni is a member of the delta-epsilon group of the Phylum Proteobacteria (Heuvelink *et al.*, 2009). Although the species taxonomy within the genus has not been fully resolved, genetically *C. jejuni* is most closely related to *C. coli*. Current estimates, based on a molecular clock of base substitution rates, predict that *C. jejuni* and *C. coli* diverged from a common ancestor during the Neolithic period and may have occurred following the domestication of pigs (Wilson *et al.*, 2009). Pinpointing the precise time of speciation is especially difficult as bacteria do not fossilize easily and when they do, their morphological characteristics are insufficient for accurate taxonomic placement. At present, it is believed that *C. jejuni* and *C. coli* are converging based on patterns of genetic exchange (Sheppard *et al.*, 2008).

1.2. Etiology of campylobacteriosis

Campylobacteriosis is an inflammation of the intestinal tract characterized by diarrhea, abdominal pain, fever, nausea, and vomiting (World Health Organization; http://www.who.int/water_sanitation_health/diseases/campylobacteriosis/en/). Infection by *C. jejuni* has been demonstrated to occur following ingestion of as few as 500 to 800 cells (Baker *et al.*, 2006; Tustin *et al.*, 2011). As these observations were based on single cases, they may not be representative of the general population and campylobacteriosis may require a higher dose of *C. jejuni*. A recent clinical trial using human subjects suggests that the number of *C. jejuni* cells required to cause disease is higher than the

minimum amount of cells required to establish colonization as only 50 % of volunteers receiving 10^5 CFU of *C. jejuni* 81-176 developed the disease state (Baker *et al.*, 2007). The human intestinal microbiota is incredibly complex and varies substantially from one person to another (Robinson *et al.*, 2010). A healthy intestinal microbiota may provide colonization resistance in which host colonization with foreign organisms is less likely due to competition from the native gut flora.

1.3. *C. jejuni* pathophysiology

Once ingested, *C. jejuni* cells must pass through the stomach and into the intestine where the bacterium incites disease. The incubation time prior to the onset of symptoms is commonly between 24 and 48 h, but has also been documented to occur up to 7 days following ingestion (Blaser *et al.*, 1987; Wood *et al.*, 1992). The predominant symptoms early in the course of infection are malaise, myalgia, and cramping abdominal pain which can be generalized or localized, and sometimes difficult to discern from acute appendicitis (Blakelock and Beasley, 2003). Other early symptoms may include fever, headaches and vomiting (Acheson and Allos, 2001). Shortly after the onset of abdominal cramps, diarrhea typically follows. The severity of diarrhea varies substantially between individuals, with some experiencing enteritis with mild watery diarrhea, and others experiencing severe enteritis with bloody diarrhea characteristic of colitis (Blaser, 1997). In immunocompetent individuals, symptoms usually subside in approximately 7 days (Zilbauer *et al.*, 2008), but hospitalization can be required in severe cases.

Once established in the intestine, *C. jejuni* can survive within mucus of the intestine or invade the host intestinal epithelium or both (Wassenaar and Blaser, 1999). It is thought that the spiral shape and motility of *C. jejuni* aids in its ability to penetrate

mucus and is thus essential to epithelial invasion and intestinal colonization. Once in contact with the host epithelium, adhesion is the first step in pathogenesis followed by invasion of epithelial cells. A variety of extracellular/transcellular proteins are thought to play a role in adhesion, including the autotransporter CapA (Ashgar *et al.*, 2007), the fibronectin binding outer-membrane protein CadF (Konkel *et al.*, 1990), the surface exposed lipoprotein JlpA (Jin *et al.*, 2001), and the periplasmic binding protein PEB1 (Pei and Blaser, 1993). Invasion of intestinal epithelial cells occurs with the tip of *C. jejuni* first, followed by its flagellar end (Krause-Gruszczynska *et al.*, 2007). The invasion process is microtubule-dependent and not actin filament dependent (Dasti *et al.*, 2010). *C. jejuni* does not escape the vacuole it uses to enter host cells (Kopecko *et al.*, 2001). Virulence of *C. jejuni* is largely dependent on the flagellar apparatus, which consists of a basal body, hook, and filament. The flagellar apparatus of *C. jejuni* functions as a type III secretion system, exporting effector proteins into the host cell, including the cytolethal distending toxin (CDT) which functions as a DNAse. When the toxin gains access to the nucleus, CDT arrests the cell cycle by causing breaks in gDNA and subsequently induces cell death (Zilbauer *et al.*, 2008).

1.4. Campylobacteriosis treatment and secondary complications

Campylobacteriosis is self-limiting in most cases (Wassenaar and Blaser, 1999). Relapse occurs in as many as 20% of infected individuals and is normally milder than initial infection (Crushell *et al.*, 2004). Macrolides are the antibiotics of choice for severe or prolonged enteritis as well as extraintestinal manifestations of infection such as septicemia (Zilbauer *et al.*, 2008).   Complicated sequalae including premature birth and abortions in pregnant women, Gullian-Barré Syndrome (GBS), reactive arthritis (ReA)

(Hannu *et al.*, 2004), inflammatory bowel syndrome (IBS) (Kalischuk and Buret, 2010) and inflammatory bowel disease (IBD) (Gradel *et al.*, 2009) have been documented in patients following infection with *Campylobacter* spp. (Crushell *et al.*, 2004).

1.5. Campylobacteriosis outbreaks and control measures

The majority of campylobacteriosis cases reported are sporadic; however, outbreaks in both natural and engineered environments have been documented. However, recent evidence indicates that mini-outbreaks are common in southwestern Alberta (Inglis *et al.* unpublished). Outbreaks have been associated with consumption of raw cow's milk (Heuvelink *et al.*, 2009), raw peas (Gardner *et al.*, 2011), fresh chicken (Pearson *et al.*, 2000), water (Clark *et al.*, 2003) and contact with mud (Stuart *et al.*, 2010).

Water is a common point of contamination preceding *C. jejuni* outbreaks. In 1986, an epidemic of campylobacteriosis occurred in Greenville, Florida. This outbreak was attributed to a failure of chlorination, an unlicensed water treatment operator, and exposed treatment towers. The exposed treatment towers were frequented by birds, 37 % of those tested were later discovered to harbour *C. jejuni* (Sacks *et al.*, 1986). In 1985, an outbreak in southern Ontario resulted from the contamination of well water by spring run-off following heavy rains (Millson *et al.*, 1991). A second outbreak occurred in a nearby town, Walkerton, ON in 2000 when surface water containing livestock waste from farms contaminated the town water supply (Clark *et al.*, 2003). Of note, the Walkerton outbreak was linked to *C. jejuni* from cattle.While the role of waterborne infection in campylobacteriosis outbreaks is well understood, less is known concerning waterborne sporadic disease.

Consumption of undercooked chicken is believed to be a prominent source of infectious *C.jejuni* in campylobacteriosis outbreaks (Sheppard *et al.*, 2009). In New Zealand, there have even been calls for a ban on the sale of fresh chicken (Baker *et al.*, 2006). Of note, freezing chicken reduces viable *C. jejuni* populations by greater than two log units. However, a freezing requirement would represent a substantial cost to the poultry industry by requiring significant investment in equipment suitable for freezing and storing large quantities of fresh product. This cost would undoubtedly be passed onto the consumer in the form of increased prices. A unique scenario arose in Belgium in 1999 to ascertain the importance of poultry products in campylobacteriosis rates. Due to dioxin-contaminated feed components, chicken and egg products were withdrawn from the Belgium market. Retrospective studies estimate that the poultry ban resulted in the decline of *C. jejuni* infections by 40% (Vellinga and Van Loock, 2002). Similarly, an outbreak of avian influenza in the Netherlands in 2003 resulted in extensive culling of poultry in the region. This coincided with a decrease in campylobacteriosis cases, particularly in regions most impacted by the culling (Friesema *et al.*, 2012). These studies suggest a link between poultry and campylobacteriosis; however, it is uncertain whether the decline in campylobacteriosis cases was a result of the elimination of a source of direct infection or a reservoir. Furthermore, the market withdrawal of poultry did not eliminate infections indicating the existence of other significant reservoirs of infectious *C. jejuni*.

The most comprehensive campylobacteriosis control measures to date were implemented in Iceland following an outbreak in 1999 (Tustin *et al.*, 2011). Control measures were very broad and included technical, organizational, and policy changes. Measures at farms producing poultry included producer education, enhanced biosecurity and on-farm surveillance for *C. jejuni*. Changes at the poultry processing stage included a

leak-proof packaging policy, mandatory freezing of products originating from *Campylobacter* positive poultry flocks, and changes to the poultry processing. Furthermore, the Icelandic government implemented a consumer education program and an inter-organizational response committee (Tustin *et al.*, 2011). Overall, the combined measures were successful in reducing rates of campylobacteriosis. Interestingly, despite all of these measures, the rate of campylobacteriosis did not decline below pre-outbreak levels. This fact led researchers to speculate that there is likely an underlying environmental factor involved in the epidemiology of *C. jejuni* (Tustin *et al.*, 2011).

1.6. Epidemiology of campylobacteriosis

C. jejuni is a zoonotic pathogen found in birds and mammals, including livestock such as poultry, cattle, sheep, poultry, and pigs (Kärenlampi *et al.*, 2007; McCarthy *et al.*, 2007; Young *et al.*, 2007). Although *C. jejuni* has been associated with sporadic cases of diarrhea in chickens, it is generally considered a commensal in both wild and farm animals. *C. jejuni* is believed to infect humans through a variety of routes including the consumption of raw or under cooked meat, untreated drinking water, and raw or improperly pasteurized milk. The mode of transmission is the fecal-oral route. Thus, individuals in direct contact with afflicted animals, or animals carrying the pathogen, such as abattoir workers, are at an increased risk of infection (Blaser *et al.*, 1987).

The incidence of campylobacteriosis in southwestern Alberta is higher than both the provincial (AH; http://www.health.alberta.ca/documents/Notifiable-Diseases-Report-2004.pdf) and national (PHAC; http://dsol-smed.phac-aspc.gc.ca/dsol-smed/ndis/index-eng.php) averages, however, reasons for the high rates of campylobacteriosis in this region are unknown.

1.7. Environmental persistence

Once voided from suitable hosts, *C. jejuni* may be subjected to harsh environmental conditions including UV irradiation, oxidation, desiccation, freeze/thawing, and hypotonic/hypertonic stress. Furthermore, cells face competition for resources from other bacteria and predation. *C. jejuni* was once thought to persist poorly in extraintestinal habitats; however, using advanced culture techniques, *C. jejuni* is commonly isolated from surface waters and it has been demonstrated to remain culturable for up to four months in microcosm water held at 4 °C without aeration (Rollins and Colwell, 1986). *C. jejuni* will enter a viable but non-culturable (VBNC) state in response to unfavourable conditions (Baffone *et al.*, 2006). This state is characterized by reduced metabolic activity, a change in cell morphology (from spiral shaped to coccal), and the inability to be cultured directly. Suspensions of VBNC *C. jejuni* cells have a variety of morphologies including intact coccal, degenerated coccal and spiral forms; such suspensions are able to colonize mice (Jones *et al.*, 1991) and it is thought the intact coccal cells are actually VBNC. This is consistent with the recent microbial scout hypothesis, which proposes that microorganisms exit dormancy in a stochastic manner as a long term survival strategy (Buerger *et al.*, 2012; Epstein, 2009).

1.8. Adaptations of non-endospore forming gram negative bacteria promoting survival outside of traditional host organisms

Non-endospore forming gram negative bacteria were traditionally considered to be poor persisters in comparison to gram positive bacteria and Archaea. This is due to their less robust cell wall in comparison to gram positive bacteria and Archaea, which have adaptations, including heat stable proteins and/or a thick layer of peptidoglycan, to

withstand harsh environmental conditions such as desiccation, extreme temperatures, and barometric pressure. Notably, the putatively poor environmental persistence of many gram negative animal pathogens including the Proteobacteria, has led to the conclusion that these bacteria must be ingested by a susceptible host shortly after expulsion from an infected animal. Recent research has demonstrated that some Proteobacteria can persist for prolonged periods extraintestinally via a number of remarkable adaptations. One example is that of *Salmonella enterica* (the causative agent of salmonellosis), which is able to not only attach and survive on, but can also replicate within living plants by invading open stomata (Kroupitski *et al.*, 2009; Shirron and Yaron, 2011). Another example is that of *Legionella pneumophila* (the causative agent of legionnaires disease). This bacterium is able to invade the free-living freshwater protist *Acanthamoeba castellani*. This amoeba is the only known host apart from mammals within which *L. pneumophila* is able to replicate (Cirillo *et al.* 1994). Remarkably, the ability to invade and replicate within *A. castellani* enhances the ability of *L. pneumophila* to invade human epithelial cells and macrophages (Cirillo *et al.*, 1994). Furthermore, *L. pneumophila* has been observed to survive encystment within *Acanthamoeba polyphaga* making it resistant to high levels of chlorine (Kilvington and Price, 1990). Whether plants or protists harbour other bacterial pathogens and serve as vectors in transmitting bacterial pathogens from animal to animal has not been extensively examined.

2. Protists in aquatic ecosystems

Protists are defined as eukaryotes with a unicellular level of organization without differentiation into tissues (Adl *et al.*, 2005). Protists are the most ancient eukaryotes, believed to have emerged between 1.8 and 2.2 billion years ago (Knoll *et al.*, 2006). They

are considered to be the most biologically diverse group of organisms on the planet (Farmer and Habura, 2010). Protists range in size from 0.2 μm to 2.0 mm and can be found in forest soils (Adl and Gupta, 2006), freshwater bodies of water including rivers (Kiss *et al.*, 2009), streams (Ribblett *et al.*, 2005), lakes (Lefranc *et al.*, 2005), and oceans (Not *et al.*, 2009). Protists are important in nutrient cycling (including the mobilization of trace nutrients) and controlling bacterial populations (Figure 1.1).

2.1. Identification of protists in aquatic ecosystems

Although the importance of protists in aquatic ecosystems is known, little research has focused on their temporal distribution in freshwater rivers. Traditionally, surveying ecosystems for protists was enormously time consuming and required a great deal of expertise as protists were identified one at a time by light microscopy. Recently, 18S rDNA sequencing of total DNA extracted from a sample has been employed to great success in protistan surveys of aquatic ecosystems. Sequencing combined with a simple BLAST (Basic Local Alignment Search Tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi) search takes a fraction of the time to identify constituients of a sample compared to traditional identification methods.

2.2. Role in nutrient cycling

Perhaps one of the most often cited roles of protists includes nutrient cycling. In aquatic systems, protists are important members of the microbial loop. Protists have a range of trophic states including autotrophy, phagotrophy, and mixotrophy. Autotrophic protists, with the exception of cyanobacteria, are the smallest phototrophic organisms on the planet and perhaps the most numerous. Autotrophic protists, bacteria, and other

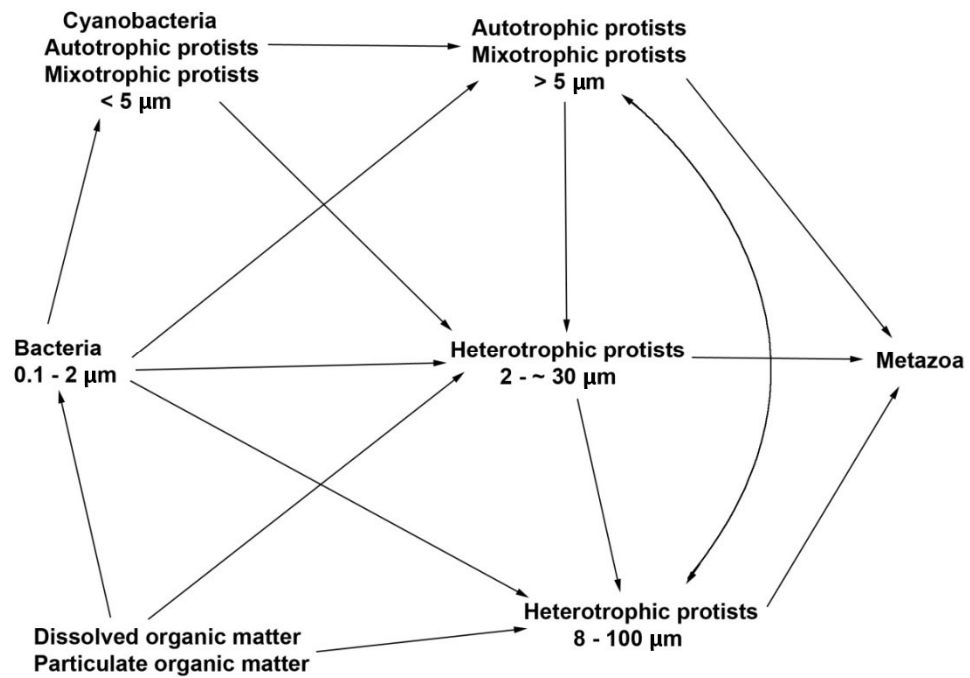

Figure 1.1. Graphical depiction of the microbial food web in aquatic ecosystems (Ducklow, 1994; Sherr and Sherr, 2002).

phagotrophic protists are grazed by phagotrophic protists, which obtain energy via the digestion of phagocytosed microorganisms. Mixotrophic protists employ a combination of heterotrophy and autotrophy and can be either dominant phagotrophs capable of autotrophy or *vice versa*. Phagotrophic and mixotrophic protists are collectively defined as protozoans. In freshwater systems, protozoans are thought to regulate bacterial population densities via predation. Protozoans also feed on other protists as well as metazoan eggs and small crustaceans. Furthermore, they are a significant food source for metazooplankton and are a major source of regenerated nutrients through the excretion of nitrogen, phosphorus, and trace metals including iron (Sherr and Sherr, 2002). Despite their importance in freshwater ecosystems, protists have not been extensively studied in this environment.

2.3. Controlling bacterial populations

Phagotrophy in protists is an ancient feeding mechanism and is deeply rooted in the eukaryotic tree of life and thus widespread among protistan lineages. Phagotrophy in protists is a significant source of bacterial mortality and many bacteriophagous protists display selective feeding habits. This may be due to the fact that the digestion of gram positive cells is more time consuming than gram negative cells (González *et al.*, 1990). Interestingly, bacteria have evolved complex measures to avoid predation by phagotrophic protists. Phenotypic characteristics that may have evolved in bacteria in response to grazing by protists include cell-cell communication (quorum sensing), cell miniaturization, biofilm formation, cell wall structure, filamentation, motility patterns, and toxin production (Pernthaler, 2005). Many of these characteristics also function as virulence factors in other organisms. The coincidental evolution hypothesis postulates that

virulence factors arose from adaptation to other niches. In the case of human bacterial pathogens, the theory suggests that virulence evolved in response to the selective pressure of protistan grazing (Adiba *et al.*, 2010).

2.4. Role of protozoans in the persistence and transmission of bacterial pathogens

Interest in the role of protists in the environmental persistence and transmission of pathogenic bacteria stemmed from the work of King et al. in 1988. In this study, several coliform bacteria including *Citrobacter freundii*, *Enterobacter agglomerans*, *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella oxytoca* and *Klebsiella pneumonia*, and bacterial pathogens *Salmonella enterica* Typhimurium, *Yersinia enterocolitica*, *Shigella sonnei*, *Legionella gormanii* and *C. jejuni* were incubated in the presence of the amoeboid protozoan *Acanthamoeba castellani* and ciliate protozoan *Tetrahymena pyriformis* and then subjected to different concentrations of chlorine (King *et al.*, 1988). All bacteria tested exhibited increased resistance to chlorine when co-cultured with one or both of the protozoans examined and all bacterial pathogens tested were 50-fold more resistant to chlorine when co-cultured with *Tetrahymena pyriformis* (King *et al.*, 1988). The authors speculated that the enhanced survival observed was the result of engulfment of bacteria by the protozoan cells, that this mechanism is an evolutionary precursor for pathogenicity, and is a survival mechanism for bacteria in inhospitable aquatic environments (King *et al.*, 1988).

2.5. Mechanisms of bacterial survival

Amoebae and bacteria have evolved together over millions of years and have developed some interesting symbioses. The social amoeba *Dictyostelium discoideum* (i.e.

a ‘slime mould’) has been shown to employ a unique survival strategy under depleting nutrient conditions that has been referred to as primitive farming behavior. As food sources are depleted, *D. discoideum* stops feeding on bacteria and incorporates bacterial cells into its fruiting body. Following dispersal, the bacteria are released, effectively seeding a new crop of food for the amoeba (Brock *et al.*, 2011). Bacterial entry into protozoans occurs by phagocytosis, in which a small vacuole called a phagosome is formed following attachment of bacteria to the protozoan cell wall. Bacterial survival in protozoans relies on either the bacteria escaping from the phagosome, by preventing phagosomes from binding to lysosomes, or surviving within phagolysosomes (Scola and Raoult, 2001). Exploitation of nearby cells via invasion of the cytoplasm is not restricted to bacteria-protozoa interactions as it has also been observed in bacteria invading other bacteria, such as the case with *Bdellovibrio bacteriovorus* (Rendulic *et al.*, 2004). In addition to protists, there are numerous examples of bacteria avoiding digestion in mammalian macrophages (Molmeret *et al.*, 2004; Oyston, 2008; Rosenberger and Finlay, 2003). *Mycobacterium tuberculosis*, an obligate intracellular pathogen resides within phagosomes and diminishes their ability to fuse with lysosomes but not early phagosomes (Sturgill-Koszycki *et al.*; Sturgill-Koszycki *et al.*, 1994). By retaining the ability to fuse with early phagosomes, *M. tuberculosis* has a readily available source of transferrin, a source of iron packaged for delivery throughout the body (Johnson and Wessling-Resnick, 2012).

The facultative intracellular pathogen *L. pneumophila* is perhaps the best studied bacterium in terms of its ability to exploit protozoans. *Legionella spp.* have been isolated from environmental protozoans on many occasions (Newsome *et al.*, 1998). Entry of *L. pneumophila* into the protozoans *A. castellani*, *Hartmannella veriformis* and *Naegleria*

lovaniensis is a receptor mediated process in which initial attachment is mediated by a galactose/N-acetylgalactosamine (Gal/GalNAC) lectin (Declerck *et al.*, 2007; Venkataraman *et al.*, 1997). Following adhesion, *L. pneumophila* enters the protozoan by cytoskeleton rearrangement (Abu Kwaik *et al.*, 1998). A portion of uptake (~10 %) is mediated by coiling phagocytosis, however, little is known about this process (Abu Kwaik *et al.*, 1998). Similar to *M. tuberculosis*, the bacterial phagosome does not fuse with lysosomes (Bozue and Johnson, 1996). The phagosome is initially surrounded by host cell vesicles and mitochondria (Abu Kwaik, 1996). Over the next 4 h following entry, the phagosome containing *L. pneumophila* is surrounded by a multilayer membrane derived from the rough endoplasmic reticulum (Abu Kwaik, 1996). At this point, *L. pneumophila* undergoes intracellular replication; however, it is uncertain if the delay in replication is the result of a lag phase associated with environmental adaptation or reflects the time necessary to recruit required host cell organelles (Abu Kwaik *et al.*, 1998). After replicating, *L. pneumophila* produces a pore in the replication vacuole which induces oncotic cell death of the amoeba resulting in the liberation of *L. pneumophila* (Gao and Abu Kwaik, 2000).

2.6. The current status of *C. jejuni*-protist interaction

Since the original paper by King et al. (1987) relatively little work has been conducted on the role of freshwater protists in the persistence of *C. jejuni*. In the early 1990s, much work focused on *L. pneumophila*. Not until 2005 were additional studies conducted examining a potential association between *C. jejuni* and freshwater protists (Axelsson-Olsson *et al.*, 2005; Snelling *et al.*, 2006; Snelling *et al.*, 2005). The early work by Snelling *et al*. (2005) was largely circumstantial as they isolated *C. jejuni* and protozoa

from broiler drinking water. Furthermore, only broilers exposed to drinking water in which *C. jejuni* was detected were colonized by *C. jejuni*, which suggests water-borne transmission. They also demonstrated that *C. jejuni* survived significantly longer when co-cultured with *A. castellani* than when in a planktonic state. Axelsson-Olsen et al. (2005) yielded similar results in their persistence assays with *A. polyphaga*. Their work also suggested the presence of *A. polyphaga* resulted in the resuscitation of VBNC *C. jejuni* (Axelsson-Olsson *et al.*, 2007). Another research group later provided data suggesting that *C. jejuni* cells internalized by *A. castellani* and *T. pyriformis* under laboratory conditions (i.e. ideal growth conditions for each organism) were able to colonize broilers (Snelling *et al.*, 2008). It is important to note that these observations were made under idealized conditions and may not be representative of what is likely to be encountered in the environment as a high ratio of *C. jejuni* to protist cells was used, as well as a large inoculum for the broilers.

Since the co-culture work with the amoebae *A. polyphaga* and *A. castellani*, and the ciliate *T. pyriformis*, an array of protists capable of phagotrophy including the stramenopile *Dinobryon sertularia*, the euglenozoan *Euglena gracilis,* and the heterolobosean *Naegleria americana* have been shown to prolong the survival of *C. jejuni* in co-culture (Axelsson-Olsson *et al.*, 2010). The above studies did not address whether the prolonged survival observed was the result of the protists creating a low oxygen environment more suitable for the survival/growth of *C. jejuni* through respiration or whether *C. jejuni* cells were internalized within these protists. To date, no investigations into the mechanisms by which *C. jejuni* persist in co-culture with protists have been published.

CHAPTER TWO

Hypotheses

Campylobacteriosis is the leading cause of bacterial enteritis in Canada and other developed nations and gaining a better understanding of the epidemiology of *C. jejuni* is of the utmost importance for the development of practices to reduce infection rates (Nachamkin *et al.*, 1998; Spiller *et al.*, 2000). Southwestern Alberta, the region formerly known as the Chinook Health Region (CHR) has high rates of campylobacteriosis within its human inhabitants when compared to the Canadian average, and the number of infections is increasing three times faster than population growth (Inglis *et al.*, 2005). Furthermore, recent studies conducted by our group have shown that large numbers of individuals infected with *C. jejuni* are not diagnosed. Cattle, chicken, sheep, and wild birds have all been shown to harbor significant amounts of *C. jejuni*, which has also been isolated from environmental sources such as agricultural runoff, rivers, and streams (Hudson, 1999; Inglis *et al.*, 2005; Jones, 2001; Newell and Fearnley, 2003; Stanley and Jones, 2003). Undercooked meat, including chicken, beef, and pork, untreated water, unpasteurized milk, and fecal-oral transmission are known pathways by which humans become infected with *Campylobacter* (Brown *et al.*, 2004). Water-borne outbreaks of campylobacteriosis are relatively common with the most infamous Canadian outbreak occurring in Walkerton, Ontario in 2000 (Clark *et al.*, 2005). Most water-borne outbreaks are attributed to inadequate water treatment. In the former CHR in 2008-2009, our research group showed high rates of campylobacteriosis throughout the spring, summer, and fall (Figure 2.1). This observation coupled with the substantially higher rates of campylobacteriosis in southern versus central and northern Alberta suggests that the environment of southwestern Alberta influences the epidemiology of this disease.

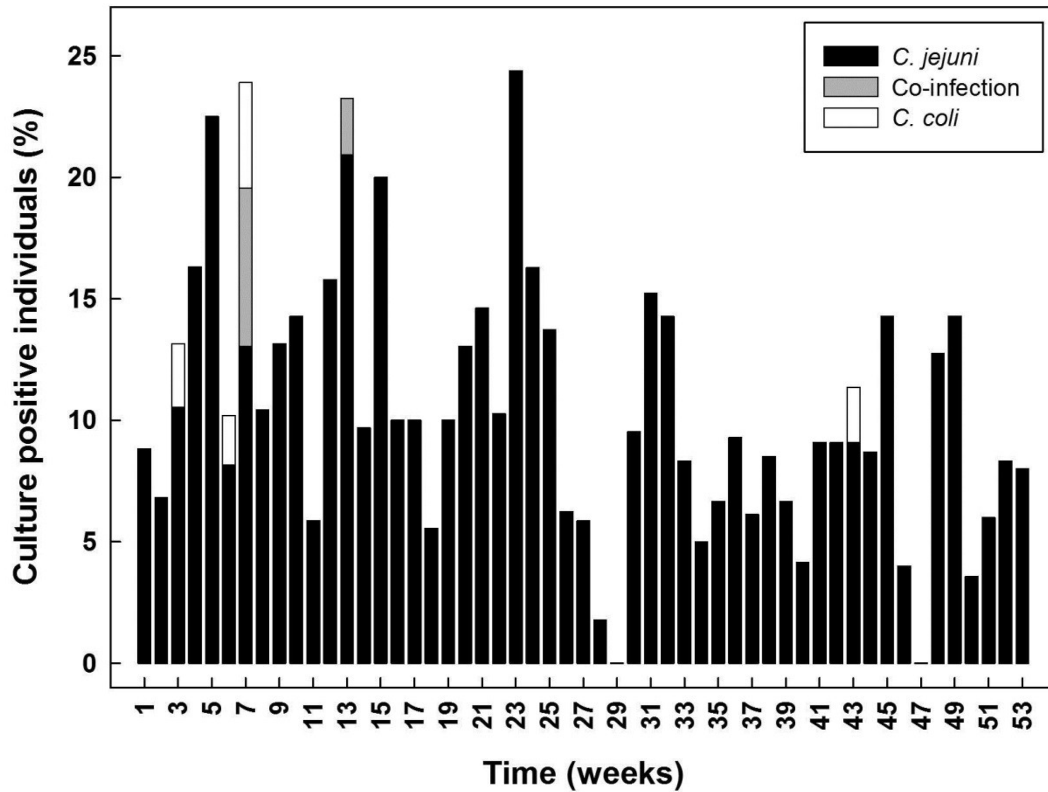

Figure 2.1. Prevalence of individuals in southwestern Alberta that were culture positive for *C. jejuni*, *C. coli*, or both (co-infection) by week over a 1-year period from May 2008 to May 2009 (Inglis *et al.*, unpublished).

C. jejuni is commonly isolated from river water, including river water within the Oldman River watershed (Walters *et al.*, 2007). This bacterium is a highly evolved enteric pathogen, and it is putatively incapable of replication extraintestinally (Vliet and Ketley, 2001). Furthermore, *C. jejuni* is thought by many not to persist in the environment. However, this is inconsistent with the high rates of campylobacteriosis observed in the southwestern Alberta and with the frequent isolation of *C. jejuni* from river water. In addition to our poor understanding of the impact of water-borne *C. jejuni* on epidemiology of outbreaks, the mechanisms by which this bacterium persists in water are poorly understood. Campylobacteria are capable of entering a VBNC state under unfavorable conditions (Rollins and Colwell, 1986). The VBNC state has been well documented for many bacterial species and is generally accepted as a persistence stage as opposed to the onset of cellular death (Oliver, 2005). VBNC *C. jejuni* has been reported to resuscitate following passage through a vertebrate host (Baffone *et al.*, 2006), and Axelsson-Olsson et al. (2008) demonstrated that VBNC *C. jejuni* may regain culturability following co-culture with the fresh water amoeba *A. polyphaga*, however, they could not definitively rule out the possibility of residual cultivable cells.

To date, there have been no investigations in the southwestern Alberta in relation to the persistence of bacterial pathogens in water resulting from the exploitation of protists. Due to the higher resistance to chlorine and UV irradiation exhibited by some bacteriophagous protists, such an interaction could have significant implications for the health of residents of southwestern Alberta. In addition, some species of protozoa themselves are pathogenic (i.e. keratitis and encephalitis incited by *Acanthamoeba* spp.), and many protozoa are able to withstand levels of chlorine and UV irradiation higher

than that used in water treatment plants to produce potable water supplies (King *et al.*, 1988). Given the above, it may be possible for pathogenic bacteria, such as *C. jejuni*, to enter and persist in water distribution systems within free-living freshwater protozoa. To our knowledge, protistan community structure in southwestern Alberta has never been investigated in depth. Even on a global level, very few studies have examined the community structure of protists in river water and none have been conducted in Canada. Furthermore, few studies have been conducted to elucidate the mechanisms by which *C. jejuni* persists in freshwater environments. It is also unknown whether these same mechanisms allow the bacterium to bypass chemical and UV deactivation within free-living freshwater protozoa in water treatment plants and subsequently infect humans. Given the high rates of campylobacteriosis, southwestern Alberta is an ideal agroecosystem to conduct such studies. As part of this thesis**,** the following hypotheses were formulated:

1. *C. jejuni* is present in southwestern Alberta rivers and exhibits a seasonal trend of abundance and persistence similar to other waterborne pathogens.
2. A diverse community of viable protists, including phagotrophic protists are present in southwestern Alberta rivers and the composition of the protistan community varies seasonally.
3. The ability of *C. jejuni* to exploit phagotrophic protists is dependent on the bacterium possessing a functional flagellar export apparatus, the capability to communicate in a density dependent manner (quorum sensing), and the ability to produce the cytolethal distending toxin (CDT).

CHAPTER 3

Temporal distribution of *C. jejuni* in river waters of southwestern Alberta and evaluation of specialized culture-based isolation methods

ABSTRACT

Southwestern Alberta experiences high rates of campylobacteriosis yet the source of infectious *C. jejuni* cells responsible is as yet unknown. Water is thought to play a role in outbreaks as well as sporadic cases yet isolation of *C. jejuni* from river water for comparison is challenging. This may be due at least in part to the cells being present in a VBNC or stressed/injured state and unable to grow on traditional isolation media. In this study, seven enrichment techniques were evaluated for their ability to isolate *C. jejuni* from river waters in southwestern Alberta. Samples were collected on a weekly basis over a 1-year period from five sites including Willow Creek (one site), the Oldman River (three sites) and the Little Bow river (one site). The majority of isolates (93%) were from river water samples collected between December 17, 2008 and March 11, 2009 and were collected near waste water outflows in Fort McLeod and Lethbridge. The majority of *C. jejuni* isolates (72%) were recovered using two methods (ASBE and KM) with 21 and 20 isolates recovered, respectively. Real-time quantitative PCR applied to DNA extracts from river water samples indicates fluctuating densities of *C. jejuni* cells with no seasonal trends. Furthermore, there was no correlation between the density of *C. jejuni* cells and physicochemical properties of water (e.g. precipitation, river flow rate, dissolved oxygen, temperature, turbidity and chlorophyll-a).The presence of high copies of *C. jejuni* detected by qPCR when culture-based approaches were negative suggests an abundance of VBNC cells which may play a significant role in the epidemiology of campylobacteriosis.

3.1 INTRODUCTION

Campylobacter jejuni is the predominant etiological agent of campylobacteriosis, which is the leading cause of bacterial enteritis among developed nations. In a recent study, the annual cost of acute gastrointestinal illness (AGI), including hospital time and missed paid employment was estimated to be $3.7 billion in Canada (Public Health Agency of Canada; http://www.phac-aspc.gc.ca/publicat/ccdr-rmtc/08vol34/dr-rm3405b-eng.php#ref). Campylobacteriosis is characterized by diarrhea, vomiting malaise and can lead to serious secondary complications such as Gullian-Barré Syndrome (GBS) (Acheson and Allos, 2001), reactive arthritis (ReA) (Hannu *et al.*, 2004), irritable bowel syndrome (IBS) (Kalischuk and Buret, 2010) and inflammatory bowel disease (IBD) (Gradel *et al.*, 2009). It is believed that *C. jejuni* infection is considerably underdiagnosed, with estimates as high as 49 actual cases for every confirmed case (Thomas *et al.*, 2006).

C. jejuni infection trends in temperate regions resemble that of classical waterborne pathogens, with a peak in the early spring, a trough in the summer and a smaller peak in fall (Jones, 2001). Recent surveillance studies conducted in southwestern Alberta support this trend within inhabitants of this region and also suggest infection rates significantly higher than previously thought (Inglis *et al.*, 2005; Inglis *et al.*, unpublished data). The source of infection is difficult to pinpoint because many *C. jejuni* infections are sporadic; also greater sampling in clinical settings compared to environmental isolation obfuscates source attribution. Water systems have been associated with campylobacteroisis outbreaks (Clark *et al.*, 2003; Richardson *et al.*, 2007) and are also thought to play a role in sporadic infection. The presence of *C. jejuni* in river water is thought to be exclusively due to fecal contamination as *C. jejuni* is not believed to be able to replicate apart from warm-blooded hosts and laboratory culture media (Abulreesh *et*

al., 2006; Jones, 2001). Currently, little is known with regards to the seasonal occurrence of *C. jejuni* river water in southwestern Alberta, however, the vast collection of clinical isolates combined with high rates of infection make it an area of interest for environmental studies preceding molecular epidemiological investigations

Recovery of *C. jejuni* from water is challenging as the bacterium often occurs in low densities and may be in a VBNC state (Rollins and Colwell, 1986), thought by many to be a physiological response to adverse conditions. Cells may also be injured as a result of exposure to a variety of environmental stressors including low temperature, drying, osmotic shock, and predation (Wu, 2008). VBNC cells, by definition, cannot be cultured using laboratory techniques. Injured cells, however, can be recovered through the use of specialized enrichment techniques.

The objectives of this study were to: (1) evaluate various culture-based techniques for isolation of *C. jejuni* from water samples; (2) expand the collection of environmental *C. jejuni* isolates from southwestern Alberta for future epidemiological studies; (3) determine the prevalence of *C. jejuni* in southwestern Alberta rivers over a 1-year period using quantitative PCR; and (4) examine physicochemical properties of river water in relation to the prevalence of *C. jejuni*.

3.2 MATERIALS AND METHODS

Sample collection and processing

Southwestern Alberta is a semi-arid ecosystem dominated by a short-grass grassland ecosystem. The region contains the headwaters of the Oldman River and its tributaries which constitute the primary watershed of the region. A total of five river sites in the Oldman watershed were sampled on a weekly basis over a 1-year period (April

2008 to May 2009). Three of the five sites were along the Oldman River from west to east (49°44'35.15"N 113°22'27.60"W; 49°43'5.10"N 112°51'56.97"W; 49°51'24.41"N 112°37'27.10"W), and are subjected to an increasing gradation of inputs from west to east. The remaining two river sites were tributaries of the Oldman River: Willow Creek (49°45'15.32"N 113°24'23.72"W) and the Little Bow River (49°54'5.22"N 112°30'24.19"W) (Figure 3.1). Latitude and longitude coordinates of sampling sites were determined using Google Earth (version 6.1.0.5001, Google Inc., [http://earth.google.com/]).

Samples were collected at 1-week intervals commencing May 7, 2008 through to May 13, 2009. At each site, samples of approximately 800-900 mL were obtained using 1 L Nalgene bottles attached to the end of a sampling pole; water was collected at a depth of ≈ 20 to 30 cm in the flowing portion of the river. Samples were stored on ice and processed within 6 h of collection.

Flow rate, precipitation, temperature, pH, turbidity and chlorophyll-a measurement

Temperature and dissolved oxygen were measured at each sample site at the time of sample collection using a handheld Accument AP74 (Fisher Scientific, Nepean, Ontario, Canada). Turbidity and pH were measured in the lab using a Lamotte handheld Turbidity Meter 2020e (Cole Parmer, Vernon Hills, IL) and Accument AB15 pH meter (Fisher Scientific), respectively. Chlorophyll-a was determined according to the ISO 10260 protocol. Briefly, 250 mL of River water was filtered through a 0.45 µm GF/F filter (Whatman, Florham Park, NJ). The filter was then added to a 50 mL falcon tube containing 20 mL of 90 % ethanol, heated to 75 °C, and the sample resuspended by gently

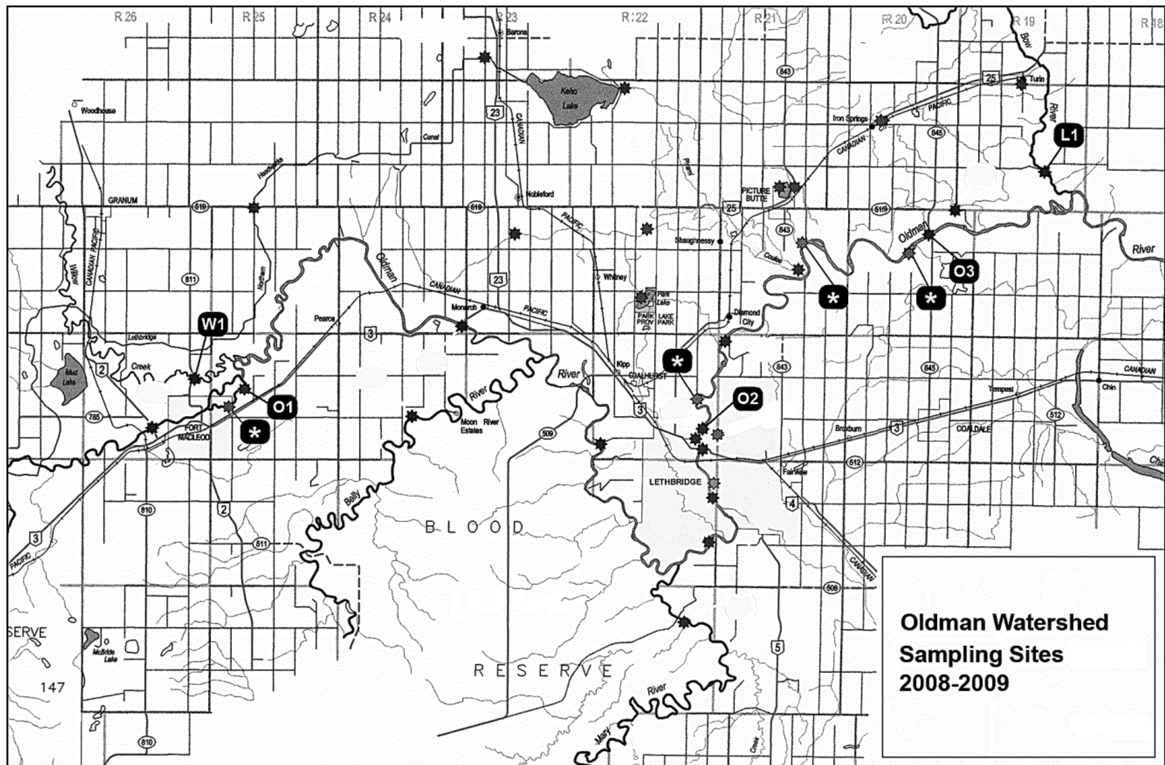

Figure 3.1. Map of southwestern Alberta showing river sampling sites along the Oldman River (O1-O3), Willow Creek (W1), and Little Bow River (L1). Wastewater outflows for the Town of Fort MacLeod, City of Lethbridge, Town of Picture Butte, and Town of Coaldale are marked with asterisks from left to right, respectively. (Source: Alberta Environment and Sustainable Resource Development.)

mixing the tube by inversion. The tubes were then cooled to room temperature and the filter removed with forceps. Large particulates were pelleted such that they did not interfere with the assay by centrifuging at 6 000 x g for 5 min. Following centrifugation, 1 mL of supernatant was transferred to a disposable cuvette and the OD_{665} and OD_{750} measured in relation to 90 % ethanol (blank). In a separate tube, 5 mL of supernatant was acidified by the addition of 5 µL of 3 M HCl and then the OD_{665} and OD_{750} were measured by spectrophotometry and chlorophyll-a calculated as per ISO 10260 guidelines. River flow rates were obtained from the Alberta Environment and Sustainable Resource Development (ESRD) website (http://www.environment.alberta.ca/apps/basins/default.aspx) for Willow Creek at ESRD site 05AB046 (Highway 811 bridge), for the Oldman River at ESRD site 05AD007 (near Lethbridge), and for the Little Bow River at ESRD site 05AC023 (near the confluence of the Little Bow and Oldman River) at each sampling time. Total precipitation data for the week leading up to each sampling time was calculated from data obtained from ESRD weather data collection sites located at Fort McLeod (05AB813), Lethbridge (05AG806) and Barnwell (05AG801).

Water sample processing for *C. jejuni* isolation

River water (250 mL) was filtered through a 0.45 µm GF/F pre-filter (Whatman) followed by a 0.22 µm Iso-Grid final filter (Neogen Corp., Lansing, MI) under vacuum using a six-place filtration manifold (Advantec MFS Inc., Dublin, CA) fitted with an Iso-Grid filtration unit (Neogen Corp.). The two filters per sample were then combined in a 50 mL Falcon tube with 20 mL 1 X phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4; pH 7.4), and vortexed to detach material

from the filter. Filters were subsequently removed and the suspension centrifuged at 14 900 x g for 15 min at 4°C. The top ≈ 17 mL of supernatant was discarded and pellets were re-suspended in the remaining 3 mL of PBS.

Specialized isolation methods and PCR-based identification of *C. jejuni*

C. jejuni was isolated using a variety of specialized culture-based methods including direct plating and enrichment. For direct plating (KS), 25 µL of the filtered suspension from each sampling site and time was spread on Karmali agar plates containing selective supplement SR167 (Oxoid Ltd., Nepean, ON) in duplicate and incubated at 37 °C in a microaerobic environment (5 % O_2, 30 % H_2, 10 % CO_2 and N_2 balance). Bolton enrichment (BE) involved adding 150 µL of the filtered suspension from each sampling site and time to 2 mL of Bolton broth (Oxoid Ltd.) containing Bolton supplement (Oxoid Ltd.) in a 10 mL test tube. The tubes were then incubated in a microaerobic atmosphere generated by CampyPacs (BBL Microbiology Systems, Cockeysville, MD) at 175 rpm using a ramped protocol consisting of 30 °C for 3 h, 37 °C for 2 h, and 40 °C for 24 h. Following the enrichment period, 10 µL from each tube was streaked onto Karmali agar plates containing selective supplement and incubated microaerobically at 37 °C for 48 h. For *Arcobacter* selective isolation broth enrichment (ASBE), 100 µL of the filtered suspension from each sampling site and time was inoculated into a test tube containing 2 mL of *Arcobacter* selective isolation broth (Houf *et al.*, 2001; Van Driessche *et al.*, 2003) in duplicate and incubated at 30 and 37 °C. At 24 h and 48 h (ASBE), 10 µL of the enrichment broth was streak plated onto Columbia agar supplemented with 5 % sheep blood (CSB) which was then incubated microaerobically for an additional 6 days at the corresponding temperature to the enrichment culture;

cultures were examined at 24 h intervals. One hundred µL from each site and time was added to 2 mL of JM broth (Johnson and Murano, 1999). The cultures were then incubated at 30 °C and 37 °C microaerobically, and processed at 24 and 48 h by streaking 10 µL onto JM agar; the JM plate was then incubated under the same conditions as the originating tubes. Cultures were examined for growth at 48 h and 6 days. Membrane filtration (MF) was accomplished by spreading 200 µL of the suspension on a 0.45 µm filter (Neogen Corp.) previously placed on a CSB plate. The medium and filter were then incubated microaerobically at 37 °C for 1 h. After the 1 h incubation, filters were carefully removed and cultures were incubated microaerobically at 37 °C for 5 days; cultures were examined at 24 h intervals.

Unless otherwise indicated, incubations were performed in air tight culture jars under microaerobic conditions (5 % O_2, 30 % H_2, 10 % CO_2 and N_2 balance). Cells from presumptive *C. jejuni* positive colonies were examined microscopically for physical characteristics (size, shape, motility) typical of campylobacteria. Cells of presumptive campylobacteria were then streaked onto CSB and incubated microaerobically for 48 to 72 h. Isolates were stored in Columbia broth with 30 % glycerol at -80 °C. In an attempt to minimize the collection of clones, a maximum of two discrete colonies of a single morphology indicative of campylobacteria for a given sampling site and time were glycerol stocked.

Table 3.1. Primers for the identification of *C. jejuni* isolates and quantitative detection of *C. jejuni* by qPCR from river water including an internal amplification control (IAC)

Primer	**Sequence (5'-3')**	**Source**
MD*map*A1Upper	CTATTTTATTTTTGAGTGCTTGTG	(Denis *et al.*, 2001)
MD*map*A2Lower	GCTTTATTTGCCATTTGTTTTATTA	(Denis *et al.*, 2001)
Cj*hip*OF	AAATAGGAAAAACAGGCGTTGT	Inglis *et al.*, unpublished
Cj*hip*OR	TATCATTAGCCTGTGCAAGACC	Inglis *et al.*, unpublished
*Dap*O-1f	ACGACACAACCTGTCGCAAGGG	Inglis *et al.*, unpublished
*Dap*O-1r	ATGCCATATCCTGTAGCTGT	Inglis *et al.*, unpublished
IAC_F	GGTATGCTAGCCCCGCTTAGGGT	This study
IAC_R	TGCTCCAGAAAAGATGTCCAGCGG	This study

Colony PCR of presumptive *Campylobacter* isolates

All presumptive *C. jejuni* isolates were screened using a *C. jejuni* specific primer set targeting the *map*A gene (Table 3.1) (Denis *et al.*, 2001). A small colony of freshly growing cells was suspended in 100 µL of sterile 1/10 TE buffer (10 mM Tris-Cl, 1mM EDTA, pH 8.0) of which 2 µL was used as template. PCR was performed in 20 µL reactions including 1 X Qiagen PCR buffer, 0.2 mM dNTPs, 0.5 µM of each primer, 0.1 µL (5 U/ µL) Hotstar taq polymerase (Qiagen Inc., Mississauga, ON), 2 µg bovine serum albumin (BSA), 2 mM $MgCl_2$ and 2 µL template. Thermal cycling included a pre-treatment at 95 °C for 15 min for enzyme activation followed by 35 cycles of 30 s at 95 °C, 1.5 min at 58 °C, and 1 min at 72 °C, and a final extension step for 10 min at 72 °C. PCR products were then resolved using the QIAxcel capillary electrophoresis system. As it was later confirmed that the *map*A primer set amplified some *C. coli* and *Arcobacter butzleri*, *map*A positive isolates were later screened for the *hip*O gene using PCR primers Cj*hip*OF and Cj*hip*OR (Table 3.1) (Inglis *et al.*, unpublished). Isolates positive for both PCR reactions were identified as *C. jejuni*.

Real-time Quantitative PCR (qPCR)

Development of internal amplification control (IAC). To prevent false negative results (e.g. due to the presence of PCR inhibitors or failed DNA extraction), a synthetic IAC was designed. The IAC target was based on a 247 bp region unique to *Pyrococcus yayanosii* CH1, a hyperthermophilic, obligate piezophile recently isolated from a hydrothermal vent in the Pacific Ocean (Jun *et al.*, 2011). *Pyrococcus yayanosii* CH1 was selected as surface waters are inhospitable to this organism and the natural habitat is located deep in the ocean and thus there should be no potential for a background signal.

Primers were designed using the primer3 add on in Geneious 5.3 (Drummond *et al.*, 2010) and the 247 bp target was synthesized and cloned into the pIDTSMART-AMP plasmid (Integrated DNA Technologies Inc., Coraville, IA). Prior to extracting DNA from processed river water samples, 5 µL of IAC stock containing 2 x 10^5 IAC copies per µL was added to each sample. In the event no *C. jejuni* was detected by qPCR and the IAC did not amplify from the same sample, it would be attributed to PCR inhibition rather than a true negative result.

DNA extraction and quantitation of *C. jejuni* and the IAC. DNA was extracted using the PowerLyzer PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) according to manufacturer's protocol with the exception that the Qiagen TissueLyser LT was used for homogenization. Quantification of *C. jejuni* was performed using qPCR with the *Dap*O-1f and *Dap*O-1r primers (Table 3.1) in 20 µL reaction volumes containing 2 X Quantitect SYBR Green PCR master mix (Qiagen Inc.), 0.5 µM of each primer, 2 µg BSA and 2 µL template. Thermal cycling included a pre-heating step at 95 °C for 15 min followed by 94 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. Following amplification steps, a melting curve was generated by heating the reaction from 55 °C to 95 °C and measuring the fluorescence at 0.5 °C increments. A standard curve was prepared based on 10-fold serial dilutions of *C. jejuni* ATCC 33560 genomic DNA. The total amount of *C. jejuni* DNA present in the sample was determined with the knowledge that the *Dap*O primers target the nucleic acid sequence of a predicted protein, Cjprotein3 which is a single copy gene. qPCR for the IAC target was performed in a separate reaction with the IAC_F and IAC_R primer set using the same master mix as stated above. The thermal cycling was identical with the exception that an annealing temperature of 64 °C was used.

Statistical analysis

Physicochemical properties and *C. jejuni* densities determined by qPCR for each sampling site were tested for normality using the UNIVARIATE procedure in SAS (SAS Institute Inc.; Cary, NC, USA). Normality of each parameter was assessed by descriptive statistics (mean, mode, skewness and kurtosis) as well as the Shapiro-Wilk test for normality. As the data was not normally distributed, bivariate non-parametric (Spearman) correlation coefficients were calculated using the CORR procedure in SAS (SAS Institute Inc.).

3.3 RESULTS

Culture-based isolation of *C. jejuni* from river water sites

A total of 1099 potential *C. jejuni* were isolated, based on colony morphology and microscopy, over the 1-year study period. Of the collected isolates, 137 (12.5 %) were *map*A positive. Fifty-seven of the 137 *map*A positive isolates were *hip*O positive and deemed to be *C. jejuni* (5.2 % of total isolates). Nearly all (93.0 %) of the *C. jejuni* isolates were recovered from the Oldman river sampling sites, O1 (25 isolates) and O2 (28 isolates), both located downstream of the Fort McLeod and Lethbridge wastewater treatment outflows, respectively. One *C. jejuni* isolate was recovered from each O3 and L1, and no *C. jejuni* was isolated from site W1 (Table 3.2). The ASBE and KS isolation methods outperformed the other isolation methods as they yielded the greatest number of isolates (21 and 20, respectively); these methods were followed by BO_{37} with 11, MF with three, BO_{30} and JM_{37} each with one and JM 30 with 0 *C. jejuni* isolates (Table 3.2). The majority of confirmed *C. jejuni* isolates (51 out of 57 isolates) were collected between December 17, 2008 and March 11, 2009 (Figure 3.2).

Culture-independent detection and quantification of *C. jejuni* by qPCR

Quantitative PCR results for *C. jejuni* using the novel *Dap*O primer set showed a sporadic occurrence of *C. jejuni* in river water throughout the sampling period for all sites tested in southwestern Alberta (Figure 3.3). At many of the sampling times, no *C. jejuni* was detected; then at adjacent times high copy numbers (≈$1x10^2$-$5x10^4$ per 100 mL) were present. There was also no apparent seasonal trend at any of the sampling sites (Figure 3.3). The greatest amount of *C. jejuni* estimated at each site for a given sampling time was $8.3x10^3$, $2.9x10^3$, $1.9x10^4$, $1.2x10^3$, and $4.7x10^4$ cells per 100 mL corresponding to sites W1, O1, O2, O3 and L1. However, none of the observed maxima for *C. jejuni* copy number predicted by qPCR at each sampling site corresponded to the same sampling time (September 17, 2008, July 23, 2008, March 11, 2009, April 22, 2009, and September 3, 2008 for sites W1, O1, O2, O3 and L2, respectively) (Figure 3.3). The IAC was detected in all samples (data not shown) and thus samples in which no *C. jejuni* was detected by qPCR were considered legitimate negatives.

Physicochemical properties of water samples and statistical analyses

Large rainfall events, high flow rates and high turbidity were observed in the spring (Figures 3.5-3.6). There was little change in pH throughout the study period with measurements between 7.57 and 8.65. Dissolved oxygen ranged from 8.01 to 12.5 mg/L, and chlorophyll-a ranged from 0 to 24.9 mg/m^3 (data not shown). As not all parameters were normally distributed, non-parametric Spearman rank correlation coefficients were calculated to examine the relationship between the occurrence of *C. jejuni* (estimated by

Table 3.2. Culture based isolation of *C. jejuni* from river water sampling sites. Sites are: Oldman River downstream of Fort McLeod (O1); Oldman River downstream of Lethbridge (O2); Oldman River under the Highway 845 bridge (O3); Little Bow River near the confluence with the Oldman River (L1); and Willow Creek north of Fort McLeod (W1). See Figure 3.1 for additional information on the sites.

	Isolation method						
Sample site	**KS**	**ASBE**	**BO_{37}**	**BO_{30}**	**JM_{37}**	**MF**	**Total**
Willow Creek (W1)	0	0	0	0	0	0	0
Oldman River (O1)	8	10	4	0	1	2	25
Oldman River (O2)	11	10	5	1	0	1	28
Oldman River (O3)	1	0	2	0	0	0	3
Little Bow River (L1)	0	1	0	0	0	0	1
Total	20	21	11	1	1	3	57

Figure 3.2. Temporal isolation of *C. jejuni* for each culture-based isolation method.

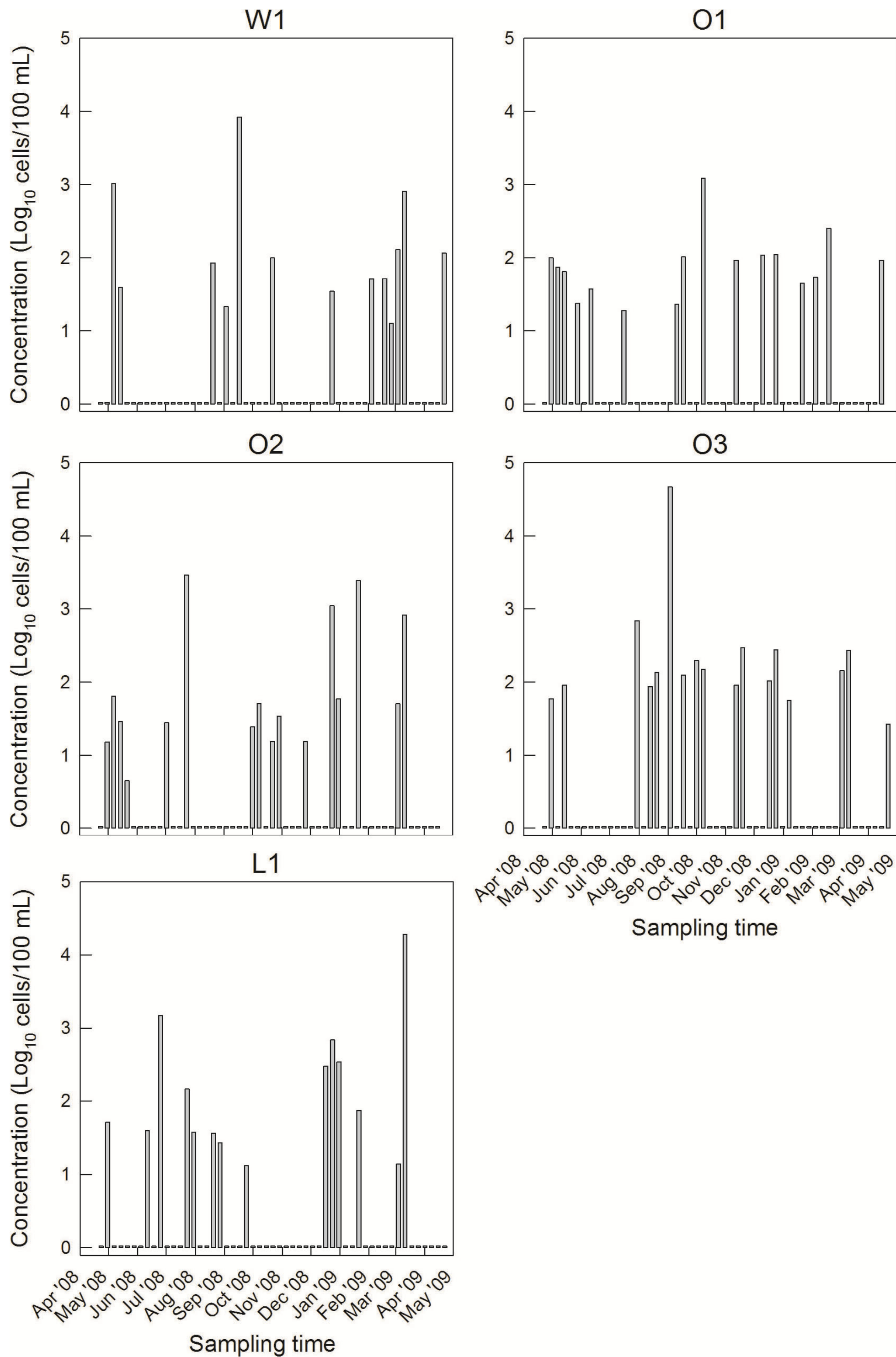

Figure 3.3. Estimation of *C. jejuni* cell densities (Log_{10} cells per 100 mL) in river water by qPCR. Sites are: Oldman River downstream of Fort McLeod (O1); Oldman River downstream of Lethbridge (O2); Oldman River under the Highway 845 bridge (O3); Little Bow River near the confluence with the Oldman River (L1); and Willow Creek north of Fort McLeod (W1). See Figure 3.1 for additional information on the sites.

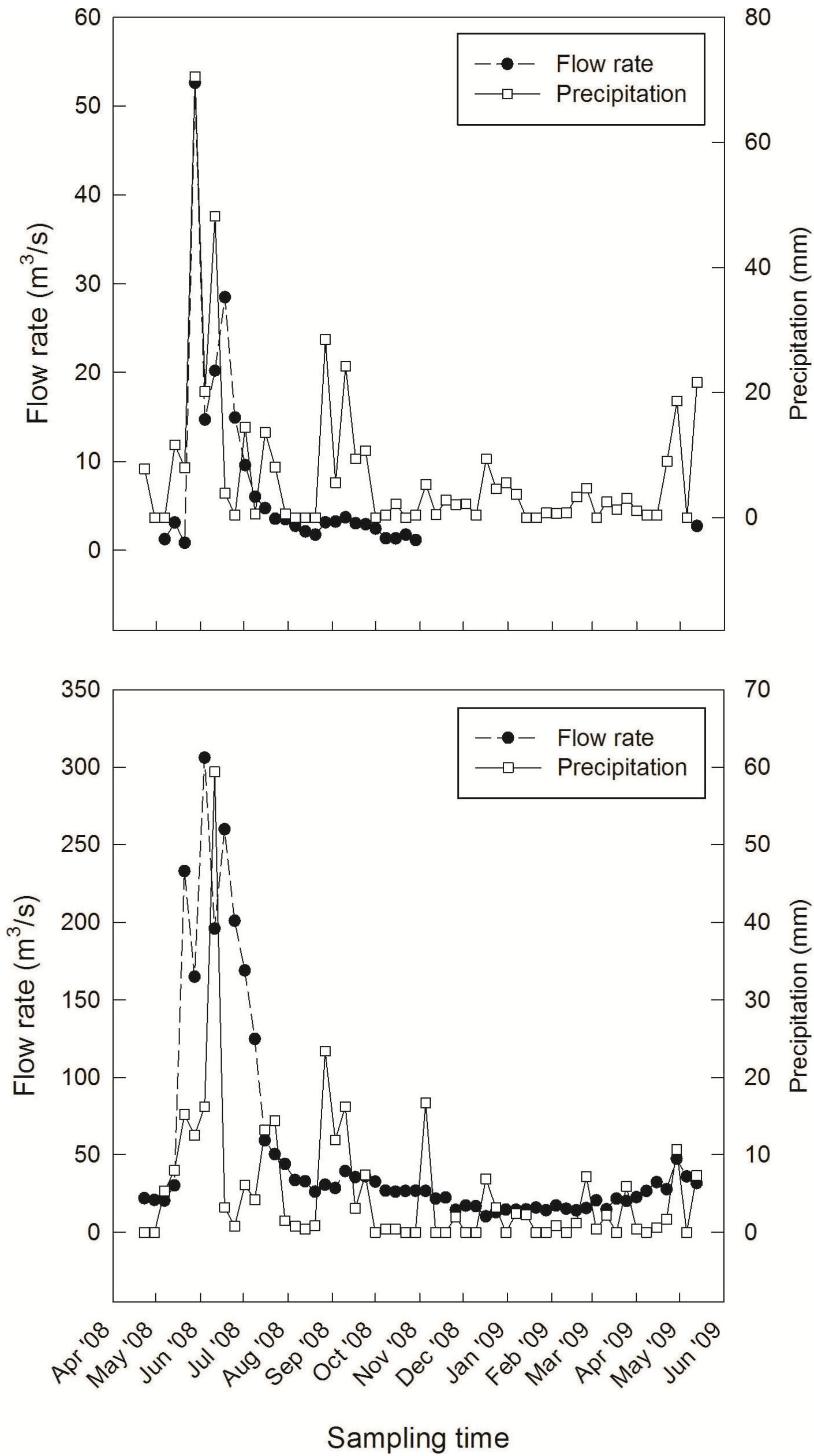

Figure 3.4. Representative flow rate in m^3/s (filled circles) and total precipitation in mm (unfilled squares) for Willow Creek (W1) (top) and Oldman River (O1) (bottom) over the study period.

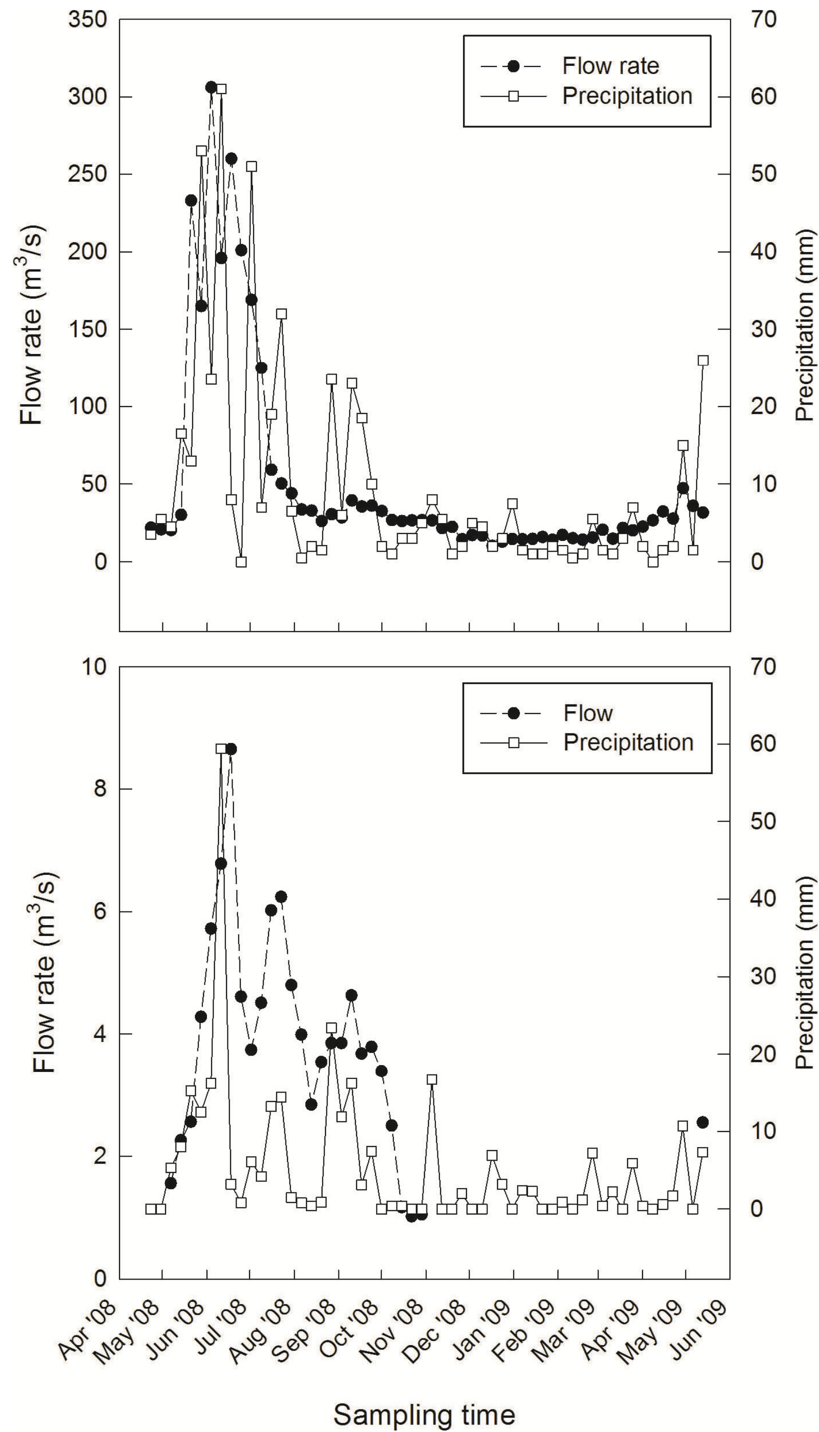

Figure 3.5. Representative flow rate in m^3/s (filled circles) and precipitation in mm (unfilled squares) for sites O2 and O3 (top) and site L1 (bottom) over the study period. Sites are: Oldman River downstream of Fort McLeod (O1); Oldman River downstream of Lethbridge (O2); Oldman River under the Highway 845 bridge (O3); Little Bow River near the confluence with the Oldman River (L1); and Willow Creek north of Fort McLeod (W1). See Figure 3.1 for additional information on the sites.

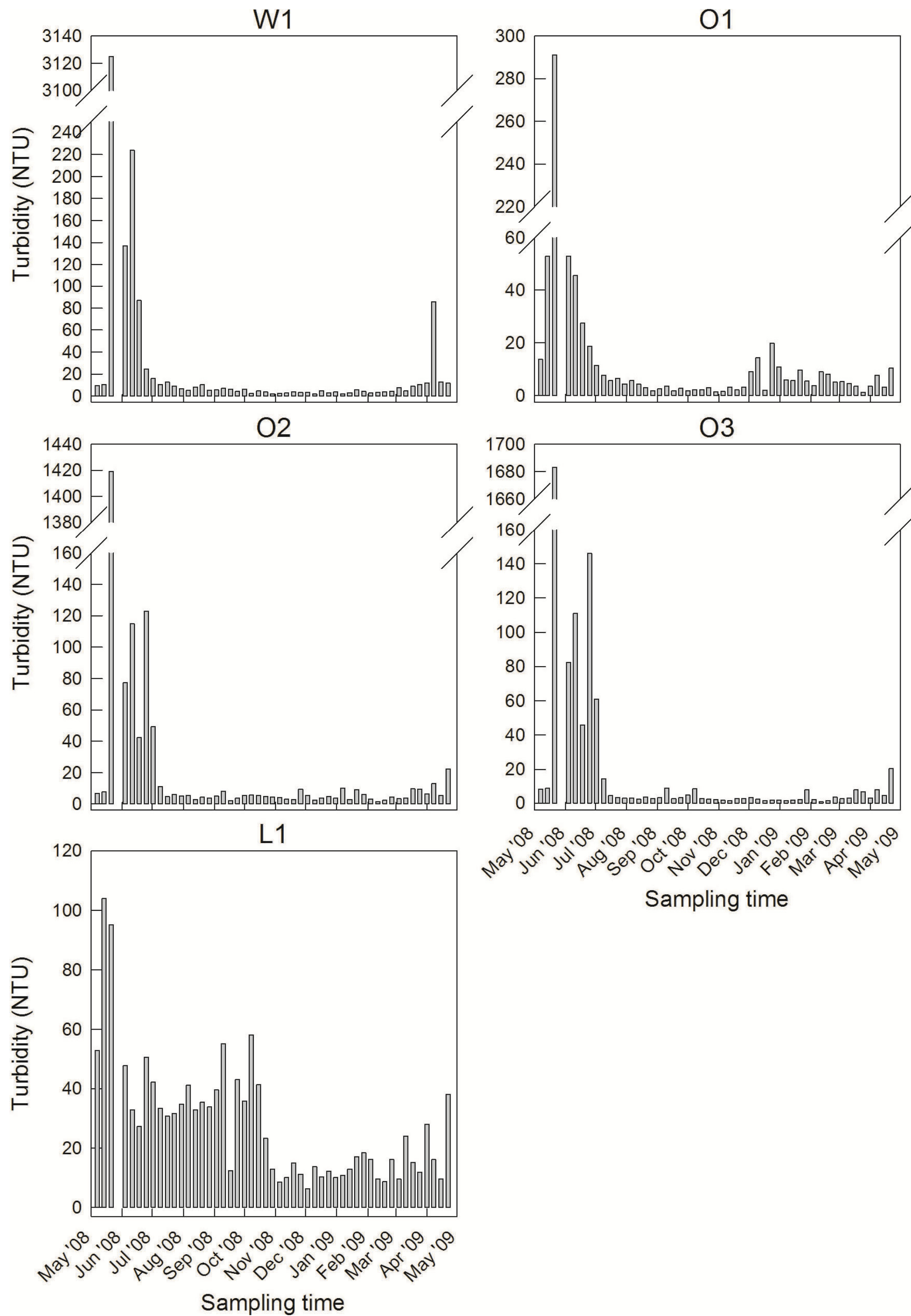

Figure 3.6. Turbidity (NTU) at each of the water sampling sites over the study period. Sites are: Oldman River downstream of Fort McLeod (O1); Oldman River downstream of Lethbridge (O2); Oldman River under the Highway 845 bridge (O3); Little Bow River near the confluence with the Oldman River (L1); and Willow Creek north of Fort McLeod (W1). See Figure 3.1 for additional information on the sites.

Table 3.3. Spearman correlation coefficients (CC) and p-values (p) calculated for *C. jejuni* abundance (estimated by qPCR) and the physicochemical parameters for N measurements throughout the study period for each of the five sampling sites. Statistically significant correlations ($p \leq 0.05$) are marked with an asterisk.

Site	Statistic	Flow rate	Precipitation	pH	Turbidity	Dissolved oxygen	Chlorophyll-a	Water temperature
	CC	-0.322	-0.056	-0.178	-0.024	0.193	0.092	0.515
W1	P	0.109	0.689	0.216	0.871	0.390	0.684	0.156
	N	26	53	50	50	22	22	9
	CC	-0.138	-0.218	-0.165	0.130	0.326	0.039	-0.517
O1	P	0.331	0.120	0.257	0.372	0.138	0.857	0.154
	N	52	52	49	49	22	24	9
	CC	-0.088	-0.016	-0.067	-0.082	-0.094	-0.159	-0.121
O2	P	0.531	0.911	0.643	0.573	0.679	0.448	0.739
	N	53	53	50	50	22	25	10
	CC	-0.085	0.067	-0.124	-0.047	-0.105	-0.012	0.757
O3	P	0.547	0.639	0.395	0.747	0.643	0.956	*0.011
	N	52	52	49	49	22	24	10
	CC	-0.205	-0.072	0.058	0.048	0.169	0.221	0.034
L1	p	0.316	0.609	0.687	0.742	0.453	0.299	0.925
	N	26	53	50	50	22	24	10

qPCR) and the various physicochemical properties recorded during the study period (Table 3.3). Spearman rank correlation coefficients indicate that there was no correlation between *C. jejuni* concentration and any of the physicochemical parameters measured with the exception of water temperature which was positively correlated ($p = 0.011$) with water temperature at Oldman River site O3.

3.4 DISCUSSION

Campylobacteriosis is the most common cause of enteric disease in Alberta (Alberta Health; http://www.health.alberta.ca/documents/Guidelines-Campylobacteriosis-2011.pdf) and provincial rates of disease are highest in the CHR (Alberta Health; http://www.health.alberta.ca/documents/Notifiable-Diseases-Report-2004.pdf); yet at present, the source of *C. jejuni* isolates infecting humans in this region is uncertain. Water has been observed to play an important role in the transmission of *C. jejuni* in outbreak scenarios (Clark *et al.*, 2003) and is also associated with sporadic infection (Taylor *et al.*, 1983). For molecular epidemiological investigations to be effective, the organism of interest must first be isolated from both infected individuals and the environmental source of the infectious agent. This is of particular importance for pathogens such as *C. jejuni*, which is considered a generalist as it colonizes and/or infects a broad range of hosts (Gripp *et al.*, 2011). Our laboratory has acquired a large collection of *C. jejuni* isolates from inhabitants of the CHR and molecular typing efforts are underway. However, at present, a substantial gap exists in the isolate collections of environmental *C. jejuni* from the CHR. Consequently, this hinders the ability of researchers to attribute molecular signatures from clinical *C. jejuni* isolates to environmental sources.

The isolation of *C. jejuni* from water is challenging as not only is there a large amount of background including fecal coliforms (Hyland *et al.*, 2003) and native waterborne bacteria; but also a number of bacterial species closely related to *C. jejuni*, such as *Arcobacter* spp., *Helicobacter* spp. and other *Campylobacter* spp. that grow under similar conditions. *C. jejuni* is also present at low concentrations, thus requiring filtration or centrifugation to concentrate water samples. To facilitate the recovery of injured cells from the environment, many enrichment methods have been developed. The widely adopted Bolton broth contains animal blood to offset toxic effects induced by oxygen exposure (Bolton *et al.*, 1984), to which sub-lethally injured *C. jejuni* cells are particularly vulnerable (Humphrey, 1988). Temperature also plays an important role on the recovery of injured *C. jejuni*, Humphrey and Muscat (1989) demonstrated that incubation at 37 °C rather than 43 °C or a combination of the two temperatures enhances the recovery sub-lethally injured *C. jejuni* from water samples as opposed to incubation at 43 °C alone (Humphrey and Muscat, 1989). It is also important to use multiple enrichment methods as different enrichment methods have been shown to bias the recovery of *C. jejuni* subtypes (Williams *et al.*, 2012).

Despite the use of appropriate incubation temperatures and a variety of complex growth media, *C. jejuni* was seldom isolated from river water in the CHR. The KS and ASBE methods performed best at isolating *C. jejuni* from the river water. Of the confirmed *C. jejuni* isolates, the majority (93%) were from samples collected between December 17, 2008 and March 11, 2009 from sites downstream of the Fort McLeod (n=25) and Lethbridge (n=28) waste water outflows. This seasonal trend is consistent with observations from other culture-based studies worldwide including studies of Morecambe Bay and the river Lune in England, where the amount of sunlight per day showed a

negative correlation with *Campylobacter* numbers (Jones, 2001). While it is tempting to attribute the isolates downstream of waste water outflows to human waste, there were no isolations in the spring and autumn to accompany spikes observed in isolation of *C. jejuni* from humans within the study area (Inglis *et al.*, unpublished data). Furthermore, at the time of sample collection, the Lethbridge waste water treatment facility irradiated all effluent prior to discharge while the Fort McLeod waste water treatment facility did not; yet *C. jejuni* was isolated from both sites. Increased grazing pressure from protists during the warmer months may have contributed to the reduced abundance of *C. jejuni*. However, it is not known how *C. jejuni* responds to grazing in aquatic ecosystems. It may follow that the VBNC state also protects from grazing by modification of surface structures, as a result, protists may preferentially ingest active *C. jejuni* rather than cells in the VBNC state.

The use of molecular-based detection methods for bacterial pathogens has received increased attention in recent years due to the ease of use and ability to detect low concentrations of the target DNA molecule. The data also suggests that at times throughout the study period, *C. jejuni* contamination of river water within the CHR is extremely high in comparison to other studies using similar techniques. For example, a recent study conducted in river water in southern Ontario estimated 103 *C. jejuni*, *C. coli* and *C. lari* cells per 100 mL, although PCR inhibition was not addressed and this number is an estimate for the three organisms combined (Van Dyke *et al.*, 2010). Consequently, the true concentration of *C. jejuni* may be even higher. Van Dyke et al. (2012) utilized PCR primers targeting the 16S rRNA gene, which is usually restricted to metagenomic studies as the 16S rRNA gene is present in all bacteria yet is variable enough to allow for species delineation in most cases. Targeting the 16S rRNA gene is likely why they were

unable to differentiate between the three *Campylobacter* spp. and highlights the need for primers capable of discerning *C. jejuni* from closely related species. The *Dap*O qPCR primer set developed in the present study was validated against a panel of *Campylobacteria* and revealed 100 % specificity for *C. jejuni*. With the exception of water temperature at Oldman River site O1, Spearman correlation coefficients yielded no correlation between the abundance of *C. jejuni* and any of the physicochemical parameters included in the study. This is not entirely unexpected, as *C. jejuni* does not replicate outside warm-blooded hosts; however, a previous study investigating fecal coliforms in this region observed the highest fecal coliform counts following heavy rainfall events and thus suggesting the contribution of surface run-off to spikes in water contamination (Hyland *et al.*, 2003). In the present study, qPCR suggests a sporadic presence and abundance of *C. jejuni* which may be the result of a diverse group of source inputs (waterfowl, agricultural runoff, waste water) in southern Alberta. It is also possible that *C. jejuni* is not uniformly distributed in the water and thus collecting a small sample from one location in the water column may result in more variance between samples.

Ethidium monoazide (EMA) and propidium monoazide (PMA) have previously been used to limit PCR amplification to DNA present in cells with intact membranes and thus considered live. It was not used in the present study as low concentrations of *C. jejuni* were expected based on previous studies and monoazide treatment has been previously shown to penetrate a proportion of living bacterial cells (Nocker *et al.*, 2006) including *C. jejuni* (Flekna *et al.*, 2007). The inability to isolate *C. jejuni* at times when it was present according to qPCR considered together with the observation that there was no conspicuous change in quantitative enumeration during times of increased isolations suggests the presence of VBNC cells. It is unlikely the molecular signatures for *C. jejuni*

originated from free DNA as (1) the water samples were filtered through a 0.22 μM filter which would not retain free DNA and (2) it is inconsistent with current data indicating entrance into the VBNC state (Baffone *et al.*, 2006). Evidence for resuscitation of VBNC *C. jejuni* by passaging through a vertebrate host (Jones *et al.*, 1991) and inoculation into embryonated eggs (Cappelier *et al.*, 1999) has been observed, however, enrichment techniques are insufficient at recovering VBNC cells. The VBNC state in bacteria is widely considered to be a survival strategy activated when exposed to environmental stresses or nutrient limitation (Oliver, 2010). The microbial scout hypothesis suggests that VBNC cells serve as scouts by "waking up" in a stochastic manner, independent of environmental cues (Buerger *et al.*, 2012). The waking up of the scout allows it to survey the environment and if unable to replicate, only the scout perishes rather than the entire cell population. Conversely, if nutrients are present then the scout is able to replicate and potentially induce nearby cells to exit dormancy. The hypotheses above coupled with the observation that *C. jejuni* remains culturable for longer periods of time in microcosm water held when at low temperatures (Thomas *et al.*, 2002), may in part explain why the majority of *C. jejuni* isolated were during the winter months. Although advanced culture-based techniques were utilized, in the event *C. jejuni* enters a VBNC state when it enters river water it would not be recovered.

In conclusion, the culture-based and culture independent detection methods used in this study increased current knowledge of the distribution of *C. jejuni* in river waters in southwestern Alberta. The combined data suggests the presence of VBNC cells and physicochemical properties of river water had no influence on the abundance of *C. jejuni*. Isolates collected during this study may be used in the future for epidemiological studies.

CHAPTER 4

Seasonal diversity of planktonic protists in southwestern Alberta rivers over a one year period revealed by T-RFLP and 18S rDNA clone libraries[1]

ABSTRACT

The temporal dynamics of planktonic protists in river water has received limited attention despite their ecological significance and recent studies linking phagotrophic protists to the persistence of human pathogenic bacteria. Using molecular-based techniques targeting the 18S rRNA gene, we studied the seasonal diversity of planktonic protists in southwestern Alberta rivers (Oldman River Basin) over a one year period. Nonmetric multidimensional scaling analysis of terminal restriction fragment length polymorphism (T-RFLP) data revealed distinct shifts in protistan community profiles that corresponded to season, rather than geographical location. Community structures were examined using clone library analysis; HaeIII restriction profiles of 18S rDNA amplicons were used to remove prevalent solanaceous plant clones prior to sequencing. Sanger sequencing of the V1-V3 region of the 18S rDNA clone libraries from the spring, summer, fall and winter supported the T-RFLP results, and showed marked seasonal differences in protistan community structure. The spring library was dominated by Chloroplastidae (29.8 %), Centrohelida (28.1 %), and Alveolata (25.5 %) while the summer and fall libraries primarily contained fungal clones (83.0 % and 88.0 %, respectively). Alveolata (35.6 %), Euglenozoa (24.4 %), Chloroplastida (15.6 %), and

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[1] A version of this chapter was published as: Thomas M.C., Selinger L.B., Inglis G.D. (2012). Seasonal diversity of planktonic protists in Southwestern Alberta Rivers over a 1-year period as revealed by terminal restriction fragment length polymorphism and 18S rRNA gene library analyses. *Applied and Environmental Microbiology*, **78**(16):5653-5660.

Fungi (15.6 %) dominated the winter library. These data demonstrate that planktonic protists, including protozoa, are abundant in river water in southwestern Alberta, and conspicuous seasonal shifts occur in community structure.

4.1 INTRODUCTION

Protists play an integral role in aquatic ecosystems. They are responsible for primary production, mobilizing trace nutrients, and controlling bacterial populations yet they remain understudied. They form a complex group of organisms spanning all eukaryotic kingdoms and vary substantially in size, shape, and motility. These characteristics were once relied upon exclusively by taxonomists for classification, however, the complexity of protistan morphology demands years of study and experience for accurate taxonomic placement. Furthermore, morphological-based identification is time consuming and not always accurate. Recent studies have shown that organisms that were once considered to be of single morphospecies have highly variable 18S rRNA gene sequences and represent many distinct species (Kim *et al.*, 2004). Conversely, protists possessing distinctly different morphologies (i.e. considered to be different morphospecies) have been shown to possess identical 18S rDNA sequences (Logares *et al.*, 2007). As a result, protistologists now rely on molecular-based methods, such as: terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), and 18S rRNA gene libraries to study the community structure of these organisms.

The majority of protistan studies conducted to date have focused on protists in oceanic ecosystems (Diez *et al.*, 2001b; Edgcomb *et al.*, 2011; Edgcomb *et al.*, 2002; Emilio O. Casamayor *et al.*, 2002; Li *et al.*, 2011; Not *et al.*, 2009), as they produce

upwards of half of the world's oxygen and are the basis of aquatic food webs. Freshwater systems (Kiss *et al.*, 2009) and water distribution systems (Valster *et al.*, 2009) are attracting more attention as of late, due in part to experimental evidence linking freshwater protists with the protection of human pathogens (King *et al.*, 1988). Phagotrophic protists (i.e. protozoans), such as *Acanthamoeba spp*., have been shown to play a role in the persistence of human pathogens, including *Campylobacter jejuni* (Axelsson-Olsson *et al.*, 2005; Snelling *et al.*, 2008; Snelling *et al.*, 2005), *Legionella pneumophila* (Abu Kwaik *et al.*, 1998; King *et al.*, 1988), *Mycobacterium avium* subspecies *paratuberculosis* (Gardner *et al.*, 2011), and *Vibrio cholerae* (Abd *et al.*, 2009; Sandström *et al.*, 2010), and thus may play a role in transmission of infectious cells to humans. This presents a significant risk for public health as *Acanthamoeba* cells are highly resistant to UV irradiation and oxidative treatments, and they may survive water treatment processes (King *et al.*, 1988). To date, limited research has examined protists in freshwater ecosystems (Carrias *et al.*, 1996; Creer, 2010; Kiss *et al.*, 2009; Kopylov and Kosolapov, 2011; Nolte *et al.*, 2010; Ribblett *et al.*, 2005; Shi *et al.*). Given the potential importance of protists in freshwater ecosystems to public health, it is of the utmost importance that we achieve a better understanding of planktonic protistan diversity.

According to the Public Health Agency of Canada's Notifiable Diseases On-Line (http://dsol-smed.phac-aspc.gc.ca/dsol-smed/ndis/index-eng.php) and Alberta Health`s 2004 Notifiable Diseases Report (http://www.health.alberta.ca/documents/Notifiable-Diseases-Report-2004.pdf), southwestern Alberta has substantially higher rates of campylobacteriosis than the Canadian and Provincial averages (Inglis *et al.*, 2011); however, reasons for the high rates of campylobacteriosis in this region remain enigmatic. Waterborne transmission of *C. jejuni* has been suggested to be an important factor in the

epidemiology of sporadic campylobacteriosis. Recent laboratory studies showing prolonged persistence of *C. jejuni* in the presence of freshwater protozoans suggest a possible link to waterborne transmission (Axelsson-Olsson *et al.*, 2005; Snelling *et al.*, 2005). Consequently, there is a need for studies investigating the diversity of protists in freshwater systems.

We hypothesized that rich protistan communities exist in river water in southwestern Alberta and community structures will differ among sampling sites, and between seasons. The following objectives were constructed to test this hypothesis: (i) to longitudinally study freshwater protistan diversity in river water in southwestern Alberta (Oldman River Basin) over a one year period, and (ii) identify protists in this region which may be contributing to the environmental persistence of human pathogens.

4.2 MATERIALS AND METHODS

Ethidium monoazide validation

(i) Culture of protists. *Acanthamoeba polyphaga*, *Tetrahymena pyriformis*, *Chlamydomonas moewusii* and *Euglena gracilis* were selected for ethidium monoazide (EMA) validation. *Acanthamoeba polyphaga* was cultured in PYG growth medium at 30 °C and 100 rpm for 1 week and then for an additional week in fresh growth medium. *Tetrahymena pyriformis* was grown in PPG growth medium (Culture Collection of Algae and Protozoa, Oban, Scotland) for 48 h at 28 °C. *Chlamydomonas moewusii* and *E. gracilis* were grown for 1 week in Ward's basic culture medium (Wards Scientific, Rochester, NY) and a modified salts growth medium (Russell *et al.*, 2004) with exposure to a south-east facing window. Cells were washed once by centrifuging at 1000 x g for 10 min followed by re-suspension of the cells in 1 X PBS. Cell concentrations were

estimated using a haemocytometer, and viability was verified with trypan blue staining. Cultures were adjusted to a final concentration of 5 x 10^4 cells per mL, and were divided into four 200 µL samples in 2 mL tubes for each protist.

(ii) EMA treatment. For each protist, two arbitrarily-selected samples were incubated at 100 °C for 20 min (heat-treated), and the remaining two samples were maintained at 4 °C for 20 min (non-heat-treated). Ethidium monoazide (EMA) is a photo-reactive cross-linker that binds irreversibly to free DNA thereby inhibiting PCR amplification (Rudi *et al.*, 2005), and EMA (3 µL; Molecular Probes) was added to one heat-treated and one non-heat-treated sample (final concentration of 100 µg mL^{-1}). Following the addition of EMA, samples were placed on ice in the dark for 5 min, and tubes (lids open) were then exposed to light emitted from a 500 W halogen light bulb for two 1-min intervals; the light source was situated 10 cm from the top of the tubes and samples were vortexed between light exposures. All samples were then stored at -20 °C until subsequent processing.

(iii) DNA extraction and PCR amplification. DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen Inc.) according to the manufacturer's instructions with the exception that the final elution volume was reduced to 50 µL. PCR was performed using 5 µL of extracted DNA. The PCR primers, Euk1A (5'-CTGGTTGATCCTGCCAG-3') and Euk516R (5'-ACCAGACTTGCCCTCC-3') were used; these primers amplify variable regions V1 to V3 of the 18S rRNA gene that corresponds to positions 4 to 563 of the *Saccharomyces cerevisiae* (Accession AY251630) 18S rRNA gene. PCR conditions were as described by Diez et al. (Diez *et al.*, 2001a). PCR products were resolved by capillary electrophoresis using the MED250 protocol on a QIAxcel capillary system (Qiagen Inc.).

River water sample collection and processing

Samples were collected from the same sites as mentioned in Chapter 3, but at 4-week intervals, commencing May 7, 2008 through to May 13, 2009. At each site, samples of approximately 800-900 mL were obtained using 1 L Nalgene bottles attached to the end of a sampling pole; water was collected at a depth of ≈20 to 30 cm in the flowing portion of the river. Samples were stored on ice and processed within 6 h of collection. Samples (250 mL) were filtered through a 0.45 µm GF/F pre-filter (Whatman) followed by a 0.2 µm Iso-Grid final filter (Neogen Corp.) under vacuum using a six-place filtration manifold (Advantec MFS Inc.) fitted with an Iso-Grid filtration unit (Neogen Corp.). The two filters per sample were combined in a 50 mL Falcon tube with 20 mL PBS (0.1 M pH 7.2), and mixed vigorously to detach cells. Filters were subsequently removed, and the suspension was then centrifuged at 14 900 x g for 10 min at 4 °C. The top 17 mL of the supernatant was discarded, and pellets were re-suspended in the remaining 3 mL of PBS. Two hundred µL aliquots were dispensed into each of two 2-mL tubes, and EMA was added to samples as described above. Following EMA treatment, samples were stored at -20 °C until subsequent processing.

DNA extraction and quantification

DNA was extracted from samples using the QIAamp DNA Stool Mini Kit (Qiagen Inc.) according to manufacturer's protocol with the exception that the lysate volume transferred was doubled to maximize DNA yield; subsequent reagent volumes for DNA precipitation and washing were also doubled. DNA extractions were quantified using the Nanodrop ND-3300 fluorimeter (Thermo Scientific) with Hoecsht dsDNA labeling (Life Technologies, Carlsbad, USA) using Calf thymus dsDNA as a quantification standard.

PCR conditions for T-RFLP community profiling

PCR was performed using 5 ng of DNA or 5 µL for samples in which the DNA concentration was less than 1 ng/µL. The PCR primers used were FAM-Euk1A and Euk516R. To repair single stranded fragments following PCR which may result in pseudo-T-RFs (Egert and Friedrich, 2003; Egert and Friedrich, 2005), samples were incubated with Klenow polymerase (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocol. Post Klenow-treatment, samples were digested with HaeIII and HhaI (Invitrogen) according to the manufacturer's specifications prior to ethanol precipitation and separation by capillary electrophoresis on an ABI-3130 Genetic Analyzer in two independent runs (Applied Biosystems, Foster City, USA).

Analysis of T-RFLP community profiles

T-RFLP data quality was manually inspected in Genemapper (Applied Biosystems) and was subsequently imported into T-REX (Culman *et al.*, 2009). T-REX was used to standardized total peak height across samples (i.e. to compensate for small differences in the amount of digested DNA resolved per sample), and to filter 'noise' according to the method described by Abdo et al. (Abdo *et al.*, 2006); three standard deviations were used as the threshold for discriminating between noise and true peaks. Profiles were aligned using a clustering threshold of 0.5 (Smith *et al.*, 2005). Peaks not present in replicate T-RFLP profiles were removed from subsequent analyses. Non-transposed (T-RFs as columns, samples as rows), and transposed (samples as rows, T-RFs as columns) presence-absence data matrices were exported from T-REX for generation of a four-way Venn diagram using a custom Excel macro (Microsoft Inc., Redmond, WA, USA) and for use in BioNumerics (Applied Maths, St-Martin-Latem, Belgium),

respectively. BioNumerics was used to generate a distance matrix using the Bray-Curtis distance measure which formed the basis for the group significance test (Costa *et al.*, 2009), and three dimensional Non-metric Multidimensional Scaling (NMS) analysis was conducted using the MDS procedure in SAS (SAS Institute Inc.). NMS is an ordination technique which arranges individual data points (in this case, T-RFLP profiles) in three dimensional space based on similarity or dissimilarity; data points which cluster together are more alike than those further apart.

Emulsion PCR and clone library construction

Sampling periods throughout the year were categorized as spring (March 21-June 20), summer (June 21-September 21), fall (September 22-December 21), and winter (December 22-March 20) based on the dates for equinoxes (March 20 and September 21) and solstices (June 20 and December 21) as per the US Naval Observatory (http://www.usno.navy.mil/USNO/astronomical-applications/data-services/earth-seasons).

Community DNA from representative sampling times for the spring (late April), summer (late August), fall (mid October), and winter (late December), based on clustering of T-RFLP profiles with NMS, were pooled and amplified with the primers Euk1A and Euk516r in an oil-in-water emulsion, as per the EMBL-90 protocol described by Williams (Williams *et al.*, 2006). Emulsion PCR was performed to reduce PCR biases, such as the tendency to form 1:1 product to template ratios due to heteroduplex formation and underrepresentation of sequences, which have mismatches between primer and template sequences (Bru *et al.*, 2008; Polz and Cavanaugh, 1998). Following amplification, the emulsion was broken by two extractions with diethyl ether, followed by one extraction with ethyl acetate and two additional extractions with diethyl ether.

PCR products were purified using the Qiagen PCR purification kit (Qiagen Inc.) and subsequently ligated into the pGEM-T Easy vector (Promega) at a 3:1 vector:insert ratio. Chemical transformation of *Escherichia coli* JM109 (Promega) was performed according to the manufacturer's protocol. Blue-white screening was used to differentiate colonies with inserts from those without; white colonies were picked into 96-well plates containing LB medium supplemented with 4 % glycerol and 100 µg mL^{-1} ampicillin using the QPIX robot (Genetix, San Jose, USA) prior to screening. Approximately 500 clones from each library were pre-screened by HaeIII restriction digest of M13F (5`-GTAAAACGACGGCCAG-3`), M13R (5`-CAGGAAACAGCTATGAC-3`) PCR amplified products. Digested fragments were sized using the QIAxcel capillary system. Clones matching the restriction profile of prevalent solanaceous plants (≈80-90 % of the clones) were excluded from further analyses.

Clone library sequencing and analysis

Sequencing was performed by a single pass with the M13F primer on an ABI-3130 Genetic Analyzer (Applied Biosystems). Sequences were imported into Geneious v5.1 (Drummond *et al.*, 2010), trimmed manually and screened for chimeras using Bellerophon (Huber *et al.*, 2004). Putative chimeras were then examined on an individual basis in Pintail (Ashelford *et al.*, 2005) with *S. cerevisiae* (Accession AY251630) used as the reference. Sequences were aligned with the SINA alignment service using the SILVA SSU release 106 database (Pruesse *et al.*, 2007), imported into Geneious and manually curated. Alignments were then exported and OTUs determined using the MOTHUR software package (Schloss *et al.*, 2009) with a 1 % sequence divergence cut-off (Bailly *et*

al., 2007; Valster *et al.*, 2009). The 18S rDNA sequence data were deposited in the GenBank database under the accession numbers JX068881 to JX069077.

The degree in which microbial communities differed based on 18S rDNA sequence was assessed using the Unifrac P-test (Hamady *et al.*, 2010). All 198 18S rDNA protistan sequences from river water, along with *Giardia lamblia* (ATCC 50803) as the outgroup, were aligned within the SILVA database and imported into MOTHUR to remove common gaps prior to tree building in Geneious. A rooted phylogenetic tree was constructed from the trimmed alignment using the Neighbor joining method and the Tamura-Nei distance metric. The Newick formatted tree was then analyzed using Unifrac (Hamady *et al.*, 2010). Chao1 and ACE species richness estimates were performed using MOTHUR (Schloss *et al.*, 2009).

4.3 RESULTS

Ethidium monoazide validation

A substantial reduction in PCR amplicon band intensity (ranging from a weak product to no detectable product) was observed for samples heated at 100 °C for 20 min and exposed to EMA for all protists tested (Figure 4.1). Unheated samples treated with EMA yielded PCR products slightly less intense than no-EMA samples indicating minimal penetration of live cells.

T-RFLP community profiles

T-RFLP profiles targeting the 18S rRNA gene were determined for 70 river water samples from the Oldman watershed of southwestern Alberta. Considerable variability in the number of T-RFs was observed among samples and T-RF numbers ranged from 17 to

112 T-RFs (Figure 4.2). The total number of unique T-RFs detected throughout the study was 331. The greatest number of T-RFs for any given season were detected during the spring (253 T-RFs), and winter (236 T-RFs) (Figure 4.3). Protistan diversity was reduced in the summer and fall, and 127 and 107 T-RFs were observed, respectively. The T-RFLP fingerprints had high heterogeneity with a Beta diversity of 6.59, as determined with T-REX. NMS analysis of T-RFLP profiles revealed a pattern of seasonal relatedness independent of sample location; the three-dimensional plot exhibited a stress of 14 (Figure 4.4). The group significance test supported the clustering of samples into distinct groups by season ($P \leq 0.05$); however, there was higher variation in the spring-summer, spring-winter, and fall-summer comparisons (Table 4.1). T-RFs exclusive to spring, summer, fall, and winter sampling periods were 59, 8, 6, and 46, respectively (Figure 4.3). A total of 59 T-RFs were common to all four seasons.

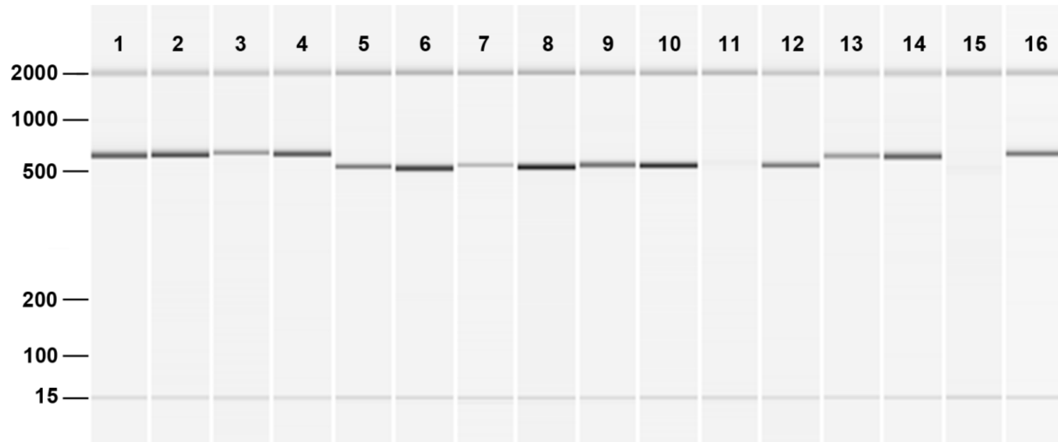

Figure 4.1. QIAxcel capillary electrophoresis of 18S rDNA PCR amplified samples of *Acanthamoeba polyphaga* (lanes 1-4), *Tetrahymena pyriformis* (lanes 5-8), *Chlamydomonas moewusii* (lanes 9-12), and *Euglena gracilis* (lanes 13-16). EMA-treated samples are presented in odd numbered lanes, and non-EMA-treated samples are presented in even numbered lanes. The first two lanes for each protist are cells that were not heat-treated, whereas the second two lanes per protist are cells that were heat treated. The markers on the left side of the image correspond to fragment size (bp).

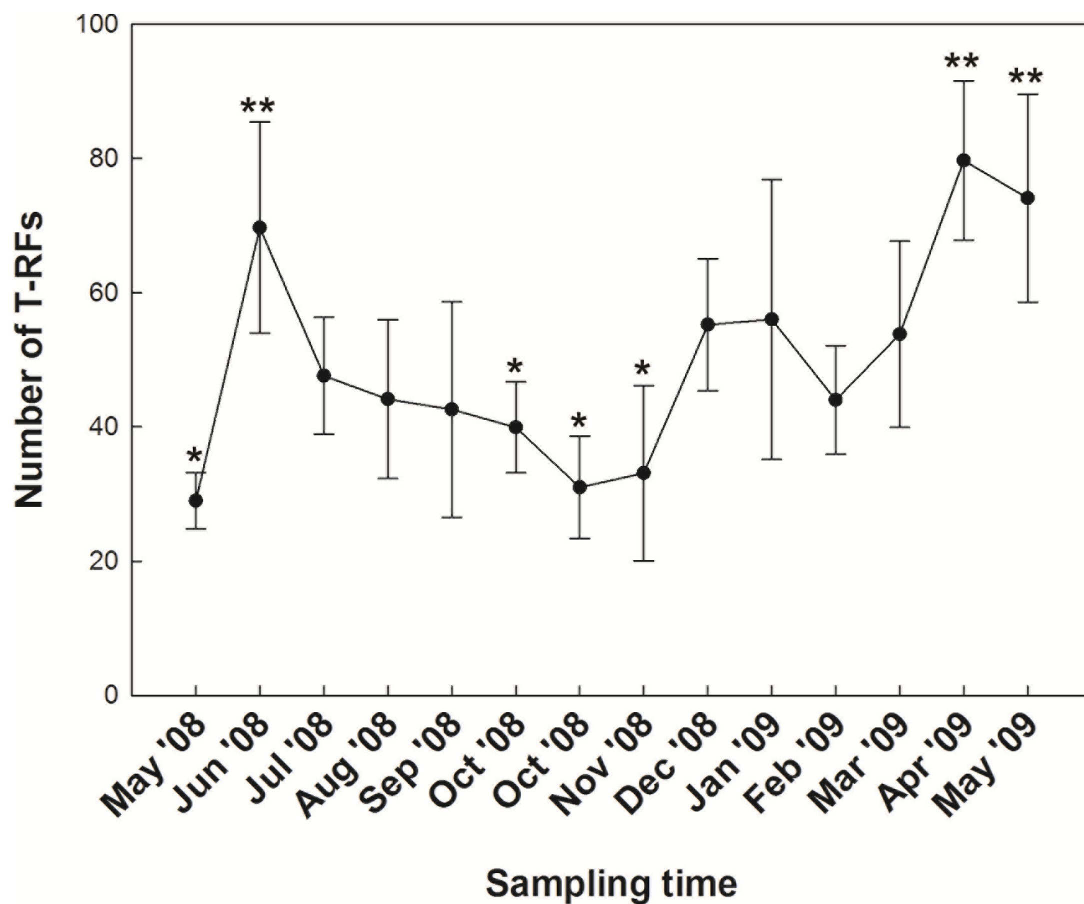

Figure 4.2. Number of terminal restriction fragments over a one year sampling period (May of 2008 to May of 2009). Vertical bars associated with means represent the standard deviation across the five sample sites. Means denoted with a single asterisk differ ($P \leq 0.05$) from means indicated by a double asterisk.

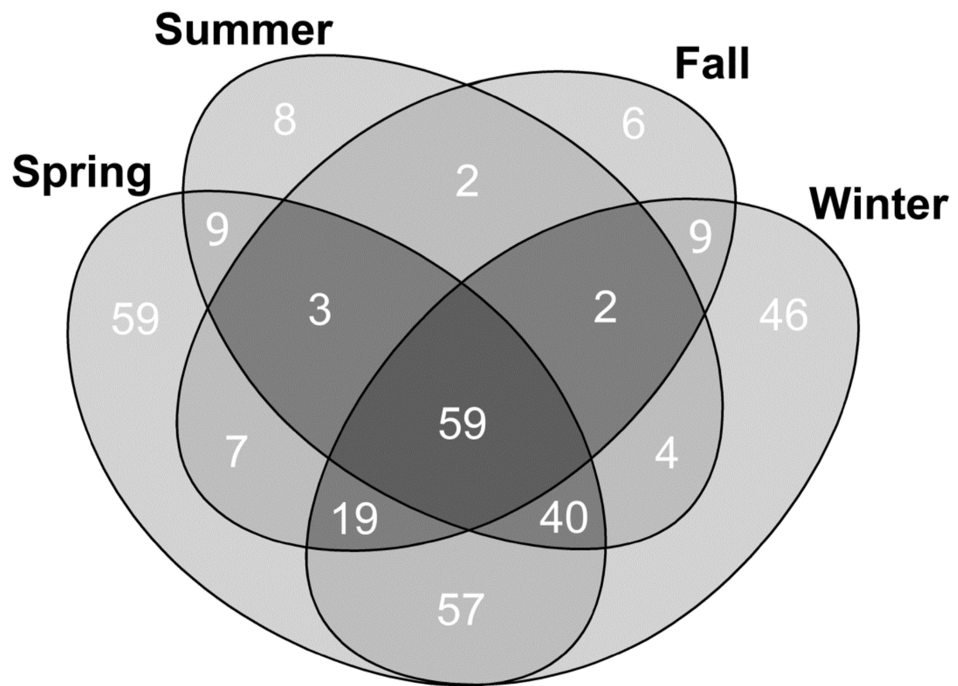

Figure 4.3. Four-way Venn diagram of unique terminal restriction fragments detected in river water by season.

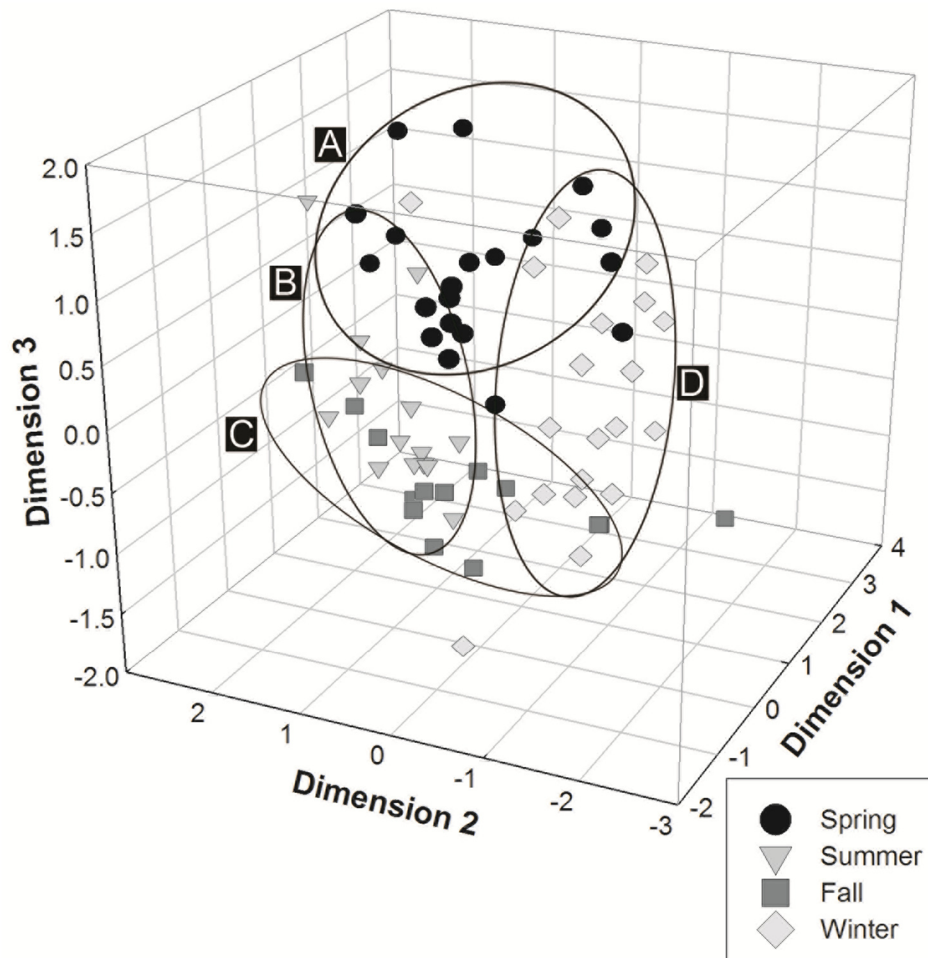

Figure 4.4. Non-metric multidimensional scaling plots of T-RFLP community profiles from river water (stress = 14) with ellipses A-D encompassing 90 % of respective markers by season; markers that appear close together are more similar than distant markers.

Table 4.1. A pairwise significance matrix of protist T-RFLP community profiles of seasonally partitioned data. Values indicated with an asterisk are significantly different ($P \leq 0.05$).

	Spring	**Summer**	**Fall**	**Winter**
Spring	-	0.391	< 0.001*	0.131
Summer	< 0.001*	-	< 0.001*	< 0.001*
Fall	0.017*	0.125	-	< 0.001*
Winter	< 0.001*	< 0.001*	< 0.001*	-

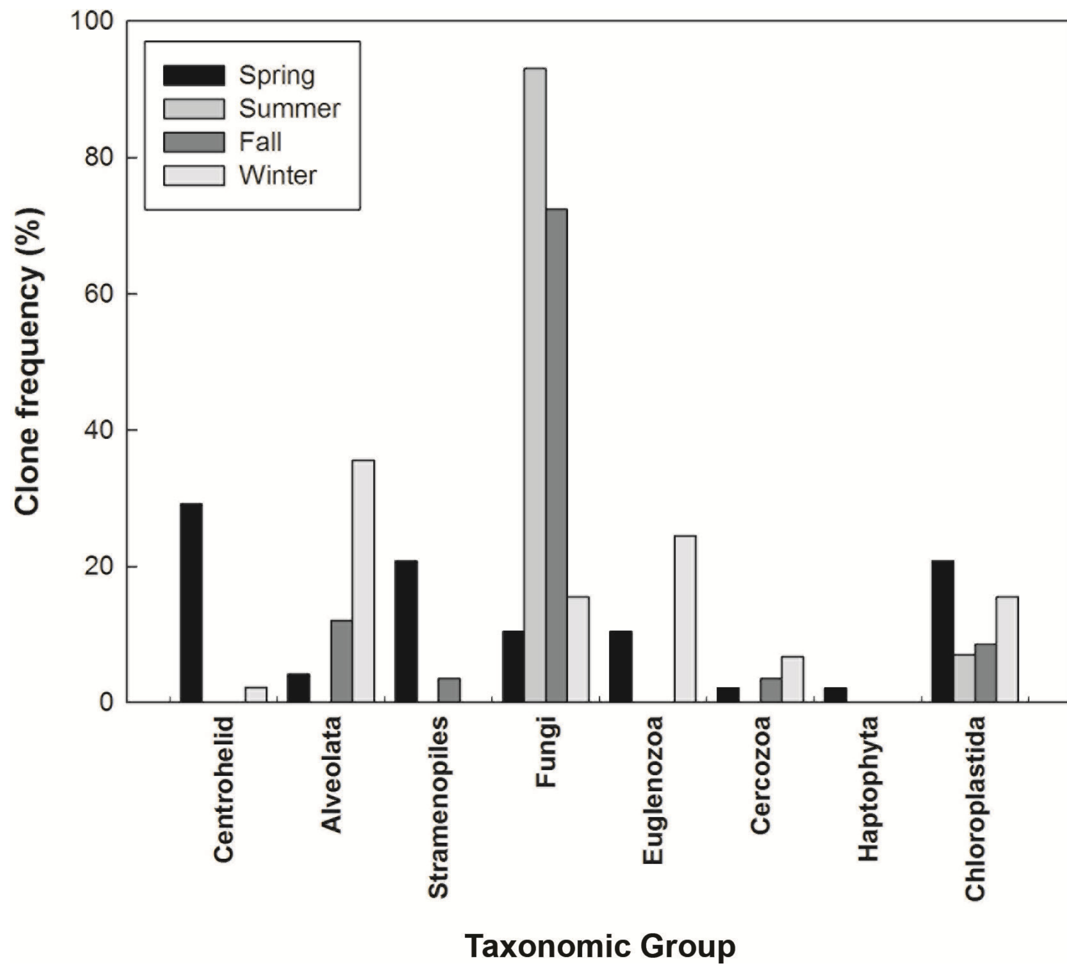

Figure 4.5. Distribution of 18S rDNA clones (%) at the first rank taxonomic level (Adl *et al.*, 2005) by season.

Clone libraries

Eighty five operational taxonomic units (OTUs) from 198 18S rDNA sequences were observed in river water. The spring library was dominated by Centrohelid, Stramenopiles, and Chloroplastida clones (≈29, 20, and 20 %, respectively) (Figure 4.5). Summer and fall libraries were dominated by Saccharomycetes, primarily *Candida spp.* (Figure 4.5), which accounted for 83 % and 88 % of the clones, respectively. Alveolata (33 %), Euglenozoa (23 %), Chloroplastida (14 %) and fungal (14 %) clones were most common in the winter library. ACE predicted 94, 29, 131, and 79 OTUs for the spring, summer, fall, and winter libraries respectively while Chao1 predicted 87, 15, 97, and 37 (Table 4.2). The Unifrac p-test confirmed differences between seasonal clone libraries ($p < 0.001$).

Table 4.2. Summary of 18S rDNA clone libraries including ACE and Chao1 non-parametric richness estimates.

			ACE			Chao1		
Library	**Clones**	**OTUs**	**Mean**	**Lower**	**Upper**	**Mean**	**Lower**	**Upper**
Spring	61	29	94	51	217	87	48	208
Summer	46	8	29	10	274	15	9	50
Fall	58	32	131	65	332	97	55	219
Winter	47	21	79	52	130	37	25	79

4.4 DISCUSSION

The temporal dynamics of protistan community structure associated with freshwater systems, particularly rivers, have yet to benefit from the explosion of molecular-based investigations into microbial ecology and biogeography. Such studies have mostly focused on Eubacteria and Archaea, using 16S rDNA sequences as a target, and to a lesser extent, oceanic picoeukaryotes (i.e. protists ≤ 0.2 μm in size) targeting 18S rDNA sequences. Using T-RFLP and clone library analysis, we observed conspicuous temporal shifts in protistan community structure in southwestern Alberta rivers.

NMS analysis of T-RFLP profiles revealed high similarity between sampling sites over time as samples clustered according to time (season) rather than location. This occurred despite the fact that sampling sites were located in areas subject to differing natural (i.e. organic matter, embankment) and anthropic (i.e. up/downstream from waste water treatment plant effluent, proximity to highways, agricultural runoff, and urban vs. rural sites) factors. Anthropoic factors, in particular, have been shown to have pronounced effects on microbial planktonic communities in freshwater systems (Frost, 2001). The stress of the ordination was 14, which indicates that the ordination is satisfactory and is a good representation of the T-RFLP data (McCune and Grace, 2003).

There are currently two predominant models of protistan biogeography: the moderate endemicity model (Foissner, 1999, 2008) and the ubiquity model (Tauxe *et al.*, 1988). The ubiquity model proposes that protists are cosmopolites and can be found anywhere in which their niche requirements are met, whereas the moderate endemicity model postulates that while the majority of protists are cosmopolites, a significant amount (roughly one third) exhibit a limited biogeographical range (Foissner, 2008). Despite the high variance in T-RF richness between sampling sites at any given time, the collective

seasonality of T-RFLP community profiles suggests that a stable core group of protists exists in river water in southwestern Alberta. As such, our results for protists in river water are consistent with the moderate endemicity model. Similarly, Nolte *et al.* (2010) observed seasonality in the distribution of protists in Lake Fuschlsee (Salzhammergut, Austria) (Not *et al.*, 2009); they observed that changes in community structure were not merely quantitative but also qualitative as taxa observed in one month would drop below the detection threshold the following month.

T-RFLP proved to be instrumental in providing a snapshot of the 18S rDNA community and assisted in the selection of sites for Sanger sequencing in the current study. Due to the costs associated with sequencing clone libraries, it was not logistically possible to process all 70 samples in this manner. In previous studies using T-RFLP of 16S rDNA communities, T-RFs were identified via *in silico* restriction digests of sequences from known databases (Adiba *et al.*, 2010). However, recent evidence suggests that this is not a robust analysis method due to differential migration of T-RFs with variable pyrimidine content and inaccurate sizing resulting from differential migration of the LIZ labeled size standard and FAM labeled PCR products (Kaplan and Kitts, 2003). Furthermore, multiple taxa may occupy a single T-RF. As these biases are consistent between samples and replicate runs, T-RFLP remains a valid method for community profiling. Given that the identification of T-RFs based on *in silico* analyses of sequence databases is not reliable, we selected sites for Sanger sequencing based on the similarities and differences in T-RFLP profiles, rather than specific protistan assemblages inferred from T-RFs.

The study of protists in freshwater systems, especially rivers, poses the added challenge that terrestrial and aquatic plant matter and microeukaryotes are present in high

numbers and there are no PCR primers capable of excluding them while retaining the ability to amplify a broad array of protists. We observed that all 18S rDNA libraries generated in the current study were dominated by clones later identified as a solanaceous plant, perhaps that of a common agricultural weed in the study area (Blackshaw, 1991). As plants have been shown to have between 500 and 40,000 copies of the 18S rRNA gene per diploid cell (Zia *et al.*, 2003), a single contaminating cell could severely hinder the detection of protists. Our sample processing regime may have benefitted from a pre-filtration step; however, it is uncertain if this would have impacted the recovery of large or attached protists.

In our study, we used EMA to prevent PCR amplification of non-viable protists. EMA has been used extensively in studies involving prokaryotes (Rudi *et al.*, 2005), however, to our knowledge this is the first study regarding the ecology of protists to use EMA for the differentiation of live from dead cells. Previous uses of EMA involving eukaryotic cells includes distinguishing viable from dead cells using flow cytometry (O'Brien and Bolton, 1995) and removing contaminating eukaryotic DNA from yeast extract (Rawsthorne and Phister, 2009). EMA has been documented to penetrate some bacterial species with intact cell membranes (Pearson *et al.*, 2000) which could result in the underestimation of diversity; however, prior to this study it was unclear if this occurs with protists. Our results indicate that while there may be some penetration of live cells, EMA is suitable for reducing DNA from dead cells in the study of protistan diversity. In some ecosystems, such as human fecal samples, a large proportion of microbial cells are not viable and community analyses in the absence of EMA may result in a dramatic overestimation of community richness and even inaccurate conclusions. For example,

concluding no change in community composition of all organisms when there may be a marked shift in *viable* organisms (Olsen *et al.*, 2001).

There was a significant disparity between the T-RFLP and clone library analyses in measured community richness. For example, 331 unique T-RFs were observed compared to only 89 OTUs. Furthermore, richness estimates based on Chao1 and ACE suggest additional taxa are present. This could be due to the use of Sanger sequencing which often fails to fully illuminate the rare biosphere due to throughput constraints. In contrast, alternatives such as second generation sequencing platforms (i.e., 454 pyrosequencing) are prone to sequencing errors, which results in the overestimation of the rare biosphere (Fortunato *et al.*, 2011). Sanger libraries offered unmatched accuracy, and at the time of this study, Sanger read lengths more than double that of 454 pyrosequencing (Mardis, 2008); the limited pyrosequencing read lengths restrict taxonomic assignment of protists (Amaral-Zettler *et al.*, 2009). To compensate for the relatively small number of clones we could logistically sequence, we prescreened clones based on HaeIII restriction profiles to avoid sequencing clones belonging to the solanaceous plants. This allowed us to screen close to 2,000 clones. A similar method was utilized by Diez et al. (2001) to study the genetic diversity of protists in oceanic water, except the restriction digest profiles were used to group clones to maximize OTU discovery via Sanger sequencing libraries.

Heterophrys sp., the dominant Centrohelid detected in the spring (14 clones) and winter (one clone) libraries, are a group of phagotrophic protists (Cavalier-Smith and von der Heyden, 2007) and thus may affect the persistence of enteric bacteria in rivers. The spring is a particularly interesting time for studies regarding the fate of enteric bacteria due to the large amount of agricultural runoff (i.e. animal fecal matter) which has been

associated with increases in coliform bacteria (Gannon and Busse, 1989) and spikes in enteric disease (Pebody *et al.*, 1997). Phagotropic protists such as *Acanthamoeba spp.*, *Hartmanella spp.*, *Naegleria spp.*, and *Tetrahymena spp.* have been linked with the environmental persistence of a wide array of pathogenic bacteria (Brown and Barker, 1999). *Legionella pneumophila*, for example, escapes the phagosome into the cytoplasm and is able to replicate within the protozoan host (Molmeret *et al.*, 2004). While the protists mentioned above were not detected in the present study, this may be due to the common association of amoeboid protists with biofilms (Huws *et al.*, 2005). Nonetheless, studies targeting *Heterophrys sp.* in relation to the persistence of enteric pathogens in river water are warranted.

Centrohelids and Chloroplastidae dominated river water during the spring, representing 28.1% and 29.8% of clones, respectively. A dramatic decline in both was noted for the summer and fall libraries which were dominated by Fungi, specifically *Candida spp.*, a genus containing the ubiquitous human pathogens *C. ablicans* and *C. tropicalis* (Brinkman *et al.*, 2003). Kopylov and Kosolapov (2011) also reported a reduction in heterotrophic protists in early summer in the Ob river of West Siberia which corresponded with increased levels of heterotrophic bacteria. Photosynthetic protists (i.e. Chloroplastidae) face increased competition from Cyanobacteria which bloom during the increased temperatures that accompany the summer months as they enter their temperature optima (Jöhnk *et al.*, 2008). Cyanobacteria are also smaller in size, thus providing advantages over the larger Chloroplastidae such as a lower sinking rate (Walsby and Holland, 2006) and more efficient nutrient acquisition due to the lower diffusion boundary layer, which is a result of having a higher surface area to volume ratio (Ploug *et al.*, 1999). Salmanian *et al.* (2012) suggest *Candida spp.* play a role in the environmental

persistence of *Helicobacter pylori* (Schloss *et al.*, 2009), which was once considered part of the *Campylobacter* genus and is associated with gastric ulcers in humans (Pernthaler, 2005). They found bacterium-like bodies (BLBs) present in vacuoles of *Candida spp.* isolated from foods that were confirmed viable through live/dead staining. Furthermore, *Candida spp.* containing BLBs were PCR positive for the *H. pylori ure*AB gene in 9 out of 15 cases (González *et al.*, 1990).

In conclusion, we observed through the use of T-RFLP and clone library analysis that protists were common in river water in southwestern Alberta and seasonal succession trends were consistent with the moderate endemicity model. The majority of T-RFs were in the spring and winter which were dominated by Alveolata, Centrohelida, and Chloroplastidae (spring) and Alveolata, Euglenozoa, Chloroplastida, and Fungi (winter). Summer and fall were dominated by saccharomycetous fungi. Our study also illustrates some of the complications researchers face in studying protists using the 18S rRNA gene as a target.

CHAPTER 5

Interactions between the model protozoan, *Acanthamoeba polyphaga* and *C. jejuni*

ABSTRACT

Campylobacter jejuni is the leading cause of enteritis worldwide yet the degree and mechanisms by which *C. jejuni* persists in the environment are poorly understood. This study examined the interaction between *C. jejuni* and the model freshwater protozoan *Acanthamoeba polyphaga*, and specifically whether prolonged survival is common to a panel *C. jejuni* clinical isolates and if *C. jejuni* invades and exhibits cytoxicity toward *A. polyphaga*. Clinical *C. jejuni* isolates from southwestern Alberta as well as *C. jejuni* 81-176 knockout mutants deficient in the production of cytolethal distending toxin (CDT), a functional flagellar apparatus (FlaAFlaB), and quorum signalling (LuxS) were utilized to further elucidate interactions between these two organisms. All *C. jejuni* strains tested survived longer in co-culture with *A. polyphaga* than in PYG growth medium or *A. polyphaga* growth by-products. *C. jejuni* exhibited a cytotoxic effect toward *A. polyphaga* irrespective of the knockout strains tested and entry is dependent on a functional flagellar export aparatus. Culture-based methods including enrichment failed to recover *C. jejuni* following encystment and excystment of *A. polyphaga*, and the data presented suggests *C. jejuni* invades *A. polyphaga* in a manner consistent with the trigger method. Collectively, these results support the hypothesis that protozoans play a role in the persistence of *C. jejuni* in the environment.

5.1 INTRODUCTION

The degree and mechanisms by which *C. jejuni* persists in the environment are poorly understood at present. Following clearance from an infected host, it is generally believed that *C. jejuni* must be transferred directly to a subsequent host (i.e. fecal-oral transmission) or else endure harsh environmental conditions (i.e. sub-optimal temperatures, predation and osmotic stress) while awaiting ingestion by a suitable host. Recent research suggests that protozoans (phagotrophic protists) such as *A. polyphaga* and *T. pyriformis* may play a role in the environmental persistence (Snelling *et al.*, 2005) and subsequent infection of vertebrate hosts by *C. jejuni* (Snelling *et al.*, 2008). However, it is currently not known how widespread this feature is among clinical isolates and research is required to elucidate specifics of this interaction, including the mechanism of entry into protozoa, whether *C. jejuni* can survive encystment, and the role of cell-cell signalling molecules and toxins.

Bacterial invasion of living cells (i.e. inducing phagocytosis in cells that are not phagocytic under normal conditions) is currently believed to occur via one of two mechanisms; the trigger and zipper mechanisms (Cossart and Sansonetti, 2004). The trigger mechanism involves the injection of bacterial effector proteins into the host cell cytosol via type III or type IV secretion systems which triggers rearrangement of the host cell cytoskeleton thereby facilitating invasion (Alonso, 2004). The zipper method occurs in three stages starting with contact and adherence between bacterial proteins on the outer membrane (termed adhesins and invasins) and receptors on the target cell leading to receptor clustering. This leads to actin polymerization and membrane extension forming a phagocytic cup. Lastly, the phagocytic cup is closed by actin depolymerisation engulfing the bacterium (Cossart and Sansonetti, 2004).

The exact mechanism by which *C. jejuni* invades mammalian cells remains a mystery and even less is known about the invasion of protozoans. In mammalian cells, invasion by *C. jejuni* exhibits characteristics common to both the trigger and zipper methods (Ó Cróinín and Backert, 2012). During the invasion process, *C. jejuni* binds to the target cell membrane via adhesins including CadF, CapA, FlpA, JlpA, MOMP and PEP1 (Eucker and Konkel, 2012; Flanagan *et al.*, 2009), a feature common to the zipper method. Consistent with the trigger mechanism, *C. jejuni* injects effectors proteins into the host cell including CiaC, which is required for maximum invasion (Neal-McKinney and Konkel, 2012). Of particular importance to this process is the flagellar export apparatus, which is required for secreting type three secretion system effectors (T3SSEs) into the target cell (Konkel *et al.*, 2004; Young *et al.*, 2007). Lastly, *C. jejuni* invades the target cell by a process dependent on rearrangement of microtubules and association with dynein (Hu and Kopecko, 1999).

Cytolethal distending toxin (CDT) is part of the cyclomodulin family of bacterial effectors that modulate the eukaryotic cell cycle. CDT functions as a cycle inhibiting factor (CIF) that arrests the host cell cycle between the G2 and M phases by inducing breaks in host cell DNA (Pérès *et al.*, 1997) (Pickett *et al.*, 1996). Present in many enteric pathogens, CDT is thought to play a role in *C. jejuni* pathogenesis by enabling *C. jejuni* to avoid early detection by the host immune system via cell cycle arrest (Hassane *et al.*, 2003).

Quorum sensing, a population dependent cell-cell signalling system is another characteristic of *C. jejuni* that has been demonstrated to play a role in the colonization of the chicken gastrointestinal tract, cellular motility and adherence (Quinones *et al.*, 2009). The gene responsible, an orthologue of LuxS, is required for autoinducer-2 (AI-2) activity

in *C. jejuni*. AI-2 is a signalling molecule in both gram negative and gram positive bacteria and it has been shown to affect expression of an array of genes including those required for virulence (He *et al.*, 2008; Vendeville *et al.*, 2005).

The protozoan *A. polyphaga* has been shown to play an important role in the environmental survival and transmission of human pathogens (eg. *Legionella pneumophila*) (Cirillo *et al.*, 1994). Experimentation by Axelsson-Olssen et al. (2005) demonstrated that a *C. jejuni* isolated from a human patient survived longer when co-cultured with *A. polyphaga* than in culture medium alone and also suggests the co-culture may be able to resuscitate viable but non-culturable bacteria. Snelling et al. (2006) later showed *C. jejuni* internalized by *A. castellani* were able to colonize broilers. Combined, these studies suggest that protozoans play a role in the epidemiology of *C. jejuni*. However, little is known with respect to mechanisms. It is believed that protozoans played a role in the evolution of current pathogens by serving as an "evolutionary crib" (Greub *et al.*, 2004) and better understanding these interactions may improve our understanding of their pathogenesis in higher eukaryotes. The features of *C. jejuni* described above, including the flagellar export apparatus, CDT and LuxS production have not been investigated in the interplay between *C. jejuni* and protozoans. Furthermore, limited work has focused on *C. jejuni* isolates of clinical origin. Thus, the following objectives were established: (1) examine the survival of clinical *C. jejuni* isolates from southwestern Alberta in the presence of *A. polyphaga;* (2) determine the role of the flagellar apparatus, cytolethal distending toxin, and quorum sensing on invasiveness of *C. jejuni* and the cytotoxicity in *A. polyphaga*, and (3) determine if *C. jejuni* is capable of surviving encystment by *A. polyphaga*.

5.2 MATERIALS AND METHODS

Microorganisms and growth conditions

Clinical *C. jejuni* isolates (CHR 19, CHR 38, CHR 46, CHR 58, CHR 88 and CHR 96) from inhabitants of southwestern Alberta previously shown to be highly invasive (Kalischuk *et al.*, 2007), two isolates (K5E1, K4E12) recovered from a human infected with *C. jejuni* NCTC 11168, *C. jejuni* 81-176, *C. jejuni* 81-176 ΔCDT (Kalischuk *et al.*, 2007), *C. jejuni* 81-176 ΔFlaAFlaB (Kalischuk *et al.*, 2007), and *C. jejuni* 81-176 ΔLux*S* (Quinones *et al.*, 2009) were streaked onto Columbia agar (Oxoid Ltd.) supplemented with 5 % sheep's blood (CSB) and incubated for 48 h under microaerobic conditions (5 % O_2, 30 % H_2, 10 % CO_2 and N_2 balance) at 37 °C. *C. jejuni* was streaked onto fresh CSB and cultures were incubated overnight (≈ 16 h) microaerobically at 37 °C. *C. jejuni* biomass was scraped off the medium surface and suspended in 1 X PBS. The optical density was measured spectrophotometrically and adjusted to an OD_{600} of 1; this corresponded to a concentration of ≈ $1x10^9$ viable cells mL^{-1}. *C. jejuni* cell concentrations were confirmed by dilution plating. *A. polyphaga* CCAP 1501/3G was obtained from the Culture Collection of Algae and Protozoa (CCAP) (Oban, Scotland) and cultured in peptone–yeast-glucose broth (PYG medium) (Axelsson-Olsson *et al.*, 2005) at 30 °C and 100 rpm for 1 week, and then for an additional 1 week in fresh PYG medium to obtain a concentration of ~ 1×10^5 cells mL^{-1}. Cultures were centrifuged at 1 000 x g for 10 min, resuspended in fresh PYG medium and final cell concentrations confirmed using a haemocytometer.

Mutant verification

Mutants *C. jejuni* 81-176 ΔCDT and *C. jejuni* 81-176 ΔFlaAFlaB were spread on Karmali agar with 30 μg mL^{-1} kanamycin to confirm they retained the kanamycin cassette used previously for transformation (Kalischuk *et al.*, 2007). The validity of the *C. jejuni* 81-176 ΔLuxS mutant was confirmed by PCR using the primers Uplux3 (5'-TCTACTATAGGGATATCAAATTGTGAA-3') and Downlux1 (5'-CCTATTTTAGAAGCAATTTCTCTTA-3') targeting regions flanking the LuxS gene. The LuxS mutant has an insertion of ~ 800 bp containing a chloramphenicol resistance cassette. PCR reactions contained 250 μM dNTPs, 0.5 μM of each primer, 1 unit of HotStar Taq polymerase (Qiagen Inc.), 1 X reaction buffer, and 2.5 mM $MgCl_2$. PCR thermocycling conditions included incubation at 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 49 °C for 30 s, and 72 °C for 90 s, and a final extension step at 72 °C for 10 min. Amplicons were resolved by capillary electrophoresis using the QIAxcel platform (Qiagen Inc.).

Co-culture experiment with *C. jejuni* 81-176 and clinical isolates from southwestern Alberta

The survival of *C. jejuni* 81-176 and clinical isolates from diarrheic individuals living in southwestern Alberta were incubated in the presence of *A. polyphaga, A. polyphaga* growth by-products, or PYG growth medium alone (Figure 5.2). The experimental design was modeled after a previously published report (Axelsson-Olsson *et al.*, 2005). *A. polyphaga* cultures were prepared as described previously. *A. polyphaga* growth by-products were prepared by filtering the supernatant of 1 week-old cultures through a 0.2 μm filter. *A. polyphaga* cultures were seeded into 12 well plates at a final

concentration of 1 x 10^5 CFU/mL and incubated at 37 °C to allow amoebae to attach to the surfaces of individual wells. Additional control conditions included wells with PYG as well as well with PYG innoculated with *A. polyphaga*. Sample wells were then challenged at an MOI of 100:1 (*C. jejuni*: *A. polyphaga*), incubated at 25 °C under aerobic conditions and processed every 24 h for 6 days by spreading 50 µL of the co-culture on CSB plates (Oxoid Ltd.), and incubating the cultures microaerobically for 48 h at 37 °C. *A. polyphaga* in PYG, by-products, and PYG controls were included in separate wells. Sample wells in which viable *C. jejuni* was detected in at least two of the three replicates were considered positive.

Intracellular survival of *C. jejuni* 81-176 wild type and mutant strains

One mL of *A. polyphaga* culture was added to individual wells of a 12 well plate and incubated aerobically for 1 h at 37 °C to facilitate attachment of amoebae to the well bottom. Following incubation, PYG was carefully aspirated and 900 µL of fresh PYG added to each well. Wells receiving *C. jejuni* 81-176, *C. jejuni* 81-176 ΔCDT, *C. jejuni* 81-176 ΔFlaAFlaB or *C. jejuni* 81-176 ΔLuxS were inoculated with 100 µL of a cell suspension containing 1 x 10^8 CFU mL^{-1} in PBS, corresponding to an MOI of 100:1. Control wells received 100 µL PBS. Plates were incubated for 4 h at 37 °C then the medium aspirated, followed by the addition of 1 mL Pages Amoeba Saline (PAS; CCAP) buffer containing 100 µl mL^{-1} gentamicin sulfate (Sigma, St. Louis, MO) and incubated for 1 h at 37 °C to kill extracellular *C. jejuni*. Replicate samples were then processed immediately after the 1 h gentamicin sulfate treatment and at 2 h post-gentamicin treatment by washing wells twice with 1 X PAS buffer. Amoebae were then incubated at room temperature for 1 h with 1 mL of 1 X PBS buffer with 0.05 % Triton X-100 (Sigma)

to lyse the amoebae. The lysate was ten-fold serially diluted in 1 X PBS and 50 µL of each dilution was spread on Columbia blood agar (Oxoid Ltd.). Cultures were incubated at 37 °C under microaerobic conditions for 48 h prior, and CFU were enumerated at the dilution yielding 30 to 300 CFU per plate.

Contact dependent survival assay

A study was conducted to determine if the persistence of *C. jejuni* in co-culture with *A. polyphaga* is contact dependent. Microfuge tubes containing a removable insert containing a 0.1 µm filter (Millipore) were used to compare the survival of *C. jejuni* ± contact with *A. polyphaga* in PYG. PYG broth was added to each side of the filter. Treatments included: *C. jejuni* added to one side of the filter and *A. polyphaga* to the other; *C. jejuni* and *A. polyphaga* added to the same side of the filter (top and bottom); *C. jejuni* alone (bottom); and *A. polyphaga* alone (top). Tubes were incubated aerobically at 25 °C on a slight angle to facilitate fluid contact with both sides of the filter.

Cytotoxicity of *C. jejuni* 81-176 wild type and mutant strains

C. jejuni strains including the wild type and mutant strains (ΔCDT, ΔFlaAFlaB, and ΔLuxS) were each co-cultured with *A. polyphaga* in 12 well tissue culture plates at an MOI of 100:1 in the presence of PYG growth medium or PAS buffer to determine the effect of nutritional substrate on the cytotoxicity of *C. jejuni*. Control treatments included the respective *C. jejuni* strains (wild type and mutants) in (1) PYG and (2) PAS, *A. polyphaga* in (3) PYG and (4) PAS. Enumeration of live and dead *A. polyphaga* cells was determined by gently detaching *A. polyphaga* from the bottom of 12 well tissue culture plates using a cell scraper. A 360 µl sample of the cell suspension was removed and 0.4%

Trypan Blue (Sigma) was added to a final concentration of 0.04% v/v. After 1 min at room temperature, live (unstained) and dead (stained) *A. polyphaga* were enumerated using a hemocytometer.

Encystment

C. jejuni 81-176 and *A. polyphaga* co-cultures were prepared at an MOI of 100:1 and incubated for 3 h in PYG growth medium at 37 °C to facilitate invasion. The control treatment consisted of *A. polyphaga* in the absence of *C. jejuni*. Encystment was induced at 30 °C by aspirating PYG, washing twice with 1 X PAS, then adding Tris buffered encystment medium. The ratio of cysts to trophozoites (vegetative cells) was determined at 24 h intervals using an inverted microscope at 400 X magnification by averaging the counts for three arbitrary fields of view. At 96 h, remaining trophozoites were lysed by incubating cultures in 0.05 % Triton X-100 (Sigma) for 1 h at room temperature. Sample wells were washed once with PAS followed by the addition of PAS with 100 µg mL^{-1} gentamicin sulphate to kill extracellular *C. jejuni*. Cysts were washed twice with PAS buffer, and excystment was induced by the addition of PYG and monitored by microscopy. After 1 week, 100 µL of each sample was spread on CSB in duplicate and incubated microaerobically for 48 h at 37 °C. Enrichment for *C. jejuni* was performed by adding 0.5 mL of excysted cell culture to 4.5 mL Bolton enrichment broth. Enrichment tubes were incubated overnight at 37 °C and 10 µL of the enrichment broth was streaked onto Columbia blood agar in duplicate. Cultures were incubated microaerobically for 48 h at 37 °C and checked for growth of *C. jejuni*.

Experimental design and statistical analysis

All experiments were performed in triplicate with each replicate performed at a separate time in a randomized complete block design. To determine if there were statistically significant differences among means, a one-way ANOVA was performed in conjunction with Tukey's test ($\alpha = 0.05$) using the PROC ANOVA procedure of SAS (SAS Institute Inc.).

5.3 RESULTS

Amplification of LuxS gene from *C. jejuni* 81-176 and *C. jejuni* 81-176 ΔLuxS

PCR targeting regions flanking the LuxS gene confirmed the presence of an insert of approximately 800 bp, corresponding to the chloramphenicol resistance cassette, in *C. jejuni* 81-176 ΔLuxS (lane 2) relative to *C. jejuni* 81-176 wild type (lane 1) (Figure 5.1).

C. jejuni* strains survive longer in co-culture with *A. polyphaga

All of the *C. jejuni* isolates tested were detected for a greater number of days when they were co-cultured with *A. polyphaga* at 25 °C then when they were incubated with *A. polyphaga* growth by-products or PYG broth alone (Figure 5.2). Survival times in co-culture ranged from 5 to 6 days, compared to 3 to 5 days in the presence of *A. polyphaga* by-products and from 3 to 4 days in PYG medium alone.

Fate of *C. jejuni* internalized by *A. polyphaga*

There was no difference between the number of *C. jejuni* 81-176, *C. jejuni* 81-176 ΔCDT nor *C. jejuni* 81-176 ΔLuxS internalized by *A. polyphaga* ($P \leq 0.05$) (Figure 5.3). In contrast, conspicuously less ($P < 0.05$) *C. jejuni* 81-176 ΔFlaAFlaB was internalized

relative to other isolates (less than 30 colonies on the 10^0 plate). All internalized strains showed a decline ($P \leq 0.05$) at the 2 h sampling time.

Cytotoxicity of *C. jejuni* 81-176 wild type and mutant strains

Following 48 h of co-culture with *A. polyphaga*, *C. jejuni* 81-176, *C. jejuni* 81-176 ΔCDT, *C. jejuni* 81-176 ΔLuxS, showed no change in CFU mL^{-1} when incubated in the presence of PYG, however, a trend was observed in which *C. jejuni* 81-176 ΔFlaAFlaB increased in cell concentration (data not shown). In the absence of amoebae, only *C. jejuni* 81-176 ΔFlaAFlaB was detected in the PYG treatment while the rest were undetectable at 48 h. In the presence of PAS buffer, no *C. jejuni* strains were detected in wells containing amoebae nor those without amoebae after 48 h of co-culture (data not shown). The number of dead *A. polyphaga* increased ($P \leq 0.05$) when co-cultured with each of the *C. jejuni* strains tested compared to the control condition (Figure 5.4). *A. polyphaga* alone reached a higher cell concentration than when co-cultured with all mutants and wild type *C. jejuni*, but did not significantly differ from treatment with the ΔLuxS mutant (Figure 5.4).

C. jejuni* 81-176 cannot be recovered with culture-based techniques following encystment/excystment of *A. polyphaga

There was no difference in the ratio of cysts to trophozoites between *A. polyphaga* co-cultured with *C. jejuni* 81-176 and *A. polyphaga* cultured alone (Figure 5.5). Following excystment, *C. jejuni* was not detected by direct plating nor enrichment.

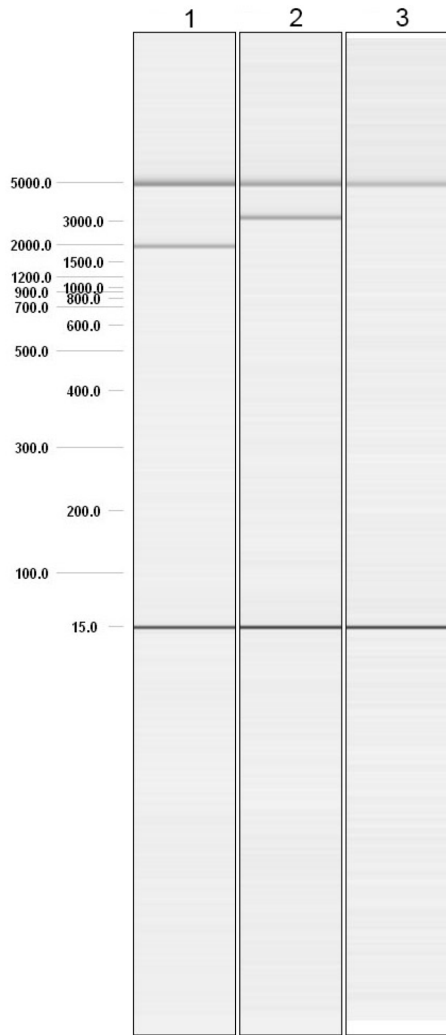

Figure 5.1. Capillary electrophoresis of PCR products from: (1) *C. jejuni* 81-176 wild type; (2) *C. jejuni* 81-176 ΔLuxS; and (3) no template control.

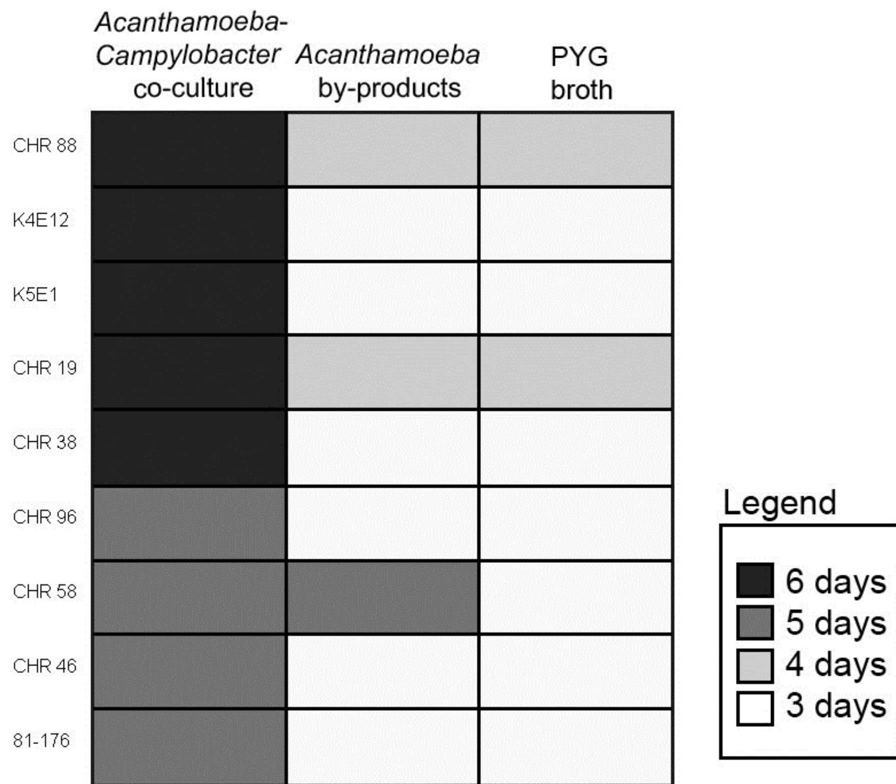

Figure 5.2. Heat map depicting the survival of *C. jejuni* isolates (days) in which two out of three replicates were culture positive following co-culture with *A. polyphaga*, *A. polyphaga* growth by-products, and PYG broth.

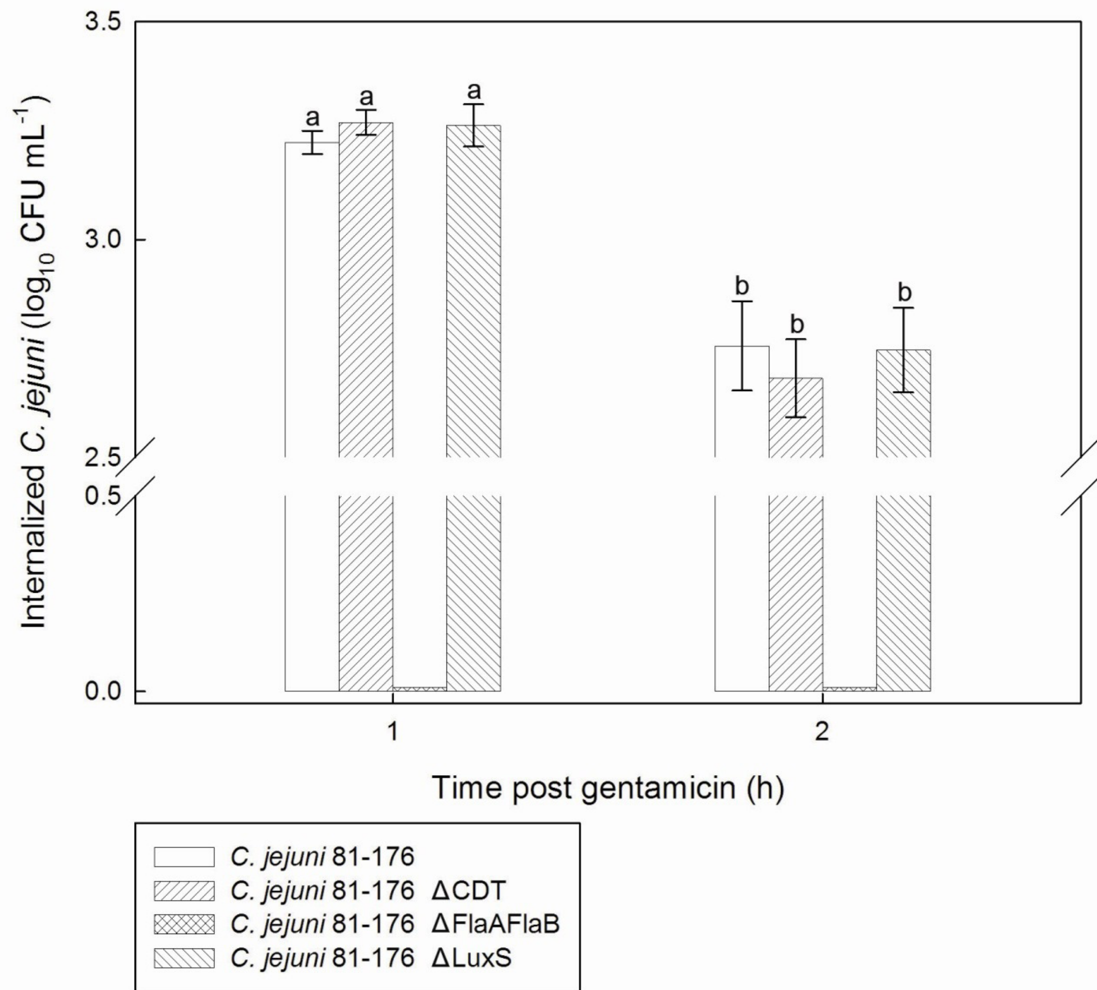

Figure 5.3. Internalization of *C. jejuni* 81-176 wild type, ΔCDT, ΔFlaAFlaB, and ΔLuxS (CFU) co-cultured with *A. polyphaga*. Vertical bars represent standard error and significant differences exist between the two time periods based on Tukey's test (α = 0.05).

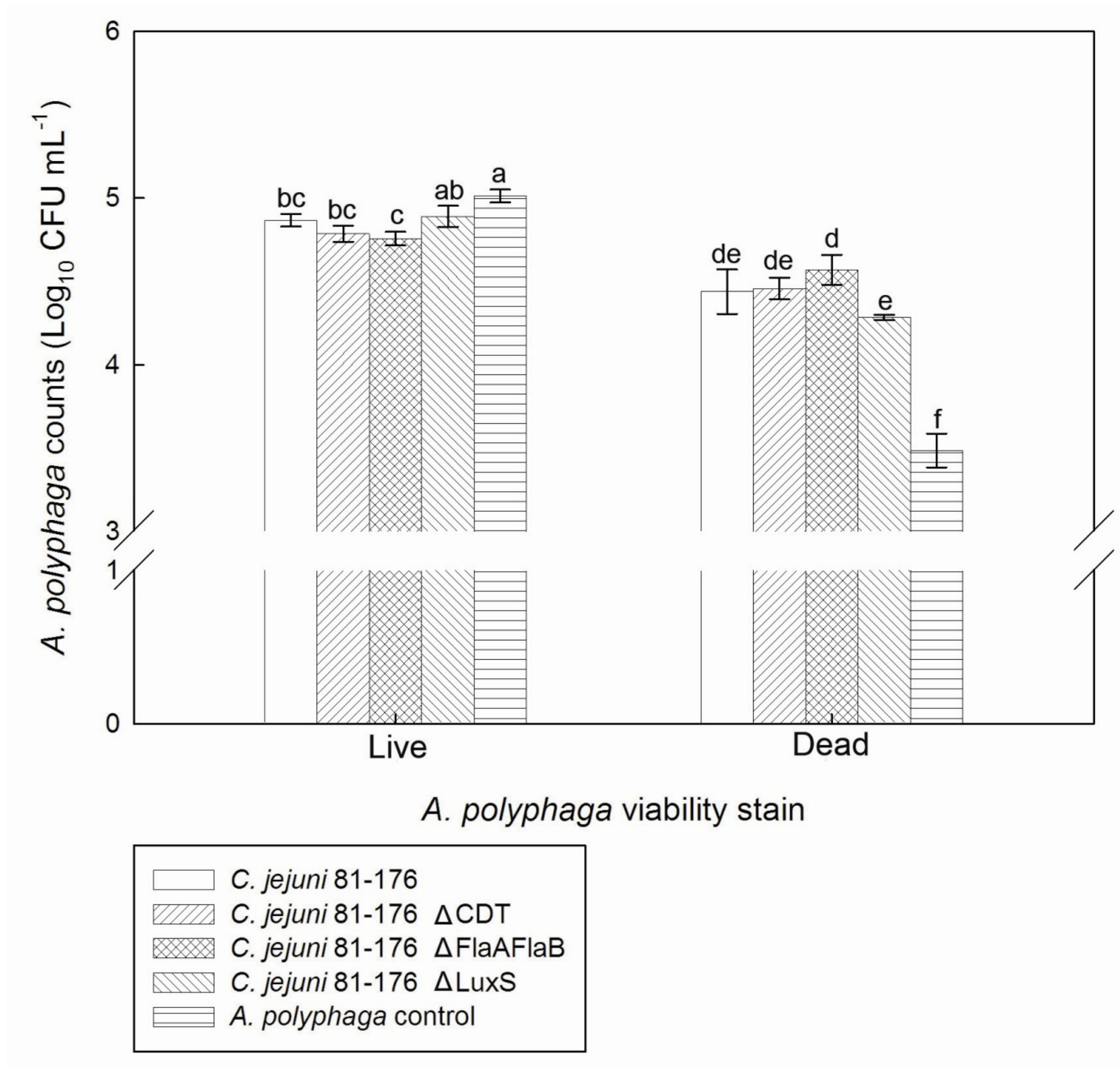

Figure 5.4. Live and dead *A. polyphaga* counts determined by trypan blue staining following 48 h of co-culture with *C. jejuni* 81-176 wild type and ΔCDT, ΔFlaAFlaB, and ΔLuxS mutants. Vertical bars represent standard error and significant differences exist between groups with no common letters based on Tukey's test ($\alpha = 0.05$).

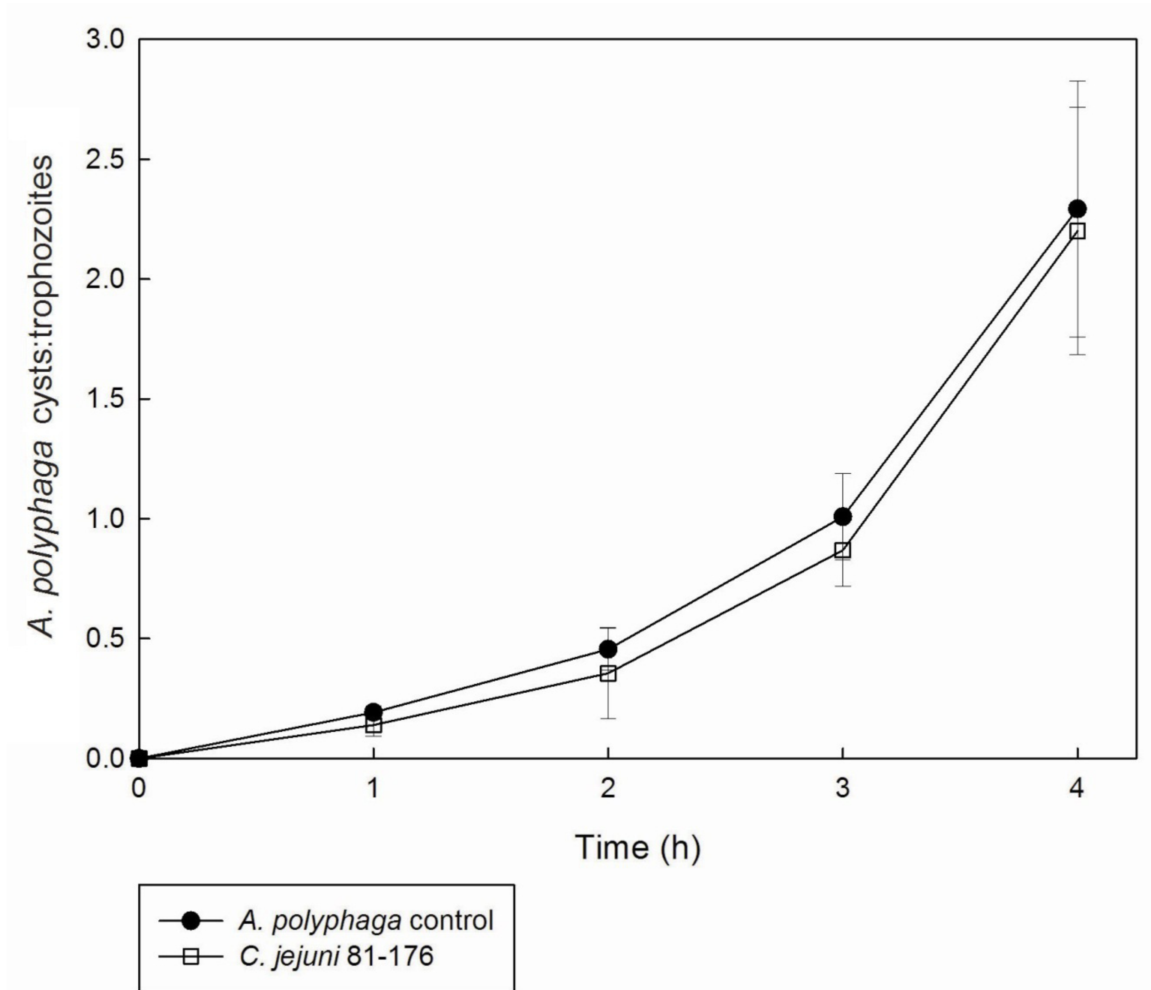

Figure 5.5. Ratio of *A. polyphaga* cysts to trophozoites when the protozoan was co-cultured with wild type *C. jejuni* 81-176 (white squares) or cultured alone (black circles). Vertical bars indicate standard error.

5.4 DISCUSSION

Investigations into the interplay between pathogenic bacteria and protozoans such as *A. polyphaga* are challenging as it is difficult to distinguish between engulfment initiated by the phagocyte and invasion by the bacterium. Engulfment and invasion mechanisms have similar characteristics (i.e. cytoskeleton rearrangement) and have not been investigated in the interaction between *C. jejuni* and *A. polyphaga*.

Prolonged survival of a clinical *C. jejuni* isolate in co-culture with *Acanthamoeba spp.* has previously been demonstrated (Axelsson-Olsson *et al.*, 2010); however, it is unknown how widespread this feature is among clinical isolates. Multiple clinical isolates from southwestern Alberta were tested as part of the present study, all of which survived longer in co-culture with *A. polyphaga* than when co-cultured with by-products or PYG medium. This data also facilitated the selection of a suitable strain for exploring the interactions between *C. jejuni* and *A. polyphaga* in greater depth. The presence of *A. polyphaga* under nutrient rich conditions (i.e. PYG growth medium) is expected to create a more suitable environment for *C. jejuni* survival as *A. polyphaga* depletes oxygen during aerobic respiration and *C. jejuni* is a microaerophile (Vandamme *et al.*, 2005). This was noted as all of the strains tested survived longer in the presence of *A. polyphaga* than PYG medium or by-products. Of note, the survival of *C. jejuni* in the presence of PYG medium and by-products was identical for nearly all strains. This may indicate that the prolonged survival is more dependent on the microaerobic environment created by *A. polyphaga* than a by-product formed by *A. polyphaga* that can be metabolized by *C. jejuni* as it is likely that the by-products were re-oxygenated during the removal of *A. polyphaga* by filtration. Trypan blue staining of *A. polyphaga* in co-culture with *C. jejuni*, the data suggests that *C. jejuni* imparts a cytotoxic effect on *A. polyhaga* as there was 1 log more

dead *A. polyphaga* when incubated in co-culture with *C. jejuni*. Further, this effect occurred irrespective of mutant status which may suggest an as yet unknown toxin or mechanism of action. Alternatively, there may have been competition for nutrients. *C. jejuni* cannot metabolize glucose (Vandamme *et al.*, 2005), but there may be competition for other nutrients, such as those present in yeast extract or peptone. To further evaluate this possibility, *A. polyphaga* was inoculated into a modified PYG medium in which yeast extract and peptone were absent; however, *A. polyphaga* was unable to grow sufficiently for analysis. An alternative approach to elucidate the specifics of this interaction included an experiment designed to investigate whether the cytotoxic effect is contact dependent. Microfuge tubes containing 0.1 µm filters were inoculated with *A. polyphaga* and *C. jejuni* in various combinations (same side or opposite sides of the filter). However, the columns leaked and were not suitable for maintaining a constant volume and sterility.

The invasion of mammalian cells by *C. jejuni* has been studied previously (Eucker and Konkel, 2012; Hu and Kopecko, 1999; Russell and Blake, 1994; Neal-McKinney and Konkel, 2012; Watson and Galán, 2008), yet the exact mechanism of invasion remains elusive. As mentioned previously, *C. jejuni* incorporates features of both the trigger and zipper methods during invasion (Ó Cróinín and Backert, 2012). *In vitro* studies show conflicting results for the role of CDT in pathogenesis, as it was not required to induce oncosis in enterocytes (Kalischuk *et al.*, 2007), yet required for maximal adherence and invasion of the HeLa cell line (Jain *et al.*, 2008). A previous study examining the effect of supernatants from cultures of CDT+ and CDT- isolates on the gastrointestinal (GI) tract of mice resulted in no significant pathology in the GI tract of CDT- isolates while mice fed supernatants from CDT+ isolates demonstrated moderate to severe pathology (Jain *et al.*, 2008). Considering the conflicting nature of previous reports in regards to the role of CDT

in pathogenesis and the potential interaction between *A. polyphaga* and *C. jejuni* in aquatic settings, we hypothesized that CDT may have a central role in the exploitation of protists. The fact that *C. jejuni* ΔFlaAFlaB was rarely recovered from *A. polyphaga* in the gentamicin protection assay (and when it was, it was detected at very low numbers) combined with the consistent recovery of *C. jejuni* ΔCDT, ΔLuxS and WT from *A. polyphaga* trophozoites suggests that *C. jejuni* invades *A. polyphaga* in a manner dependent on a functional flagellar export apparatus, which is consistent with the trigger method. In regards to *A. polyphaga*, observations suggest that non-motile *C. jejuni* is not phagocytosed by the amoebae under nutrient rich conditions. Both the wild type and mutant strains of *C. jejuni* showed a decrease in the number of bacterial cells recovered following exposure of *A. polyphaga* to gentamicin sulfate for 2 h compared to 1 h. It is unclear whether the decrease in cell concentration is due to killing of internalized *C. jejuni* by *A. polyphaga* or penetration of gentamicin. Killing by *A. polpyhaga* is unlikely as no *C. jejuni* ΔFlaAFlaB mutants were internalized and this would have been expected in the event *A. polyphaga* was actively feeding on the bacterium. Gentamicin protection assays are often limited to a few hours as gentamicin will penetrate into the cells over time (Hamrick *et al.*, 2003). Recently, bacteriophages have been proposed as an alternative to antibiotics for protection assays (Hamrick *et al.*, 2003), and may aid in future studies concerning the long-term fate of intracellular *C. jejuni*.

Persistence of *A. polyphaga* during periods of unfavourable conditions is facilitated by formation of a cyst. Other bacterial pathogens, including *Mycobacterium avium* and *L. pneumophila* have been demonstrated to survive encystment/excystment of *A. polyphaga*, after which they were recovered by direct plating (Kilvington and Price, 1990; Steinert *et al.*, 1998). In the encystment/excystment assay used herein, which is

similar to those employed previously, *C. jejuni* was not recovered following direct plating nor enrichment suggesting *C. jejuni* does not survive encystment of *A. polyphaga* or it survives by entering VBNC state.

In conclusion, the data presented here suggests that *C. jejuni* invades *A. polyphaga* in a manner consistent with the trigger method in which the T3SS (in this case the flagellar apparatus) is required for invasion. Cytolethal distending toxin and quorum sensing via AI-2 are not required for entry. *C. jejuni* does not exploit encystment of *A. polyphaga* for prolonged survival. Further studies are warranted; specifically involving fluorescence microscopy to determine the fate of internalized *C. jejuni* cells following engulfment by, and encystment of *A. polyphaga*.

CHAPTER 6

Conclusions and Future Research

At the onset of this project, little was known in regards to the seasonal distribution of *C. jejuni* and protistan community structure in river waters in southern Alberta and even less was understood in regards to the mechanisms by which *C. jejuni* persists in the presence of phagotrophic protists. The following hypotheses were investigated as part of the research reported herein:

1. *C. jejuni* is present in southwestern Alberta rivers and exhibits a seasonal trend of abundance and persistence similar to other waterborne pathogens.
2. A diverse community of viable protists, including phagotrophic protists are present in southwestern Alberta rivers and the composition of the protistan community varies seasonally.
3. The ability of *C. jejuni* to exploit phagotrophic protists is dependent on the bacterium possessing a functional flagellar export apparatus, the capability to communicate in a density dependent manner (quorum sensing), and the ability to produce the cytolethal distending toxin (CDT).

These hypotheses are interconnected; in order for protists to play a role in the transmission of waterborne *C. jejuni* to humans in southern Alberta it must be demonstrated that both *C. jejuni* and protists capable of serving as hosts for *C. jejuni* are present.

Research findings presented in Chapter 3 demonstrate that *C. jejuni* is present in southwestern Alberta rivers throughout the year. A seasonal trend in *C. jejuni* abundance was not observed by qPCR; however, the majority of culture-based isolations were during the winter months. In addition, *C. jejuni* concentrations did not correlate with

physicochemical properties of water and the environment sampled. It is possible that the PCR-based enumeration quantified a large population of VBNC cells, capable of resuscitation following ingestion by a suitable host such as humans, thereby representing a significant public health concern.

The exploration of protistan diversity presented in Chapter 4 revealed that planktonic protists, including protozoans are abundant in river water in southwestern Alberta, and that conspicuous seasonal shifts occur in the community structure. The T-RFLP data combined with clone libraries support the theory of moderate endemicity for planktonic protists in river water as NMS analysis clustered samples according to time rather than location. The spring library was dominated by Chloroplastidae (29.8 %), Centrohelida (28.1 %), and Alveolata (25.5 %), while the summer and fall libraries contained primarily fungal clones (83.0 % and 88.0 %, respectively). Alveolata (35.6 %), Euglenozoa (24.4 %), Chloroplastida (15.6 %), and Fungi (15.6 %) dominated the winter library. Sanger clone libraries suggest the genera *Heterophrys* of the centrohelid heliozoans are prevalent protozoans during the spring. These organisms are of particular interest because *Heterophrys spp.* are bacteriophagous.

A. polyphaga was used as a model protozoan to study interactions with *C. jejuni* in Chapter 5. Isolates of clinical origin and isolated in southern Alberta from diarrheic humans survived longer in co-culture than in the presence of growth media or bi-products, suggesting that phagotrophic protists may play a role in their survival outside vertebrate hosts. Data indicated that *C. jejuni* invades *A. polyphaga* in a manner consistent with the trigger mechanism in which the T3SS is required for invasion. However, cytolethal distending toxin and quorum sensing via AI-2 are not required for entry. If *C. jejuni*

exploits encystment of *A. polyphaga* as a survival mechanism outside of vertebrate hosts, it is likely in a VBNC state.

In addition to furthering our understanding of freshwater ecosystems and the epidemiology of *C. jejuni*, research findings presented herein could have implications for future water treatment facilities. It has previously been demonstrated that protozoan cysts and trophozoites are resistant to chlorination and pathogens such as *L. pneumophila*, which are able to replicate within trophozoites, have caused significant outbreaks. An example of which occurred during the summer of 2012 in Quebec in which 177 people were infected by *L. pneumophila*, 13 of which died. In Lethbridge, drinking water is UV irradiated to disinfect the water of protistan pathogens (e.g. *Cryptospordium* and *Giardia*), but the effect of commercial UV treatment on deactivation of pathogenic bacteria within protistian cells is unknown and warrants investigation. As a result, additional water treatment measures may be necessary. Furthermore, molecular signatures of a wide array of protozoans have been found in treated drinking water (Valster *et al.*, 2009), and it is currently unknown if and to what degree these protozoans protect enteric pathogens thereby contributing to the burden of disease in humans.

Future Research

The research reported in this thesis identified a number of important future opportunities. Areas warranting additional research are:

1. Due to the manner in which river water samples were processed, it is unlikely the molecular signatures detected by qPCR represented dead *C. jejuni* cells (those without intact cell membranes); however, additional studies using DNA intercalating/crosslinking

agents such as EMA or PMA to exclude these signals from downstream analyses would improve our confidence in the detection of VBNC *C. jejuni* by qPCR.

2. Molecular epidemiological studies are required to better understand the origin of *C. jejuni* isolates infecting humans in southwestern Alberta and elsewhere. Many techniques have been developed for strain typing and have varying resolving power. This includes multilocus sequence typing (MLST), restriction fragment length polymorphism or short variable sequencing of the *flaA* gene (*flaA* RFLP and *flaA* SVR, respectively), pulsed field gel electrophoresis (PFGE) and comparative genomic fingerprinting (CGF); these methods rely on subtle changes in genomic content or composition to differentiate between isolates. CGF is perhaps the most robust method developed to date with the added benefit of being relatively inexpensive, high throughput, and requires minimal specialized equipment (Taboada *et al.*, 2012). CGF relies on genes which are variably absent or present (VAP), most of which are located within 16 hypervariable regions of the *C. jejuni* genome (Taboada *et al.*, 2012). Currently, the function of these VAP genes is unclear and it is uncertain if they play a role in adaptation to specific hosts or if strains can regain this genetic information. Bacterial contingency loci, or simple sequence repeats located within open reading frames or in promoter regions, may be an added value for molecular typing in combination with the above method(s). Polymerase slippage at these sites is responsible for mutations which in the case of SSRs in promoter regions, act as a switch, turning a set of genes on or off, or in the case of SSRs in open reading frames, by potentially altering phenotype (Moxon *et al.*, 2006). *C. jejuni* isolates possessing a number mutations including single nucleotide polymorphisms within their genomes relative to the parent strain were observed during infection of a human (Inglis *et al.*,

unpublished data); it is possible that these genetic changes reflects a host adaptation . Therefore, knowing the nature of the genetic changes associated with different hosts, we may be able to infer the origin of the isolate.

3. *Heterophrys* spp. were prevalent in the protistan diversity study during the spring sampling time, when many pathogens are also at increased densities. As such, they are of interest for investigation into native species which may play a role in the persistence of pathogens. *Heterophrys* spp. are not easily obtained as there are no type strains available in the Culture Collection of Algae and Protozoa (CCAP) nor the American Type Culture Collection (ATCC). There is also very limited genomic data in the NCBI database on the *Heterophrys* genus, making the design of genus-specific primers extremely difficult. An isolate of *Heterophrys marinara* is maintained by the Woods Hole Oceanographic Institution culture collection and was isolated from sea water. As such, efforts to isolate and study *Heterophrys* spp. in fresh water are worthwhile. Not only is it an organism of interest in the protection of bacterial pathogens, but it is highly likely that *Heterophrys* spp. in fresh water represents novel eukaryotic species. Furthermore, isolation of *Heterophrys* would allow researchers to study its role in freshwater ecosystems. Centrohelid heliozoans can be distinguished based on morphology as they contain a unique organelle, the centroplast, which contains axonemes with radially extended axopodia that play a role in capturing food and can be retracted rapidly (within ~20 ms) (Bardele, 1975). In addition, many centrohelid heliozoans have a surface layer of siliceous scales (Zlatogursky, 2010).

4. During the study of protistan diversity, a significant obstacle was encountered in the use of the 18S rRNA gene as a target gene. Plant clones belonging to the Solanaceae family of plants dominated all clone libraries, making up as much as 80-90% of amplicons. This required that these clones be identified and removed prior to sequencing. In the current study, solonaceous clones were identified by restriction digest profiles; however, this was time consuming and would not be suitable for next generation sequencing approaches which bypass the cloning step.

An alternative approach may lie in the use of blocking primers (Vestheim and Jarman, 2008), which have been used in metagenomic studies to selectively inhibit the amplification of undesired templates, in this case, solanaceous plants. There are three types of blocking primers including: (1) annealing inhibiting blockers; (2) elongation arrest blockers; and (3) annealing inhibiting blockers. The most basic blocking primer design relies on small differences in sequences near annealing sites; however, this is problematic for universal binding sites, which are highly conserved. An alternate blocking primer approach includes a dual priming oligonucleotide (DPO). With a DPO, two primers are linked by five deoxyinosine nucleotides, which enables them to behave like separate primers and thus keeping a suitable T_m. The 5' end of the DPO overlaps with the annealing site for the universal primer while the 3' end overlaps with a short sequence specific for the undesired template (Vestheim and Jarman, 2008). The 3' end is modified with 2,3-diphosphoglycerate (DPG), which prevents extension of the primer.

This problem could also be approached at the post-amplification stage. For example, a restriction endonuclease that only cuts DNA of the 18S rRNA gene of Solanaceae plants could be used to screen this DNA. Following PCR amplification, one could set up a restriction digest and subsequently isolate sequences of interest (uncut

amplicons) via agarose gel electrophoresis and gel extraction. In terms of further sampling the protistan communities of southwestern Alberta rivers, next generation sequencing in combination with blocking primers could prove useful due to the massive increase in sequence data obtained and may help in the discovery of rare taxa. The deeper coverage provided by next generation sequencing would be useful in studying protistan diversity. Furthermore, as next generation sequencing is approaching the DNA read lengths obtained by Sanger sequencing will facilitate a better understanding of the biogeography of protists as deeper coverage should yield more complete community profiles.

5. In regards to the persistence of *C. jejuni* in the presence of phagotrophic protists, additional research, including the use of microcosm water and incubation temperatures closer to temperatures experienced in river water are warranted. Fluorescence microscopy and alternative protection assays (e.g. bacteriophage protection) would provide additional insight into the long term fate of *C. jejuni* following invasion.

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Appendix A.

Table A.1. 18S rDNA clones detected in the summer, fall, winter and spring clone libraries showing % similarity, organism and taxanomic rank.

Library	Clone	% Sim.	Organism	Accession	First Rank	Second Rank	Third Rank	Fourth Rank
Summer	1	99.8	*Candida tropicalis*	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	3	100	*Yarrowia lipolytica*	AY497757	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	4	99.8	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	5	99.3	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	7	99.3	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	10	99.7	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	12	99.8	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	13	99.9	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	14	99.2	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	17	99.9	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	18	100	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	20	99.8	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	21	99.6	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	22	99.5	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	23	99.6	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	25	99.7	*Trichosporon montevideense*	AB001762	Opisthokonta	Fungi	Basidiomycota	
Summer	26	99.4	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	27	99.7	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	28	99.4	*Candida tropicalis*	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	29	99.9	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	30	99.5	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	31	99.3	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	32	99.7	*Candida tropicalis*	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	35	100	*Mychonastes afer*	GQ477044	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae
Summer	36	99.9	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	37	100	*Candida tropicalis*	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	38	99.8	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	39	99.3	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	44	99.8	*Candida tropicalis*	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	46	99.9	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	47	99.6	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	48	99.8	*Candida sojae*	AB013549	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	50	99.9	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	51	99.6	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	52	99.8	*Candida tropicalis*	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	54	100	*Candida tropicalis*	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	55	99.8	*Nannochloris atomus*	AB080305	Archaeplastida	Chloroplastida	Chlorophyta	Trebouxiophyceae
Summer	56	99.6	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	57	99.9	*Candida tropicalis*	M60308	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	58	100	*Mychonastes homosphaera*	GQ477054	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae

Summer	62	99.8	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Summer	63	100	*Candida sojae*	AB013549	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	1	99.6	*Candida tropicalis*	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	2	100	*Candida tropicalis*	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	3	99.7	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	4	99.9	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	5	99.7	*Filobasidium elegans*	AB075545	Opisthokonta	Fungi	Basidiomycota	
Fall	8	97.1	*Uncultured cercozoan clone*	EU143886	Rhizaria	Cercozoa	Ebriacea	Ebria tripartita
Fall	9	99.8	*Mychonastes homosphaera*	AB025423	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae
Fall	11	99.8	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	12	99.9	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	13	99.7	*Candida tropicalis*	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	14	99	*Chlamydomonas raudensis*	AJ781313	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae
Fall	15	95.1	Uncultured eukaryote clone	FJ410617	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	16	98.7	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	17	96.2	*Epistylis chrysemydis*	AF335514	Chromalveolata	Alveolata	Ciliophora	Intramacronucleata
Fall	18	99	*Candida tropicalis*	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	19	99.7	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	20	99.7	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	21	99.8	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	22	99.4	*Mychonastes homosphaera*	X73996	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae
Fall	23	99.6	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	26	99.9	*Candida tropicalis*	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	27	99.3	*Mychonastes homosphaera*	X73996	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae
Fall	28	99.7	*Saprolegnia parasitica*	AB086899	Chromalveolata	Stramenopiles	Peronosporomycetes	
Fall	29	99.2	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	30	98.9	*Candida tropicalis*	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	31	99.8	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	32	98.6	*Rigidothrix goisery*	DQ490236	Chromalveolata	Alveolata	Ciliophora	Intramacronucleata
Fall	33	99.9	*Mychonastes homosphaera*	X73996	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae
Fall	35	99.5	*Candida tropicalis*	AF290987	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	36	96.9	Uncultured eukaryote clone	GU970807	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	37	99.4	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	38	99.5	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	39	99.2	*Candida tropicalis*	EF120592	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	40	99.5	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	41	96.9	Uncultured eukaryote clone	GU970807	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	42	99.5	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	43	99.6	*Epistylis urceolata*	AF335516	Chromalveolata	Alveolata	Ciliophora	Intramacronucleata
Fall	44	99.6	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	45	99.3	*Candida tropicalis*	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	46	98.3	*Codonella sp.*	DQ487193	Chromalveolata	Alveolata	Ciliophora	Intramacronucleata
Fall	47	99.6	*Codonella sp.*	DQ487193	Chromalveolata	Alveolata	Ciliophora	Intramacronucleata
Fall	48	98.7	*Codonella sp.*	DQ487193	Chromalveolata	Alveolata	Ciliophora	Intramacronucleata
Fall	49	99.5	*Candida tropicalis*	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	50	98.9	*Codonella sp.*	DQ487193	Chromalveolata	Alveolata	Ciliophora	Intramacronucleata
Fall	51	93.6	Uncultured marine cercozoan	FN598356	Rhizaria	Cercozoa		

Fall	52	99.4	*Candida tropicalis*	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	53	99.6	Trichosporon montevideense	AB001762	Opisthokonta	Fungi	Basidiomycota	
Fall	54	99.4	*Candida tropicalis*	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	55	99.5	*Candida tropicalis*	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	56	99.7	*Candida tropicalis*	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	57	95.2	Uncultured eukaryote clone	FJ410617	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	58	95.2	Uncultured eukaryote clone	FJ410617	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	59	98.7	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	60	99.6	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	61	99.3	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	62	99.6	*Candida tropicalis*	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Fall	63	99.3	*Candida tropicalis*	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Winter	1	99.6	Heterophrys sp. Oxford1	AY749611	Intcertae sedis	Centrohelida	Heterophryidae	
Winter	2	97.4	Uncultured eukaryote clone	AB238184	Chromalveolata	Alveolata	Apicomplexa	Insertae cedis
Winter	3	99.8	Uncultured eukaryote clone	FN393299	Rhizaria	Cercozoa	Cercomonadida	
Winter	4	96.6	Epistylis chrysemydis	AF335514	Chromalveolata	Alveolata	Ciliophora	Intramacronucleata
Winter	5	98.9	Chlamydomonas debaryana	AB542922	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae
Winter	6	99.2	Vorticella sp. SP-2009-5	GU187052	Chromalveolata	Alveolata	Ciliophora	Intramacronucleata
Winter	7	97	Eimeriidae environmental sample clone	EF024044	Chromalveolata	Alveolata	Apicomplexa	Conoidasida
Winter	8	91.3	Uncultured eukaryote clone	AM409555	Excavata	Euglenozoa	Euglenida	Heteronematina
Winter	9	99.6	Candida tropicalis	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Winter	11	99.7	Candida tropicalis strain NRRL Y-12968	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Winter	13	100	Cercozoa sp. WA28p82t6LS	EU709189	Rhizaria	Cercozoa	Cercomonadida	
Winter	14	99.6	Candida tropicalis	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Winter	15	91.4	Uncultured eukaryote clone	AM409556	Excavata	Euglenozoa	Euglenida	Heteronematina
Winter	16	98	Chlamydomonad sp. Pic 8/18 P-1w	AY220086	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae
Winter	17	97.4	Telotrochidium matiense	EF417835	Chromalveolata	Alveolata	Ciliophora	Intramacronucleata
Winter	18	96.4	Epistylis urceolata	AF335516	Chromalveolata	Alveolata	Ciliophora	Intramacronucleata
Winter	19	99.8	Uncultured stramenopile clone	FJ939068	Chromalveolata	Stramenopiles	Bicosoecida	
Winter	20	99.1	Uncultured eukaryote clone	FN393299	Rhizaria	Cercozoa	Cercomonadida	
Winter	21	99.3	Vorticella sp. SP-2009-5	GU187052	Chromalveolata	Alveolata	Ciliophora	Intramacronucleata
Winter	22	95.6	Epistylis chrysemydis	AF335514	Chromalveolata	Alveolata	Ciliophora	Intramacronucleata
Winter	23	99.3	Vorticella sp.	GU187049	Chromalveolata	Alveolata	Ciliophora	Intramacronucleata
Winter	24	91.6	Uncultured eukaryote clone	AM409555	Excavata	Euglenozoa	Euglenida	Heteronematina
Winter	25	96.1	Uncultured eukaryote clone	GU970273	Excavata	Euglenozoa	Euglenida	Heteronematina
Winter	26	99.9	Candida tropicalis	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Winter	27	97.5	Uncultured eukaryote clone	GU922505	Chromalveolata	Alveolata	Ciliophora	Unknown
Winter	28	99.7	Vorticella sp.	GU187049	Chromalveolata	Alveolata	Ciliophora	Intramacronucleata
Winter	29	97.9	Uncultured eukaryote clone	GU922505	Chromalveolata	Alveolata	Ciliophora	Unknown
Winter	30	97.4	Uncultured eukaryote clone	AB238184	Chromalveolata	Alveolata	Apicomplexa	Insertae cedis
Winter	31	99.6	Vorticella sp.	GU187049	Chromalveolata	Alveolata	Ciliophora	Intramacronucleata
Winter	32	99.1	Chlamydomonad sp. Pic 8/18 P-1w	AY220086	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae
Winter	33	91.1	Uncultured eukaryote clone	AM409555	Excavata	Euglenozoa	Euglenida	Heteronematina
Winter	34	91.3	Uncultured eukaryote clone	AM409555	Excavata	Euglenozoa	Euglenida	Heteronematina
Winter	35	99.5	Candida tropicalis	DQ515959	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Winter	36	97.6	Uncultured eukaryote clone	AB238184	Chromalveolata	Alveolata	Apicomplexa	Insertae cedis
Winter	37	91.5	Uncultured eukaryote clone	AM409556	Excavata	Euglenozoa	Euglenida	Heteronematina

Winter	38	91.3	Uncultured eukaryote clone	AM409556	Excavata	Euglenozoa	Euglenida	Heteronematina
Winter	39	98.9	Chlamydomonas debaryana	AF008240	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae
Winter	40	99.9	Candida tropicalis	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Winter	41	98.3	Chlamydomonas rapa	U70790	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae
Winter	42	91.1	Uncultured eukaryote clone	AM409556	Excavata	Euglenozoa	Euglenida	Heteronematina
Winter	43	99.6	Candida tropicalis	EU348785	Opisthokonta	Fungi	Ascomycota	Saccharomycetes
Winter	44	91.4	Uncultured eukaryote clone	AM409555	Excavata	Euglenozoa	Euglenida	Heteronematina
Winter	45	98.3	Chlamydomonas rapa	U70790	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae
Winter	46	98.7	Chlorococcum dorsiventrale	AB058303	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae
Winter	47	91.3	Uncultured eukaryote clone	AM409556	Excavata	Euglenozoa	Euglenida	Heteronematina
Winter	48	99.6	Vorticella sp.	GU187049	Chromalveolata	Alveolata	Ciliophora	Intramacronucleata
Spring	2	98.9	Uncultured sphenomonad euglenozoan	AY821957	Excavata	Euglenozoa	Euglenida	Heteronematina
Spring	4	98.8	Chlamydomonas pulsatilla	AF514404	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae
Spring	5	96.1	Uncultured eukaryotic picoplankton	EF196695	Chromalveolata	Stramenopiles	Bicosoecida	
Spring	6	96	Uncultured eukaryotic picoplankton	EF196695	Chromalveolata	Stramenopiles	Bicosoecida	
Spring	7	99.6	Uncultured chlorophyte clone	EU143983	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae
Spring	8	95.7	Uncultured Chytridiomycota clone	AJ867630	Opisthokonta	Fungi	Chytridomycetes	
Spring	9	98.3	Rhogostoma sp. DB-2002	GU970200	Rhizaria	Cercozoa	Insertae sedis	
Spring	11	99.8	Heterophrys sp. Oxford1	AY749611	Intcertae sedis	Centrohelida	Heterophryidae	
Spring	13	99.7	Heterophrys sp. Oxford1	AY749611	Intcertae sedis	Centrohelida	Heterophryidae	
Spring	14	99.4	Uncultured sphenomonad euglenozoan	AY821957	Excavata	Euglenozoa	Euglenida	Heteronematina
Spring	15	98	Heterophrys sp. Oxford1	AY749611	Intcertae sedis	Centrohelida	Heterophryidae	
Spring	16	97.2	Uncultured alveolate clone	FJ410727	Chromalveolata	Alveolata		Unknown
Spring	17	98.7	Chlorococcum dorsiventrale	AB058302	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae
Spring	18	99.5	Heterophrys sp. Oxford1	AY749611	Intcertae sedis	Centrohelida	Heterophryidae	
Spring	19	98.2	Diacronema vlkianum	AJ515246	Chromalveolata	Haptophyta	Pavlovophyceae	
Spring	20	99.8	Uncultured stramenopile clone	FJ939072	Chromalveolata	Stramenopiles	Bicosoecida	
Spring	21	99.6	Uncultured stramenopile clone	FJ939072	Chromalveolata	Stramenopiles	Bicosoecida	
Spring	22	99.8	Heterophrys sp. Oxford1	AY749611	Intcertae sedis	Centrohelida	Heterophryidae	
Spring	23	100	Heterophrys sp. Oxford1	AY749611	Intcertae sedis	Centrohelida	Heterophryidae	
Spring	25	99.9	Heterophrys sp. Oxford1	AY749611	Intcertae sedis	Centrohelida	Heterophryidae	
Spring	26	99.7	Pythiaceae sp.	FJ794931	Chromalveolata	Stramenopiles	Peronosporomycetes	
Spring	27	99.5	Heterophrys sp. Oxford1	AY749611	Intcertae sedis	Centrohelida	Heterophryidae	
Spring	28	99.9	Uncultured Chlorophyta clone	GQ844481	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae
Spring	29	99.2	Uncultured Chlorophyte clone	EU143983	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae
Spring	30	99.3	Uncultured sphenomonad euglenozoan	AY821957	Excavata	Euglenozoa	Euglenida	Heteronematina
Spring	33	91	Uncultured eukaryote clone	FJ810606	Chromalveolata	Alveolata	Ciliophora	Unknown
Spring	34	96.9	Uncultured eukaryotic picoplankton	DQ409097	Opisthokonta	Fungi	Chytridomycetes	
Spring	35	98.8	Uncultured eukaryote clone	GU919336	Rhizaria	Insertae sedis		
Spring	37	99.1	Uncultured Chytridiomycota clone	AJ867630	Opisthokonta	Fungi	Chytridomycetes	
Spring	40	99.7	Heterophrys sp. Oxford1	AY749611	Intcertae sedis	Centrohelida	Heterophryidae	
Spring	41	98.2	Uncultured chlorophyta clone	FJ410715	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae
Spring	42	95.7	Uncultured eukaryotic picoplankton	EF196695	Chromalveolata	Stramenopiles	Bicosoecida	
Spring	43	95.7	Uncultured eukaryotic picoplankton	EF196695	Chromalveolata	Stramenopiles	Bicosoecida	
Spring	45	99.6	Heterophrys sp. Oxford1	AY749611	Intcertae sedis	Centrohelida	Heterophryidae	
Spring	47	98.3	Chlorococcum sp.	AB058332	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae
Spring	49	96.7	Uncultured Chlorophyta clone	HQ191358	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae

Spring	50	99.8	Heterophrys sp. Oxford1	AY749611	Intcertae sedis	Centrohelida	Heterophryidae	
Spring	52	96.1	Uncultured eukaryotic picoplankton	EF196695	Chromalveolata	Stramenopiles	Bicosoecida	
Spring	53	99.7	Heterophrys sp. Oxford1	AY749611	Intcertae sedis	Centrohelida	Heterophryidae	
Spring	54	97	Uncultured sphenomonad euglenozoan	AY821957	Excavata	Euglenozoa	Euglenida	Heteronematina
Spring	56	96.3	Uncultured eukaryotic picoplankton	EF196695	Chromalveolata	Stramenopiles	Bicosoecida	
Spring	57	96.3	Uncultured Chytridiomycete clone	AJ867630	Opisthokonta	Fungi	Chytridomycetes	
Spring	58	99.9	Heterophrys sp. Oxford1	AY749611	Intcertae sedis	Centrohelida	Heterophryidae	
Spring	59	99.5	Heterophrys sp. Oxford1	AY749611	Intcertae sedis	Centrohelida	Heterophryidae	
Spring	61	99.5	Uncultured sphenomonad euglenozoan	AY821957	Excavata	Euglenozoa	Euglenida	Heteronematina
Spring	62	96.3	Uncultured eukaryotic picoplankton	EF196695	Chromalveolata	Stramenopiles	Bicosoecida	
Spring	63	98.6	Chlorococcum sp.	AB058333	Archaeplastida	Chloroplastida	Chlorophyta	Chlorophyceae
Spring	64	98.5	Uncultured eukaryote clone	AJ130856	Rhizaria	Insertae sedis		
Spring	68	98	Stylonychia ammermanni	FM209295	Chromalveolata	Alveolata	Ciliophora	Intramacronucleata
Spring	69	99	Uncultured eukaryote clone	GU919336	Rhizaria	Insertae sedis		
Spring	70	99.7	Auxenochlorella protothecoides	X56101	Archaeplastida	Chloroplastida	Chlorophyta	Trebouxiophyceae
Spring	71	96.2	Uncultured Chytridiomycete clone	AJ867630	Opisthokonta	Fungi	Chytridomycetes	

Appendix B. SPYDER, a new method for *in silico* design and assessment of 16S rDNA primers for molecular microbial ecology[2]

ABSTRACT

Molecular microbial ecology studies are heavily reliant on “Universal” 16S rDNA primers for elucidating microbial community structure and composition yet primer design and optimization is often overlooked. Primers which exhibit minor biases due to primer-template mismatches can substantially alter the pool of amplicons from a community DNA sample resulting in inaccurate conclusions. As a result, it is important that primers are critically evaluated against the most comprehensive datasets available prior to commencing molecular microbial community studies. We present a user friendly, Window-based method named Spyder for the *in silico* design and assessment of 16S rDNA primers. The method utilizes the Ribosomal Database Project’s probe match feature coupled with a compact program (Available at http://people.uleth.ca/~selibl/Spyder/Spyder.html) which aligns and identifies mismatches between primers and templates. To demonstrate the value of Spyder, we assessed commonly used “Universal” and Phyla specific primers and identifed primer modifications that improved coverage of target organisms by 5-42% as well as removed excessive degeneracies.

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[2] A version of this chapter was published as: Thomas M.C., Thomas D.K., Selinger L.B., Inglis G.D. (2011). SPYDER, a new method for *in silico* design and assessment of 16S rRNA gene primers for molecular microbial ecology. *FEMS Microbiology Letters*, **320**(2):152-159.

INTRODUCTION

It is estimated that over 99% of bacteria have yet to be cultured (Brooks *et al.*, 2007). While the application of molecular-based approaches has greatly increased our knowledge of microbial ecology, molecular methods are fraught with problems of their own (Forney *et al.*, 2004). The current flow for culture independent microbial community analyses stems from the work by Pace and colleagues, who described a technique for amplifying 16S rRNA genes from bulk nucleic acid extractions using "Universal" primers. Sequences are then classified and compared using phylogenetic trees (Pace *et al.*, 1985). As the vast majority of molecular ecology studies targeting microorganims depend on polymerase chain reaction, they are subject to the associated biases. Surprisingly, this is often overlooked by microbial ecologists.

The 16S rRNA gene is the gene of choice for molecular ecology studies focusing on prokaryotes due the fact that the gene is: (a) ubiquitous, (b) highly conserved, and (c) possesses enough variability to discriminate between taxa. Primers targeting the 16S rRNA gene for domain- or phyla-specific studies must adhere to a type of "Goldilocks" state; that is, not too exact in that it excludes desired species or genera, yet exact enough to prevent the inclusion of undesired contaminants in subsequent analyses. Initial primers were designed from sequence data obtained from cultured species. As a result, these primers are not comprehensive. None-the-less, many researchers still frequently utilize 'universal' primers developed in the early 1990's. Over the past two decades, sequence databases, including those containing 16S rDNA data, have expanded tremendously, and the large size of the databases presents a significant challenge to researchers wishing to design/utilize primers for bacterial ecology studies as most prokaryotic taxa within the databases have no or few cultured representatives. Furthermore, major bias exists towards

just four out of 25 phyla, namely the *Actinobacteria*, *Bacteriodetes*, *Firmicutes*, and *Proteobacteria* (Hugenholt, 2002). According to the SILVA SSU REF release 102 database (Pruesse *et al.*, 2007), these four phyla comprise nearly 86% of 16S rRNA gene sequences currently available. Despite this lack of representation by rare or under sampled phyla, sequence databases such as that used by the Ribosomal Database Project (RDP) (Cole *et al.*, 2009) Probe Match program, contain in excess of 1.6 million 16S rDNA sequences. Databases have become so large that it is impractical to manually align and analyze sequences for broad-spectrum primer design. While programs like ARB (Ludwig *et al.*, 2004) and PRIMROSE (Ashelford *et al.*, 2002) have been developed with features to assist in the design of comprehensive primers, neither have the functionality to allow the user to subjectively enter degenerate bases based on alignments. Furthermore, the computing power required to run either program on modern databases in their entirety is far beyond that of the average computer. As a consequence, partial databases containing representative sequences are often used. Lastly, ARB is a Unix based program and thus presents an additional barrier for individuals that are not well versed in the use of this operating system.

Concerning primer design, conserved regions are sought out for proper primer-template annealing; however, there is no such thing as a truly ‘universal’ primer due to the nature of the 16S rRNA gene as mutations have been accumulating throughout prokaryotic evolution. As a result, mismatches between primer and template are inevitable. It is widely accepted that mismatches between primers and targets at the 3’ end of a primer can result in no amplification or greatly reduced amplification efficiency yet the ramifications of mismatches occurring at other locations have received little attention until relatively recently. Furthermore, the assumption that a PCR reaction can tolerate two

mismatches between primer and template is often used as a baseline for *in silico* analyses; however, amplification in a multi-template PCR reaction can differ substantially making this premise an oversimplification. For example, using qPCR a single mismatch occurring from the mid-point to the 3' end between primer and target was shown to reduce amplification 1000-fold (Bru *et al.*, 2008). As such, it is critical that primers are designed with care to ensure accurate profiling of community structures. A reduction in amplification may not be an issue when dealing with pure DNA samples originating from a single organism, yet it has major consequences when interpreting 16S rDNA libraries constructed for the purpose of community analysis. Sequence databases, such as RDP (Cole *et al.*, 2009) and SILVA (Pruesse *et al.*, 2007), have grown exponentially since their inception yet many primers commonly in use today have not been assessed in relation to the massive amount of sequence data currently available. This is due in part to the fact that there are few efficient means of data mining and evaluating primers against today's massive databases. The purpose of this study was to design a user friendly Windows-based program capable of quickly analyzing data gathered from the Ribosomal Database Project Probe Match, allowing users to identify sites in which current primers are not comprehensive, and to improve upon those primers.

MATERIALS AND METHODS

Program design and implementation

The program developed (Spyder) was designed in PERL and uses the Needleman-Wunsch algorithm (Needleman & Wunsch, 1970) via dynamic programming, a type of recursion that keeps track of each previous recursive step (i.e. Smart recursion). The similarity matrix (Table B.1) is used to generate a scoring matrix based on the alignment

of primer and target. The matrix is a slight modification of a generic scoring matrix for the four bases ‘A C T G’. The modifications take into account the possibility of degenerate bases, which are often encountered in sequence databases. Degeneracies were assigned scores (Table B.1) such that the score of the degenerate base is the summation of the scores for each possible combination between the degeneracy and the corresponding base. For example, the degenerate base “H” – could be either base “A”, “C” or “T”, therefore the score for “H” is the sum of scores for “A”, “C” and “T”. The scoring matrix is used to assign scores for all positions in every possible alignment between the primer and target. Each possible alignment is scored through a trace back of the scoring matrix and the optimal alignment (i.e. that with the highest score) selected.

Primers evaluated and RDP Probe Match search parameters

Commonly used 16S rDNA primers (Table B.2) were evaluated against sequences within the RDP database (Cole *et al.*, 2009). Primer-target regions were selected according to their approximate annealing position relative to *Escherichia coli* (Genbank Accession J01695) (Figure B.1). The antisense (-) strand was selected for forward primers, and sense (+) strand for reverse primers. Regions were selected such that they ensured coverage of the primer binding site while maintaining maximal coverage of the database, which was verified by retrospective analysis of the Spyder output (instructions provided as supplemental document ‘Application of Spyder to the Ribosomal Database Project Probe Match.doc’).

Table B.1. Similarity matrix for alignment algorithm showing scores assigned to matched and mismatched nucleotide bases including scores for degeneracies[3].

	A	**G**	**C**	**T**	**R**	**Y**	**B**	**D**	**K**	**M**	**H**	**V**	**S**	**W**
A	10	-1	-3	-4	9	-7	-8	5	-5	7	3	6	-4	6
G	-1	7	-5	-3	6	-8	-1	3	4	-6	-2	1	2	-4
C	-3	-5	9	0	-8	9	4	-8	-5	6	6	1	4	-3
T	-4	-3	0	8	-7	8	5	1	5	-4	4	-7	-3	4
R	9	6	-8	-7	15	-15	-9	8	-1	1	1	5	-2	2
Y	-7	-8	9	8	-15	17	9	-7	0	2	10	-6	1	1
B	-8	-1	4	5	-9	9	8	-4	4	-4	8	-5	3	-3
D	5	3	-8	1	8	-7	-4	9	4	-3	5	0	-5	6
K	-5	4	-5	5	-1	0	4	4	9	-10	2	-6	-1	0
M	7	-6	6	-4	1	2	-4	-3	-10	12	9	7	0	3
H	3	-2	6	4	1	10	8	5	2	9	13	0	4	7
V	6	1	1	-7	5	-6	-5	0	-6	7	0	8	2	-1
S	-4	2	4	-3	-2	1	3	-5	-1	0	4	2	6	-7
W	6	-4	-3	4	2	1	-3	6	0	3	7	-1	-7	10

[3] Degeneracies listed are standard IUPAC letters where R=A or G, Y =C or T, B=C, G, or T, D=A, G, or T, K=G or T, M=A or C, H=A, C, or T, V=A, C, or G, S=G or C, and W=A or T.

Table B.2. Primers analyzed in this study including target, RDP Probe Match search target for probe analysis, and target sequence in relation to *Escherichia coli* (Genbank Accession J01695).

Primer	**Sequence**	**Target organisms**	**Approximate Seq. Target**	**RDP Search Target**	**Reference**
Act1159R	TCCGAGTTRACCCCGGC	Actinobacteria	1139-1159	1039-1259	Blackwood *et al.* (2005)
Alf28f	ARCGAACGCTGGCGGCA	Alphaproteobacteria	28-44	7-128	Ashelford *et al.* (2002)
Alf684r	TACGAATTTYACCTCTACA	Alphaproteobacteria	666-684	570-784	Ashelford *et al.* (2002)
A571F	GCYTAAAGSRICCGTAGC	Archaea	554-571	451-671	Baker *et al.* (2003)
BLS342F	CAGCAGTAGGGAATCTTC	Bacilli	352-369	222-442	Blackwood *et al.* (2005)
CFB555f	CCGGAWTYATTGGGTTTAAAGGG	Bacteriodetes	555-577	530-655	Muhling *et al.* (2008)
CFB968r	GGTAAGGTTCCTCGCGTA	Bacteriodetes	951-968	848-1068	Muhling *et al.* (2008)
Beta359f	GGGGAATTTTGGACAATGGG	Betaproteobacteria	359-378	239-569	Muhling *et al.* (2008)
Beta682r	ACGCATTTCACTGCTACACG	Betaproteobacteria	671-682	750-971	Muhling *et al.* (2008)
Beta680F	CRCGTGTAGCAGTGA	Betaproteobacteria	680-694	680-694	Overmann *et al.* (1999)
CYA361f	GGAATTTTCCGCAATGGG	Cyanobacteria, chloroplasts	361-378	241-481	Muhling *et al.* (2008)
CYA785r	GACTACWGGGGTATCTAATCC	Cyanobacteria, chloroplasts	765-785	665-885	Muhling *et al.* (2008)
9bfm	GAGTTTGATYHTGGCTCAG	Eubacteria	Sep-27	9-100	Muhling *et al.* (2008)
347F	GGAGGCAGCAGTRRGGAAT	Eubacteria	347-365	247-465	Nossa *et al.* (2010)
803R	CTACCRGGGTATCTAATCC	Eubacteria	785-803	685-903	Nossa *et al.* (2010)
HDA1	ACTCCTACGGGAGGCAGCAGT	Eubacteria	338-358	238-458	Walter *et al.* (2000)
HDA2	GTATTACCGCGGCTGCTGGCA	Eubacteria	516-536	416-636	Walter *et al.* (2000)
1350R	GACGGGCGGTGTGTACAAG	Eubacteria	1389-1407	1289-1507	Glockner *et al.* (2000)
F27	AGAGTTTGATCMTGGCTCAG	Eubacteria	8-27	7-127	Lane (1991)
F44	RGTTYGATYMTGGCTCAG	Eubacteria	10-27	7-110	Abnous *et al.* (2009)
Firm350f	GGCAGCAGTRGGGAATCTTC	Firmicutes	350-369	230-450	Muhling *et al.* (2008)
Firm814r	ACACYTAGYACTCATCGTTT	Firmicutes	794-814	794-914	Muhling *et al.* (2008)
Gamma395f	CMATGCCGCGTGTGTGAA	Gammaproteobacteria	395-404	275-495	Muhling *et al.* (2008)
Gamma871r	ACTCCCCAGGCGGTCDACTTA	Gammaproteobacteria	851-871	750-971	Muhling *et al.* (2008)
Plancto352f	GGCTGCAGTCGAGRATCT	Planctomycetes	352-379	232-452	Muhling *et al.* (2008)
Plancto920r	TGTGTGAGCCCCCGTCAA	Planctomycetes	901-920	800-1020	Muhling *et al.* (2008)
Pln903R	CTCCACCGCTTGTGTGA	Planctomycetes	903-922	783-1003	Blackwood *et al.* (2005)
1512uR	ACGGHTACCTTGTTACGACTT	Universal	1492-1512	1392-1520	Muhling *et al.* (2008)
U1406R	GACGGGCGGTGTGTRCA	Universal	1391-1407	1291-1500	Hansen *et al.* (1998)
UA1406R	ACGGGCGGTGWGTRCAA	Universal	1390-1406	1290-1500	Baker *et al.* (2003)
U341F	CCTACGGGRSGCAGCAG	Universal	341-357	241-457	Hansen *et al.* (1998)
1492R	TACGGYTACCTTGTTACGACTT	Universal	1492-1514	1392-1530	Lane (1991)
1391R	GACGGGCGGTGTGTRCA	Universal	1391-1407	1271-1491	Tanner *et al.* (1999)
1543R	GGNTACCTTKTTACGACTT	Universal	1492-1510	1392-1530	Abnous *et al.* (2009)

AAATTGAAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGTAACAGGAAGAAGCTTGCTTCTTTGCTGACGAGTGGCGGACGGGTG
AGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATG
TGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTAC
GGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAA
AGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAG
CGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCG
GTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAAT
TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACGGAAGTTTTCAGAGATGAGAATGTGCCTTCGGGA
ACCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCA
AAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTC
GCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGT
TCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGAAGT
CGTAACAAGGTAACCGTAGGGGAACCTGCGGTTGGATCACCTCCTTA

Figure B.1. *E. coli* 16S rRNA gene (Genbank Accession J01695) reference strain with approximate locations of variable regions V1-V9 (Van de Peer *et al.*, 1996) annotated using Geneious 5.1 (Drummond *et al.*, 2010).

Primer improvement

The Spyder output was analyzed manually for indels and substitutions. Those which were abundant relative to the number of available sequences for the searched region were noted and necessary degeneracies or modifications completed. Updated primers were then re-analyzed using the RDP Probe Match service to determine the effect on target and non-target sequences. Modified primers were checked using OligoCalc (Kibbe, 2007) to ensure no decrease in primer quality (i.e. similar GC content, no self-complementarity, hairpins, or 3' primer-primer complementarity).

RESULTS AND DISCUSSION

The Spyder program was able to successfully process over 1,000,000 sequences in a matter of minutes using a relatively modest computer (Core 2 Duo processor at 2.1 GHz with 4 GB of RAM). Conducting such an analysis on an aligned 16S rDNA database is not practical due to the size of current databases, which can easily exceed 100 MB. The primers analyzed matched between 48 and 97% of target sequences currently available with zero mismatches. All primers showed improved coverage by allowing two mismatches in the RDP probe match search parameters; however, the increase in coverage was often accompanied by an increase in non-specific matches for phyla and class specific probes (Table B.3). The Spyder output, which consists of a text file summarizing the location of indels and substitutions, was used to identify locations where degeneracies could be introduced to compensate for common mismatches. A second analysis using RDP Probe Match was used to evaluate the new primer and verify that it did not compromise specificity (Table B.4). OligoCalc confirmed primer quality, including suitable GC content, and the absence of self-complementarity, hairpins, and 3' primer-

primer complementarity (data not shown). The most substantial improvements were for the primers targeting Alphaproteobacteria (Alf28f), Gammaproteobacteria (Gamma395f), Bacteriodetes (CFB555f), Firmicutes (Firm350f), and Archaea (A571F) primers resulting in 22, 42, 15, 18, and 26% increases in coverage respectively while non-specific mismatches remained low (0.03-2.56%) (Table B.4).

Analysis of primers designed using ARB and Primrose (i.e. those designed by Muhling *et al.*, 2008) by Spyder indicated that these primers could be improved without sacrificing specificity by adding targeted degeneracies (Table B.4). This may be because databases are more comprehensive and/or that ARB does not include a feature for including degeneracies in the primer design (Muhling *et al.*, 2008). Spyder also identified improvements (5.9% increase) of the commonly used Eubacterial primer F27, which is the forward primer used along with R1492 for the Human Microbiome Project Sanger sequencing libraries (Turnbaugh *et al.*, 2007). The F27 primer was also the forward primer of choice in the recent survey of the microbiota of the oral cavity of healthy adults in which over 10,000 full length 16S rDNA sequences were analyzed (Bik *et al.*, 2010). In the majority of cases, Spyder determined that only the forward or reverse primer of a standard set could be improved. The lack of non-specific hits associated with the improved primer indicates that it may be beneficial to use a comprehensive universal or alternate primer to complete the pair in the event that the current primer pair possesses differential coverage. Adding degeneracies is a common method for improving primers; however, it is possible that too many degenerate sites will diminish the primers target specificity. As such, other methods to increase mismatch tolerance should also be considered such as using long primers (25+ bases long), increasing dNTP concentrations,

Table B.3. Primer sequences referenced in Table 2 with coverage values[4] from the RDP Probe Match showing target and non-target hits when allowing zero and two mismatches for target sequences.

Primer	Target	Coverage (0 mismatches)						Coverage (2 mismatches)					
		Target hits			Non-target hits			Target Hits			Non-target hits		
Act1159R	Actinobacteria	117021	127918	91.5%	520	117541	0.4%	121871	127918	95.3%	21363	143234	16.7%
Alf28f	Alphaproteobacteria	14637	20348	71.9%	1136	15773	5.6%	68537	82189	83.4%	58775	127312	71.5%
Alf684r	Alphaproteobacteria	67264	72834	92.4%	15132	82396	20.8%	72117	72834	99.0%	416755	488872	572.2%
A571F	Archaea	27235	49940	54.5%	13	27248	0.0%	48789	49940	97.7%	32126	80915	64.3%
BLS342F	Bacilli	159035	166334	95.6%	2024	161059	1.2%	165892	166334	99.7%	124856	290748	75.1%
CFB555f	Bacteriodetes	97909	111977	87.4%	350	98259	0.3%	111048	111977	99.2%	165454	276502	147.8%
CFB968r	Bacteriodetes	76106	80430	94.6%	4099	80205	5.1%	80082	80430	99.6%	527570	607652	655.9%
Beta359f	Betaproteobacteria	67711	72827	93.0%	1049	68760	1.4%	72337	72827	99.3%	432728	505065	594.2%
Beta682r	Betaproteobacteria	55567	63193	87.9%	576	56143	0.9%	60980	63193	96.5%	313186	374166	495.6%
Beta680F	Betaproteobacteria	69002	77786	88.7%	609	69611	0.8%	75889	77786	97.6%	323410	399299	415.8%
CYA361f	Cyanobacteria, chloroplasts	18114	19660	92.1%	1962	20076	10.0%	19579	19660	99.6%	234947	254526	1195.1%
CYA785r	Cyanobacteria, chloroplasts	15417	17678	87.2%	5670	21087	32.1%	17325	17678	98.0%	870004	887329	4921.4%
9bfm	Eubacteria	222934	276514	80.6%	19	222953	0.0%	271384	276514	98.1%	20	271404	0.0%
347F	Eubacteria	960483	1072340	89.6%	5	960488	0.0%	1045709	1072340	97.5%	9	1045718	0.0%
803R	Eubacteria	774763	902009	85.9%	3491	778254	0.4%	896687	902009	99.4%	45436	942123	5.0%
HDA1	Eubacteria	963956	1053254	91.5%	3	963959	0.0%	1040557	1053254	98.8%	11	1040568	0.0%
HDA2	Eubacteria	880321	1008026	87.3%	151	880472	0.0%	1003294	1008026	99.5%	41863	1045157	4.2%
1350R	Eubacteria	146679	157888	92.9%	65	146744	0.0%	157515	157888	99.8%	3837	161352	2.4%
F27	Eubacteria	190442	238647	79.8%	18	190460	0.0%	232150	238647	97.3%	19	232169	0.0%
F44	Eubacteria	223533	255862	87.4%	19	223552	0.0%	252082	255862	98.5%	22	252104	0.0%
Firm350f	Firmicutes	176441	328485	53.7%	813	177254	0.3%	303696	328485	92.5%	543316	847012	165.4%
Firm814r	Firmicutes	129057	267310	48.3%	3265	132322	1.2%	258609	267310	96.7%	118737	377346	44.4%
Gamma395f	Gammaproteobacteria	83758	137397	61.0%	9883	93641	7.2%	135597	137397	98.7%	209220	344817	152.3%
Gamma871r	Gammaproteobacteria	72715	110091	66.0%	1655	74370	1.5%	104434	110091	94.9%	190018	294452	172.6%
Plancto352f	Planctomycetes	6473	7805	82.9%	2363	8836	30.3%	6833	7805	87.5%	13238	20071	169.6%
Plancto920r	Planctomycetes	5252	5781	90.8%	325	5577	5.6%	5756	5781	99.6%	481930	487686	8336.5%
Pln903R	Planctomycetes	5028	5802	86.7%	339	5367	5.8%	5764	5802	99.3%	436890	442654	7530.0%
1512uR	Universal	41090	53451	76.9%	1239	42329	2.3%	51258	53451	95.9%	1600	52858	3.0%
U1406R	Universal	172750	181463	95.2%	4114	176864	2.3%	181103	181463	99.8%	4299	185402	2.4%
UA1406R	Universal	175555	181458	96.7%	4125	179680	2.3%	181111	181458	99.8%	4301	185412	2.4%
U341F	Universal	984652	1053693	93.4%	21649	1006301	2.1%	1049286	1053693	99.6%	44697	1093983	4.2%
1492R	Universal	25090	33353	75.2%	1151	26241	3.5%	32067	33353	96.1%	1495	33562	4.5%
1543R	Universal	35007	39073	89.6%	1279	36286	3.3%	38698	39073	99.0%	1606	40304	4.1%

[4] Coverage values are in groups of three with the first number corresponding to hits while the second is the total number of target sequences available and the third are these values expressed as a percentage.

$MgCl_2$, and annealing time, as well as using annealing temperatures below the T_m of the primers (Kwok *et al.*, 1994). PCR cycle number should also be minimized along with the pooling of multiple PCR products to reduce the high variation which is inherent in the early stages of multi-template PCR (Brooks *et al.*, 2007). Inosine, found naturally in the 5' position of the tRNA anticodon, is capable of annealing to A, C, G or T and may be added to primers to improve binding. Although the relative binding efficiencies differ, I-C > I-A > I-T ≈ I-G (Martin *et al.*, 1985), adding inosine to the 3' termini of primers has been shown to improve mismatch tolerance (Ben-Dov *et al.*, 2006). The primer Beta359f contained mismatches at the 3' terminus. To reduce the detrimental effect of this mismatch, Spyder indicated that the last guanosine could be replaced with inosine to increased coverage (Table B.4). Due to the redundancy of the genetic code, primers can be designed such that they end at DNA positions corresponding to the 1st or 2nd bases of a codon, avoiding the wobble position. These results emphasize that further analyses are necessary following conventional primer design for molecular microbial ecology as the ideal primer may not always be identified. Ultimately, primer selection should be approached with care. Current knowledge of community structures should be used as a guide for primer choice and design; multiple primers, either universal or targeting specific groups can also be used (Muhling *et al.*, 2008), though this strategy is accompanied by additional costs and analyses. Periodic reassessment of primers (e.g. using Spyder) is important as 16S rDNA databases are continually expanding and may contain biases towards primers currently in use for community analyses. Such biases are not only a direct result of insufficient design, but they are compounded as mismatched templates become less abundant as the cycle number increases (i.e. if a primer binds unfavourably to

Table B.4. Primer analysis results showing primer modifications with the percent increase in target (specific) hits relative to the old primer and percent increase in non-specific hits relative to specific hits.

Primer Name	Initial and Modified Primer Sequences[5]	Target	Coverage (0 mismatches)						% Increase target hits
			Target Hits			Non-target Hits			
Alf28f	ARCGAACGCTGGCGGCA	Alphaproteobacteria	14777	20496	72.1%	1142	15919	7.2%	
	ARCG**W**ACGCTGGCGGCA		18059	20496	88.1%	1175	19234	6.1%	22.2%
A571F	GCYTAAAGSRICCGTAGC	Archaea	27866	53025	52.6%	15	27881	0.1%	
	GCYTAAAGSRI**Y**CGTAGC		35352	53025	66.7%	23	35375	0.1%	26.9%
CFB555f	CCGGAWTYATTGGGTTTAAAGGG	Bacteriodetes	97909	111977	87.4%	350	98259	0.4%	
	CCGGAWTYA**Y**TGGGTTTAAAGGG		112607	120560	93.4%	534	113141	0.5%	15.0%
Beta359f	GGGGAATTTTGGACAATGGG	Betaproteobacteria	69126	74311	93.0%	1076	70202	1.5%	
	GGGGAATTTTGGACAATGG**I**		70690	74311	95.1%	1126	71816	1.6%	2.3%
9bfm	GAGTTTGATYHTGGCTCAG	Eubacteria	222934	276514	80.6%	19	222953	0.0%	
	GAGTTTGATY**M**TGGCTCAG		227238	282493	80.4%	19	227257	0.0%	1.9%
347F	GGAGGCAGCAGTRRGGAAT	Eubacteria	1004387	1118551	89.8%	4	1004391	0.0%	
	GGAGGCAGCAGTRRGG**R**AT		1026575	1118551	91.8%	4	1026579	0.0%	2.2%
HDA1	ACTCCTACGGGAGGCAGCAGT	Eubacteria	1022866	1115022	91.7%	3	1022869	0.0%	
	AC**W**CCTACGGG**W**GGC**W**GCAGT		1046964	1115022	93.9%	4	1046968	0.0%	2.4%
F27	AGAGTTTGATCMTGGCTCAG	Eubacteria	192454	241231	79.8%	18	192472	0.0%	
	WGA**S**TTTGAT**Y**MTGGCTCAG		203784	241231	84.5%	19	203803	0.0%	5.9%
Firm350f	GGCAGCAGTRGGGAATCTTC	Firmicutes	176441	328485	53.7%	813	177254	0.5%	
	GGCAGCAGTRGGGAAT**M**TTC		208996	370035	56.5%	1541	210537	0.7%	18.5%
Gamma395f	CMATGCCGCGTGTGTGAA	Gammaproteobacteria	87019	145211	59.9%	10330	97349	10.6%	
	CMAT**R**CCGCGTGT**R**TGAA		124289	145211	85.6%	12555	136844	9.2%	42.8%

[5] Modified primer is underlined with specific modifications in bold face.

a sequence but permits amplification, future amplification cycles will favour the ‘corrected’ sequence thus making it harder to detect the mismatch). This is particularly problematic for primer sites near the 5’and 3’ ends of the 16S rRNA gene as few studies perform amplifications originating from flanking regions.

As primers are gradually improved, they will approach true discrimination between microorganisms. *In silico* design of PCR primers has been instrumental in the design of current 16S rDNA primers and the utility of *in silico* design has been validated in the past (Baker *et al.*, 2003, Blackwood *et al.*, 2005, Muhling *et al.*, 2008). Many *in silico* PCR reactions allow two mismatches as a baseline yet this may need to be revised to a weighted system in which mismatches are assessed based on the type and location of the mismatch. The novel analysis described in this study can easily be applied as a tool to evaluate primers against sequences in the RDP database and will facilitate the identification of superior primers targeting the 16S rRNA gene.

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