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Distribution and habitat characteristics of Tubifex tubifex, intermediate host of whirling disease, in Banff National Park

Whelan, Colby

UNIVERSITY OF CALGARY

Distribution and habitat characteristics of *Tubifex tubifex*, intermediate host of whirling disease, in Banff National Park

by

Colby Whelan

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ABSTRACT

Whirling Disease was discovered in Canada for the first time in 2016, at Johnson Lake, Banff National Park. The disease is caused by the parasite *Myxobolus cerebralis* and has caused major declines in trout populations in the United States. The presence of whirling disease in Banff National Park could be detrimental to the recovery of the two native trout species, which are both listed as threatened. The parasite affects salmonid fish, but also requires a second obligate host, the oligochaete, *Tubifex tubifex*. The presence and distribution of *T. tubifex* in a waterbody are important factors to predict where *M. cerebralis* may spread. Occupancy modelling was tested as a method for surveying the distribution of *T. tubifex* at the site of first detection, Johnson Lake. *T. tubifex* were present, though had a patchy distribution with low detection probability. High inorganic carbon concentration in the lake sediment was negatively associated with *T. tubifex* presence. Two watersheds within Banff National Park were also surveyed, zero *T. tubifex* were found within the Cascade watershed, while *T. tubifex* had a patchy distribution within the Spray watershed. Habitat covariates were tested using General Linear Models, and *T. tubifex* were found to be significantly associated with low landscape level slope, and a small contributing area. These results suggest that the presence of *T. tubifex* is not ubiquitous in waterbodies in the region and understanding habitat could help discover areas with *T. tubifex* presence.

ACKNOWLEDGEMENTS

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List of Abbreviations

AIC…..…..Akaike's Information Criterion

BNP…..…….….Banff National Park

DEM…..……..Digital Elevation Model

IC…….…..…….….Inorganic Carbon

LOI…….……..Loss on Ignition

OM…….Organic Matter

TAM…….Triactinomyxon

CHAPTER 1: INTRODUCTION

1.1 OVERVIEW

In 1885 the Canadian Pacific Railroad from Eastern Canada to Vancouver was completed. To increase ridership, tourism to the scenic Canadian Rockies was promoted and Banff National Park (BNP) (Figure 1) was created to capitalize on the visitors (Robinson, 1978). As the idea of conservation gained prominence, the mandate of BNP evolved to prioritize the protection and preservation of unique natural ecosystems for the enjoyment of future generations. Initially, the availability of hatchery bred non-native trout and the poor understanding of outcomes from introducing non-native species led to stocking programs releasing rainbow, brook and brown trout in BNP (Mcnaught et al. 1999). Some of the early actions taken by National Park officials to increase the success of non-native species ultimately led to depleted native trout populations. BNP's management history includes the introduction of hatchery bred non-native trout, the persecution of native bull trout, and development without regard for aquatic ecosystems (Schindler 2000). The legacy of introduced trout continues to current day. These actions resulted in the decline of native westslope cutthroat (*Oncorhynchus clarkii lewisii*) and bull trout (*Salvelinus confluentus*). In 2016 the first confirmed occurrence of whirling disease in Canada was discovered in Banff National Park (Pers. Comm. M. Taylor, 2018). Whirling disease has the potential to exacerbate the decline of westslope cutthroat trout and bull trout (Hedrick et al. 1999). The purpose of this thesis is to provide vital information to determine the potential for whirling disease spread through BNP. By studying the first detection point at Johnson Lake, and two nearby watersheds, I will identify strengths and pitfalls of survey techniques and search to determine what habitat

features are predictive of whirling disease establishment. Managers could then apply this information to other watersheds within BNP to identify which waterbodies are at risk.

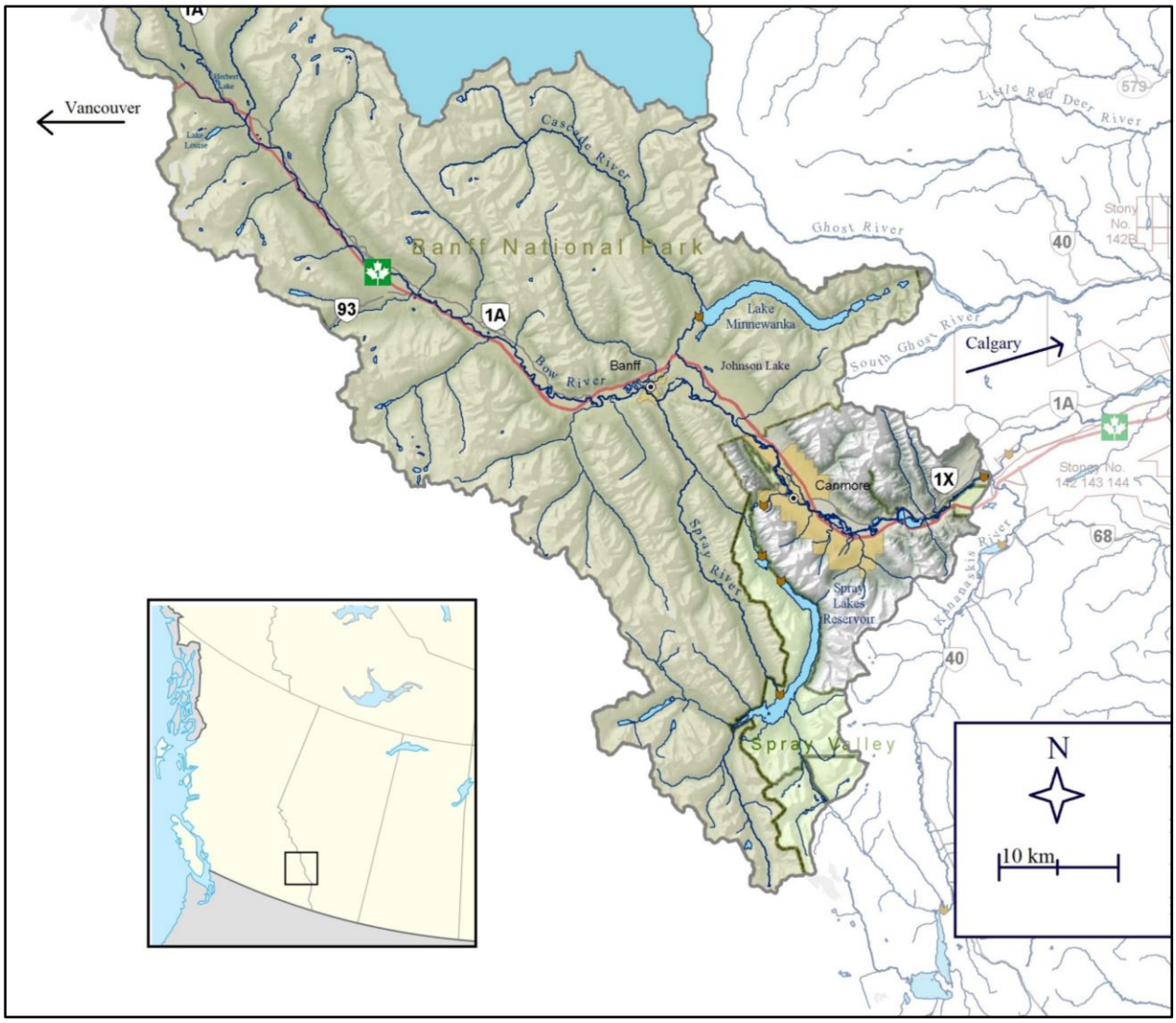

Figure 1. Southern Banff National Park, which contains the Upper Bow River watershed (tan shading), including the study sites: The Spray and Cascade watersheds and Johnson Lake. The light blue shading north of the Cascade River is the region of BNP within the Red Deer River watershed. (Modified from Bow River Basin Council, 2019).

1.2 Aquatic Ecosystems in Banff National Park

Banff National Park is home to a variety of rich and diverse aquatic ecosystems. Each year abundant snowpack at high elevation melts and trickles down in cold clear streams, which flow into spectacular hanging lakes and marshy wetlands before meeting the large valley bottom rivers

that flow to the prairies. BNP's waterbodies support life that includes two native trout species, several amphibian species, and many types of invertebrate. The waterbodies of BNP have escaped many aspects of habitat degradation, pollution, and heavy levels of exploitation characteristic of unprotected areas. Yet, native trout face many survival challenges, even in protected areas like BNP. Westslope cutthroat trout and bull trout are listed as Threatened Species under the Canadian Species at Risk Act. The greatest threat to their persistence is the past introduction of non-native trout (Schindler 2000). Starting in the early 1900's, many of BNP's waterbodies were stocked with brook trout (*Salvelinus fontinalis*), rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*). These species predate upon native trout, or compete for resources and sites to spawn (Schindler 2000). Additionally, rainbow trout and cutthroat trout can form fertile hybrids, as can brook trout and bull trout. Only a few isolated populations of unhybridized westslope cutthroat trout remain, and of those that do exist, several are located within BNP (Fisheries and Oceans Canada 2014).

Within BNP two major hydroelectric operations affect river systems. Minnewanka Dam impedes the flow of the lower Cascade River; however, the entire Cascade watershed above Lake Minnewanka is intact and undeveloped. The lower 35 km of the Spray River are regulated; Canyon Dam impounds the Spray River and creates Spray Reservoir, which diverts a large portion of the Spray River's discharge out of the watershed to a hydroelectric generating plant near Canmore AB. The redirected water eventually joins the Bow River downstream of its confluence with the Spray River. The consequences of river impoundment include reduced average water flow, reduced frequency of flushing flows, impeded connectivity, and increased siltation above and below the dam (Eaton 2000).

1.3 Whirling Disease

1.3.1 Whirling Disease History

Whirling disease originated in Central Europe but began to spread across North America in the 1950s. The disease was found in Pennsylvania in the 1950s and reached the intermountain west by the late 1980s (Bartholomew and Reno 2002). In the western United States most of the spread of the disease was due to hatchery transfers of infected juvenile trout (Bartholomew and Reno 2002). By 1987 whirling disease had spread to Colorado, where it devastated trout populations in the upper Colorado River. Whirling disease was found in Montana in 1994 (Vincent 1996); while in both states the effects were variable, mortality reached 90% in some rainbow trout populations (Nehring and Walker 1996, Vincent 1996).

1.3.2 Whirling Disease Biology

Whirling disease is a suite of symptoms that affects salmonids and is caused by the myxozoan parasite *Myxobolus cerebralis* (Sarker et al. 2015). *M. cerebralis* has a cyclical lifecycle (Figure 2) that requires the participation of an intermediate oligochaete host, *Tubifex tubifex*, which inhabits the substrate of temperate waterbodies (Wolf and Markiw 1984). Deceased infected fish release myxospores into bottom sediment (Gilbert and Granath 2003), which *T. tubifex* subsequently consume. Within *T. tubifex's* digestive system the parasite produces *triactinomyxon* spores (TAMs) (Gilbert and Granath 2003). The TAMs are released from *T. tubifex* and float passively until they attach to the skin, gills or buccal cavity of nearby fish (El-Matbouli et al. 1999). Attached TAMs grow polar filaments through the fish's body until they reach the central nervous system (El-Matbouli and Hoffmann 1998). The parasite cells then divide and feed on the cartilage that surrounds the central nervous system. These cells fuse to create the myxospore stage once again (Gilbert and Granath 2003). *T. tubifex* amplify *M. cerebralis* through its lifecycle; for every myxospore consumed a *T. tubifex* up to 40x the TAMs can be released (Stevens et al. 2001).

The consumption of myxospores by *T. tubifex* is the rate limiting step of *M. cerebralis'* lifecycle, and *T. tubifex* presence is the greatest predictor of future *M. cerebralis* invasion (Ayre et al. 2014).

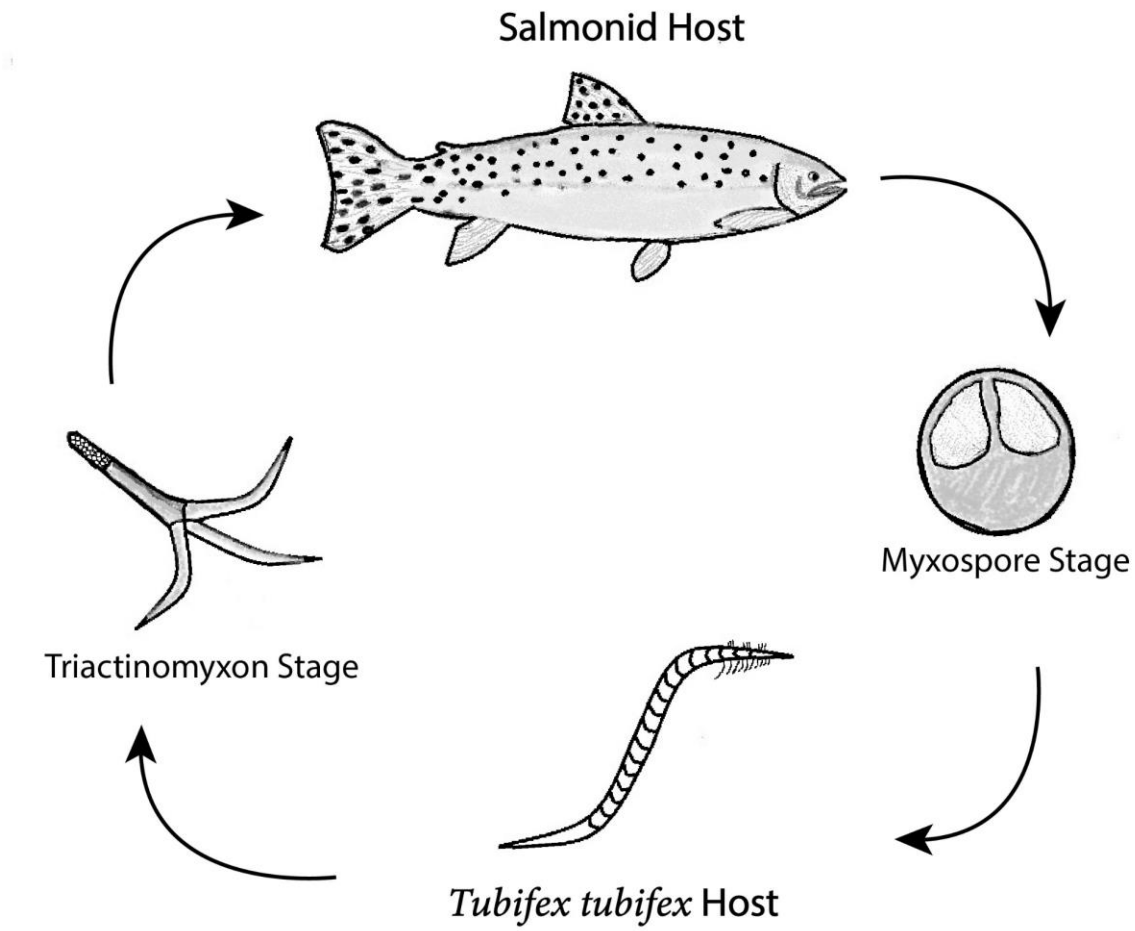

Figure 2. Cyclical lifecycle of whirling disease (*Myxobolus cerebralis*)

The symptoms of *M. cerebralis* infection can vary from no observable effects to mortality (Hedrick et al. 1999). Common symptoms include body deformities, a bent and blackened tail, and a reduced ability to swim (Halliday 1976). Caudal deformities reduce the ability to forage or avoid predators, which can lead to mortality. *M. cerebralis* selectively infects juvenile fish because their skeletal system is more rich in cartilage; antibodies are produced within 12 weeks of egg hatch but

not before irreparable cartilage damage often occurs in those infected (Ryce et al. 2005). Colder rearing temperatures among newly emerged salmonid fry result in slower ossification; the lengthened period of a predominately cartilaginous skeleton makes these fish more vulnerable to infection (Ryce et al. 2005). The parasite affects all trout species that inhabit BNP, with the exception of lake trout (*Salvelinus namaycush*) (Hedrick et al. 1999).

1.3.3 *Myxobolus cerebralis* in Banff National Park

The first known *M. cerebralis* infection in Canada was discovered at Johnson Lake in 2016. Shortly thereafter, all the major watersheds (Old Man, Bow, Red Deer, North Saskatchewan Rivers) in southern Alberta tested positive for its presence. This suggests the disease had time to spread before its presence was detected. It is unclear how the disease arrived in Canada. It was possibly transferred from an infected location on angling equipment, or through the digestive system of waterfowl or fish (El-Matbouli and Hoffmann 1991). It is unknown what effect the disease has had on fish populations in Canada. As part of a long-term strategy to prevent *M. cerebralis* spread, precautionary measures were taken across Alberta. The measures included restricting access to waterbodies, closure of infected hatcheries, and a recommendation to discard felt soled wading boots. In BNP, areas considered sensitive native trout habitat were closed to fishing. Johnson Lake was drained in the fall of 2019 as part of an ongoing effort to eliminate *M. cerebralis* from the watershed. This action will theoretically break the parasite's lifecycle by the eradication of fish, and prevent it from reproducing (Nehring et al. 2018). Elimination of *M. cerebralis* from the Johnson Lake watershed is part of a strategy to prevent its invasion into sensitive populations of native trout that inhabit the nearby Cascade River watershed. Following drainage of the lake in fall, 2019, the lake was refilled in the spring of 2020. This strategy was deemed feasible because the Johnson Lake watershed is small; however, breaking the lifecycle of *M. cerebralis* by eradication of its fish host is unrealistic in most locations.

1.4 OBJECTIVES

The purpose of my research was to determine whether conditions were present to allow *M. cerebralis* to spread throughout BNP. To determine the risk of spread, I investigated the distribution of *T. tubifex* in a location known to support *M. cerebralis* infection (Johnson Lake). I applied lessons learned from Johnson Lake to the Spray and Cascade watersheds, all within Banff National Park, and investigated *T.tubifex* habitat preferences in those sites where they were found.

In general, *T. tubifex* ecology is not well understood, nor are its habitat preferences. Furthermore, the distribution of *T. tubifex* within BNP is unknown. With such knowledge managers could characterize BNP's waters into likely and unlikely *T. tubifex* habitat. This would allow for the prioritization of waterbodies to survey and if necessary, implement management actions to reduce additional *M. cerebralis* spread.

CHAPTER 2. OCCUPANCY MODELLING OF *TUBIFEX TUBIFEX* IN JOHNSON LAKE, BANFF NATIONAL PARK

2.1 ABSTRACT

Myxobolus cerebralis, the parasite that causes whirling disease in salmonids, was recently discovered in Johnson Lake in Banff National Park. Significant resources have been applied to the recovery of native trout populations within BNP over the last 20 years, and the recent arrival of *M. cerebralis* may negatively affect their recovery. The benthic oligochaete *Tubifex tubifex* plays a crucial role in the parasite's lifecycle, and *T. tubifex's* presence is predictive of future *M. cerebralis* invasion. Occupancy modelling was selected to determine the probability of *T. tubifex* detection at any site. Johnson Lake's sediment was intensively sampled by sediment coring in a regular grid, which revealed that *T. tubifex* were present in low levels. The modelling revealed that the methods used only detected a portion of the *T. tubifex* present and indicated that *T. tubifex* favoured areas of Johnson Lake and wetland that were low in inorganic carbon. The number of *T. tubifex* encountered during the study was far below other areas studied and suggests more effort than usual may be required to detect them in newly sampled waterbodies within this region.

2.2 INTRODUCTION

2.2.1 AQUATIC BENTHIC INVERTEBRATES

Benthic invertebrates are a diverse group of organisms that consist of molluscs, oligochaetes, crustaceans, and the aquatic larval stages of many insects. Small invertebrates provide functional services to aquatic ecosystems that include food for higher trophic levels, consumption of detritus (Freckman et al. 1997, Palmer and Poff 1997, Postel and Carpenter 1997) and a valuable proxy for aquatic ecosystem health (Cairns and Pratt 1993). Monitoring methods have been developed that recognize that invertebrate taxa tolerate pollution to different extents,

and their responses to pollutants can indicate environmental contaminant concentrations (Hodkinson and Jackson 2005). Further, benthic species can themselves constitute an environmental disturbance, such as when they transmit diseases or spread as invasive species. The salmonid parasite whirling disease (*Myxobolus cerebralis*) requires the presence of the aquatic oligochaete *Tubifex tubifex* to create a sustained infection among trout populations (Wolf and Markiw 1984, Bartholomew and Reno 2002, Gilbert and Granath 2003). Past whirling disease infections have caused trout population collapses and should be considered a threat to any location where trout and *T. tubifex* co-occur (Nehring and Walker 1996, Vincent 1996).

2.2.2 Study Organism

M. cerebralis has a cyclical life cycle that was not fully understood until the participation of *T. tubifex* was discovered in 1984 (Wolf and Markiw 1984) *M. cerebralis* produces a myxospore within infected fish that is released when they die (Halliday 1976). The myxospore is consumed by *T. tubifex* where it is converted to a *Triactinomyxon* spore (TAM) that is released to further infect other fish (Wolf and Markiw 1984). Because many vectors for the spread of *M. cerebralis* exist, the presence of *T. tubifex* is indicative of the potential for a fish population to become infected (Krueger et al. 2006, Arsan et al. 2007a, Alexander et al. 2011, Zielinski et al. 2011).

T. tubifex is not ubiquitous across salmonid habitat in North America, despite its ability to occupy a wide range of waters from pristine to very polluted (Kathman and Brinkhurst 1999). *T. tubifex* has consistently been associated with fine sediment at local scales (Lazim and Learner 1987, Anlauf and Moffitt 2008). Regionally, *T. tubifex* is found in habitats associated with agriculture (Anlauf and Moffitt 2010), nutrient enrichment (Kaeser and Sharpe 2006; Allen and Bergersen 2002; Arsan et al. 2007) and reservoirs (Zendt and Bergersen 2000). *T. tubifex* prefer habitat with high levels of organic matter (OM) and leaf litter because organic substrates support

bacterial colonies that *T. tubifex* feed on (Lazim and Learner 1987, Robbins et al. 1989). *T. tubifex's* ability to migrate has not been extensively studied, however they likely travel by drifting with water current or attached to vectors such as boats or birds and mammals (Guérin and Giani 1996, DuBey and Caldwell 2004).

T. tubifex consists of six genetic lineages, yet only lineages I and III produce TAMs, which are necessary to complete *M. cerebralis'* lifecycle (Beauchamp et al. 2005b). Phenotypically the different lineages appear identical; however, the severity of *M. cerebralis* infection tends to increase as the proportion of lineage III *T. tubifex* in the population increases (DuBey and Caldwell 2004) because lineage III produces significantly more TAMs than other lineages. Lineage V *T. tubifex* can consume and deactivate myxospores, which can mitigate the effects of the presence of lineage III *T. tubifex* (Beauchamp et al. 2005a). Most populations are a mix of lineages (Nehring et al. 2013, Ayre et al. 2014); however, populations of homogenous lineage III *T. tubifex* exist (Alexander et al. 2011, Zielinski et al. 2011). Therefore, knowledge of the distribution of specific *T. tubifex* lineages is also informative for risk assessments.

2.2.3 Strategies for the Study of Invertebrates

The lakes and streams that benthic invertebrates inhabit are created by physical processes that produce a patchwork of habitat niches (Hutchinson 1993). This variation creates uneven species distributions that are difficult to predict. Distributional surveys of benthic invertebrates are a cost-effective method to collect data efficiently across large spatial scales (Wisniewski et al. 2013). Landscape-wide studies must be carefully planned to maximize the value of data due to the high cost to access remote sites (Bailey et al. 2007, Wisniewski et al. 2013). When a specimen is present but not detected it is known as imperfect detection (Wisniewski et al. 2013, Cortelezzi et al. 2017). Studies on larger bodied taxa have identified imperfect detection as a handicap to

accurate interpretation of results, yet studies on invertebrates have typically ignored imperfect detection (Kellner and Swihart 2014). Only an estimated nine percent of past invertebrate studies acknowledged imperfect detection (Kellner and Swihart 2014). Surveyor skill, weather, collection tools, organism size and patchiness all contribute to imperfect detection (Cortelezzi et al. 2017).

If investigators fail to detect their target organism due to imperfect detection it can bias model parameter estimates and mislead the interpretation of the results (Mackenzie et al. 2002, 2006, Bailey et al. 2005). The imperfect detection can result in false negatives, which can generate an underestimation of the target organism's prevalence and result in ineffective management and conservation strategies (Mackenzie et al. 2003). Imperfect detection is a concern particularly for rare and threatened species, which are often a target of management. False negatives can bias risk assessments and lead to management actions designed around false information. Ineffective management creates a false sense of security or may shift visitation and fishing pressure to more vulnerable populations. Conversely imperfect detection can lead to the application of resources to assist with the recovery of species that have healthy numbers.

2.2.4 Occupancy Modelling

Occupancy modelling was created to analyse distributions while accounting for imperfect detection (Mackenzie et al. 2002, 2003, Royle and Nichols 2003). Occupancy is the probability that a species is present in a given sampling location, whereas detection probability is the probability that a species will be detected when it is present. To account for imperfect detection, replicated detection and non-detection data are used to model species occupancy (*psi*) and detection probability (*p*) simultaneously using the following formula:

$$P(Y = y_i) = \psi \binom{K}{y_i} p^{yi}(1-p)^{K-y_i}, y_i > 0$$

$$P(Y = y_i) = \psi(1-p)^K + (1-\psi), y_i = 0$$

Where *Y* is the probability of occurrence, y_i is the number of species detections out of the total samples on a visit (*K*), and *p* is the detection probability of an organism in a single sample, given that it is present at the sampled location, and ψ represents occupancy (Mackenzie et al. 2006).

Occupancy models are used where the target organism's detection probability is less than perfect. Occupancy can incorporate habitat covariates to explain occupancy or detection estimates. A capture history can be generated with repeated surveys at each site and a detection probability can be calculated from the capture history (Mackenzie et al. 2002). Detection probability is subsequently incorporated into a maximum likelihood function to calculate the probability of occupancy across the sample area. The effect of detection probability on habitat covariates can be controlled to produce an unbiased understanding of an organism's distribution (Mackenzie et al. 2003). Consideration of detection probability has proven particularly valuable to study the distribution heterogeneity of benthic invertebrates (Wisniewski et al. 2013, Cortelezzi et al. 2017).

2.2.5 Objectives of the study

Here I present results of a study that examined occupancy, detection, and habitat factors that affect the fine-scale distribution of *T. tubifex* in Johnson Lake, Banff National Park (BNP), Alberta, Canada. In 2016 Parks Canada discovered that many fish in Johnson lake were infected with *M. cerebralis*; which provided an opportunity to study *T. tubifex* in a location known to support *M. cerebralis* infection among the fish population. Broadly, my purpose was to develop a strategy to effectively sample and model the distribution of *T. tubifex* lake-wide. Specifically, my objectives were to a) model the distribution of *T. tubifex* in a location with a known infected population of trout, b) model the habitat preferences of *T. tubifex* in Johnson Lake c) determine whether detection probability presents a difficulty for detection of *T. tubifex*. The greatest risk

factor for the sustained infection of *M. cerebralis* in a fish population is the sympatric presence of genetic lineages I or III *T. tubifex* (Zielinski et al. 2011, Ayre et al. 2014). Therefore, I also tested *T. tubifex* lineages in Johnson Lake to determine how occupancy and detection vary for both its presence, and its various lineages.

2.3 METHODS

2.3.1 STUDY SITE

The study area, Johnson Lake and Wetland (UTM: 11U 605864E x 5672800N) was a 15-hectare waterbody located in Banff National Park. It was situated on the Fairholme bench, a flat area with a southern aspect and montane forests (Figure 3). Johnson Lake was previously a wetland that was impounded in the 1930s to raise the water level by 6 m and create a more extensive waterbody for recreational opportunities. The lake had a fine sediment bottom and patchy macrophyte growth, both of which are preferred features for *T. tubifex* (Figure 3) (Lazim and Learner 1987, Anlauf and Moffitt 2008). The macrophyte community is dominated by species from the algal genus *Chara*, in some locations it forms a thick mat of vegetation over the substrate. Prior to eradication Johnson Lake supported populations of native white suckers (*Catostomus commersonii*), and non-native brook trout (*Salvelinus fontinalis*), rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*). The study also included an adjacent wetland, a 2-hectare spring-fed wetland area that drained into the west end of Johnson Lake via a low gradient stream. The wetland was drained in the spring of 2019 and allowed to refill in the spring of 2020. Prior to draining, the wetland contained small populations of brook and rainbow trout. The wetland was shallow, with a maximum depth of 1.5 m. The substrate was flocculent organic mud and the eastern

half is covered by submerged and emergent macrophytes (light green in Figure 3).

Figure 3. Aerial view of Johnson Lake and Johnson wetland, areas of heavy macrophyte growth appear lighter within both the lake and wetland (Bing Maps 2020).

2.3.2 Site Selection

The location of sample sites at Johnson Lake and its associated Wetland were determined with an overlaid grid of 40 m x 40 m cells where each cell was considered a site (Figure 4). The lake had 107 sample sites while the Wetland had 11 sites. Samples were collected from December 2016 to March 2017, while the lake surface was frozen. A mini-Glew suction corer with an internal diameter of 3.8 cm (Glew 1991) was used to collect five replicate sediment cores at each site, for a total of 590 cores. The cores were collected within 1 m^2 at the centre of each site, and depth was measured with a gradated plumb line. The top 10 cm layer of each sediment core was retained. The cores were divided with a metal slicer as they were extruded from the core tube, which cut the

cores longitudinally to create two equal halves. One half was searched for *T. tubifex* and the other was frozen for later sediment analyses

2.3.3 *Tubifex tubifex* Sorting

Samples were collected each morning and searched for oligochaetes in the afternoon. By searching unpreserved samples, I was better able to identify potential *T. tubifex* by their movement. Samples were washed in a 400 μm sieve to remove fine sediment. Large debris was rinsed into the sieve and removed. The remainder was transferred to a white bottomed tray and diluted with clean water. An illuminated magnifying glass was used to examine the sample and oligochaetes were removed and subsequently studied under a light microscope. Any oligochaete that featured chaetal hairs or bifid chaetae (Brinkhurst 1986) was removed and sent for genetic confirmation. Every tenth sample was sorted a second time by a different researcher for quality assurance. Suspected *T. tubifex* were preserved in 70% ethanol and sent to the University of Alberta Molecular Biology Service Unit for lineage confirmation by genetic analyses. DNA was analyzed following extraction from samples and amplification by qPCR following techniques described in Nehring et al. (2013). Potential *T. tubifex* were pooled by site and tested to confirm their species, and their lineage using sequences developed by Beauchamp et al. (2002). The tests for species and lineage were separate analyses, and a positive test for each was required for inclusion in further analyses. *T. tubifex* were also tested to determine if they were infected with *M. cerebralis* using a HSP70 adapted from Cavender et al. (2004).

2.3.4 Sediment Analysis

Habitat variables were selected based on review of background literature. Because of the intense effort required to collect and sort the *T. tubifex* samples only three habitat parameters were selected for study; the depth at each site, and the sediment composition indices: percent organic matter and percent inorganic carbon. Originally sediment size fractioning was considered, but was

ruled out when it was determined that the sediment from all samples could be washed through a 400 μm sieve which indicated almost all sediment would be classified into either medium sand (250-355 μm), fine sand (63-250 μm), or silt-clay (< 63 μm). *T. tubifex* tend to prefer medium to fine sediment, but don't discriminate within those categories (Lazim and Learner 1987, Anlauf and Moffitt 2008).

Half of each sample core was reserved for sediment analysis of organic matter and inorganic carbon composition via loss on ignition (LOI) following Heiri *et al.* (2000). Substrate from each of a site's five replicates were manually homogenized and then a 2 g aliquot of substrate from each was combined to create a 10 g portion that was placed in a tared crucible. Sediment was dried at 100°C for 12 hours in a drying oven (*Fisherbrand™, Isotemp™ Drying Oven*), weighed and then transferred to a muffle furnace (*Lindberg Blue Box Furnace*) where it was heated to 500°C for 4h and cooled to room temperature in a desiccator. The sediment samples were weighed, and the lost mass was considered organic matter (OM). The samples were then heated for 2h at 1000°C, then cooled to room temperature in a desiccator and re-weighed, lost mass was considered inorganic carbon (IC). All weights were measured to 1/10,000 g in a breeze-proof scale case. OM and IC were expressed as percent mass of the total sample dry mass measured after the drying oven phase.

2.3.5 Analysis

Statistical analyses were conducted in the statistical software R with the package RPresence (Mackenzie and Hines 2017). The initial analysis generated the standard *p()psi()* model, an average occupancy estimate and detection probability for all samples. OM and IC were incorporated as covariates and candidate models were run to determine the effect of each covariate,

separate and combined, on occupancy and detection probabilities. The models were ranked on suitability using Akaike Information Criterion (AIC) (Burnham and Anderson 2002).

The volume of sediment captured in each sediment core was consistent, which allowed density to be calculated at each site. Densities were not used in the models but were useful for comparisons with past studies. To calculate the density of a site the volume collected in each 10 cm long, 3.8 cm diameter cylindrical core was calculated (113.4 cm^3). Five cores were collected per site, so the core volume was multiplied by five (567.0 cm^3). The total volume collected was divided by two to account for the removal of sediment for sediment analysis (282.5 cm^3). The density of *T. tubifex* in the sediment collected was calculated for each site (# *T. tubifex* / 282.5 cm^3). The amount of sediment in a 10 cm deep 1 m^2 area was calculated (100,000 cm^3), I then extrapolated the density of *T. tubifex* in the sample to generate the theoretical density of *T. tubifex* in 1 m^2 of sediment (# *T. tubifex* / 282.5 cm^3 = *x* / 100,000 cm^3). I averaged this value across all sites in Johnson Lake and Wetland to create an average density.

2.4 Results

2.4.1 Occupancy Results

T. tubifex occupied 7 of 107 sites in Johnson Lake (Figure 4) and 6 of 11 sites in the Wetland. Of the positive detection sites, a majority (9/13) had a positive detection in only one of the five replicate samples and no sites had positive detections in more than three replicates. In total, 62 suspected *T. tubifex* were collected and 36 came from a single site in the Wetland.

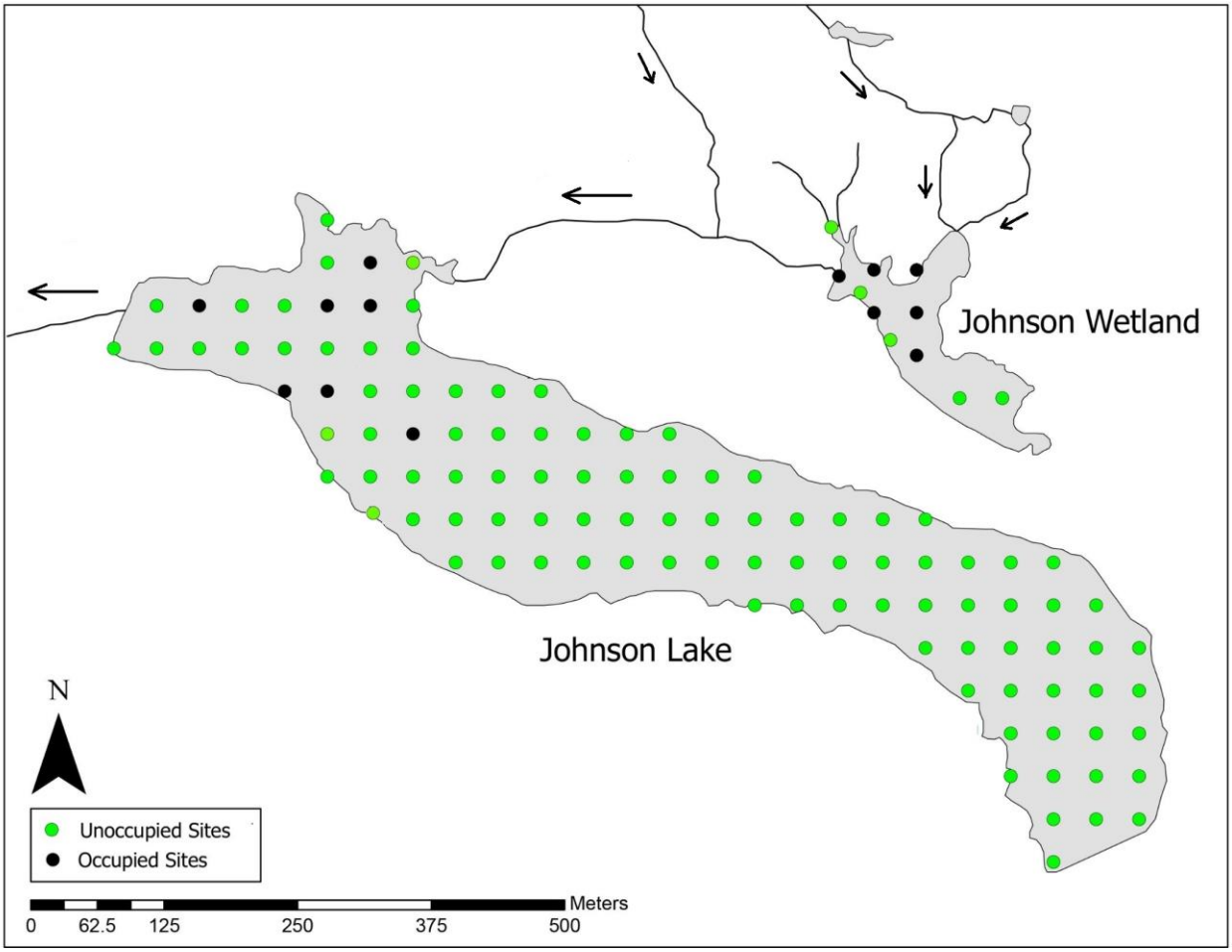

Figure 4. Results of *T. tubifex* sampling in Johnson Lake and Johnson Wetland during winter of 2016-2017, to evaluate presence of *T. tubifex* in bottom sediment. Each site consisted of five replicate samples, taken at the centre of a 40 m x 40 m cell. The arrows represent flow direction of creeks in the watershed.

The naïve or 'unadjusted' occupancy rate for the lake was 0.11, while the modelled *psi* was 0.19 and *p* was 0.16. The model ranked highest by AIC was *psi(IC)p()*, which considered inorganic carbon's association with *T. tubifex* occupancy. The effect of habitat covariates on detection probability was also modelled, but those models yielded no weight and were not included in the final summary (Table 1).

Table 1. Summary of occupancy models ranked using AIC. Inorganic carbon (IC) indicates percent inorganic carbon by dry mass, and organic matter (OM) indicates percent organic matter by dry mass. The most relevant model is indicated by the lowest DAIC and was the model that included IC.

Model	DAIC	wgt	npar	neg2ll
psi(IC)p()	0.00	0.7063	3	121.02
psi(IC+OM)p()	1.82	0.2843	4	120.84
psi(OM)p()	8.64	0.0094	3	129.66
psi()p()	27.12	0.0000	2	150.14

2.4.2 Habitat Modelling Results

The *psi(IC)p()* model carried the majority of the weight (70.63%), which indicated that it was the most appropriate model and that *T. tubifex* was negatively associated with inorganic carbon (Figure 5). The model that considered IC and OM combined also achived the threshold for consideration of a DAIC of 2.00 (Burnham and Anderson 2002) and carried nearly the remainder of the model weight (28.43 %). Depth was measured at each site but was correlated with IC , so was removed from the analysis. Depth was chosen for removal because it was considered less likely to be a true habitat preference of *T. tubifex*, compared to sediment composition. It was noted that no *T. tubifex* were found at sites with a depth greater than 2m. With the incorporation of a habitat variable, occupancy modelling can generate site-specific occupancy predictions based on whether the habitat at a site is appropriate. A heat-map of Johnson Lake and the Wetland that considers occupancy and the presence of IC was generated (Figure 6).

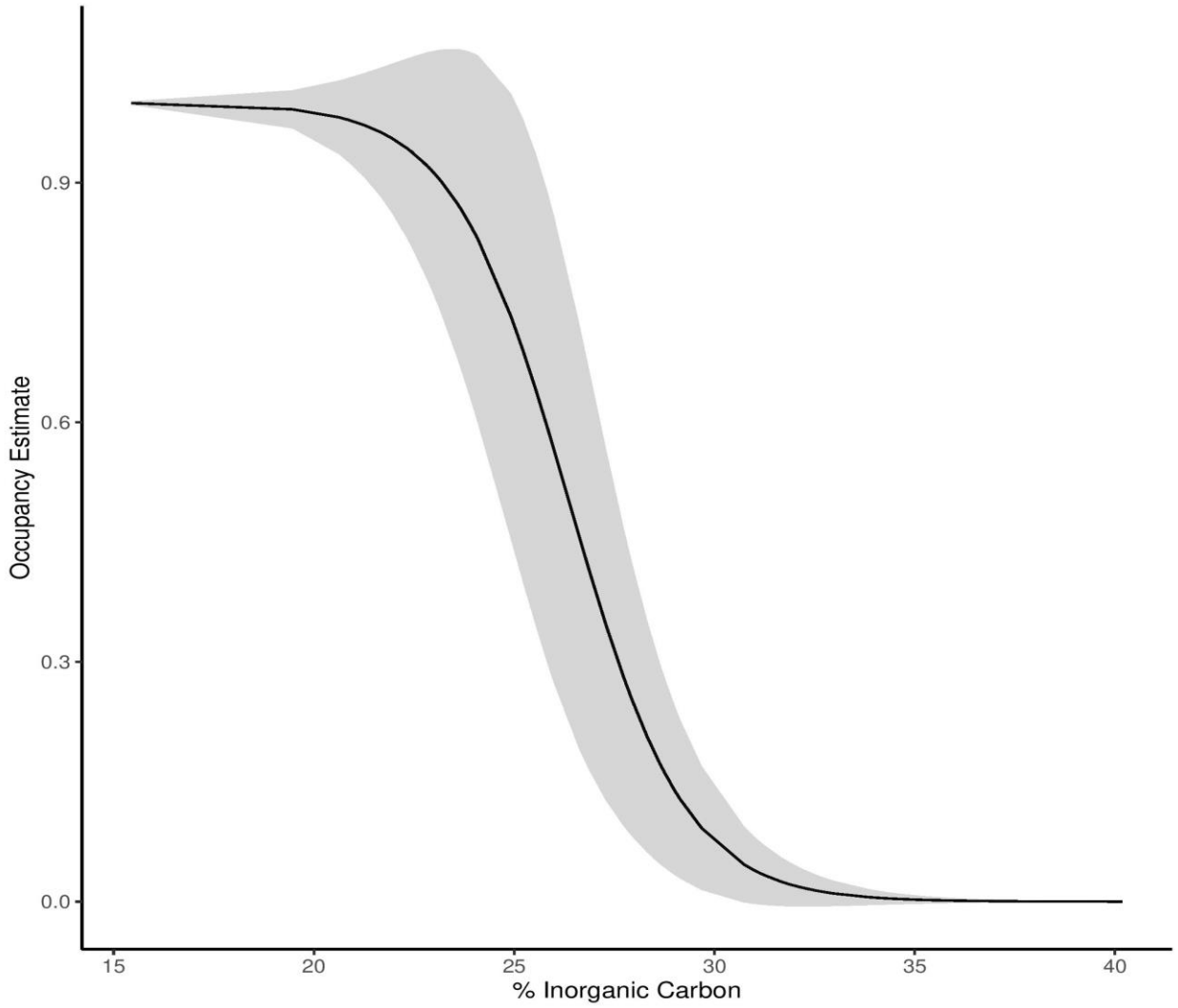

Figure 5. The probability of occupancy for *T. tubifex* in relation to inorganic carbon as a percent of sediment dry mass (solid line) for Johnson Lake and its associated Wetland. The gray shading indicates 95% confidence intervals.

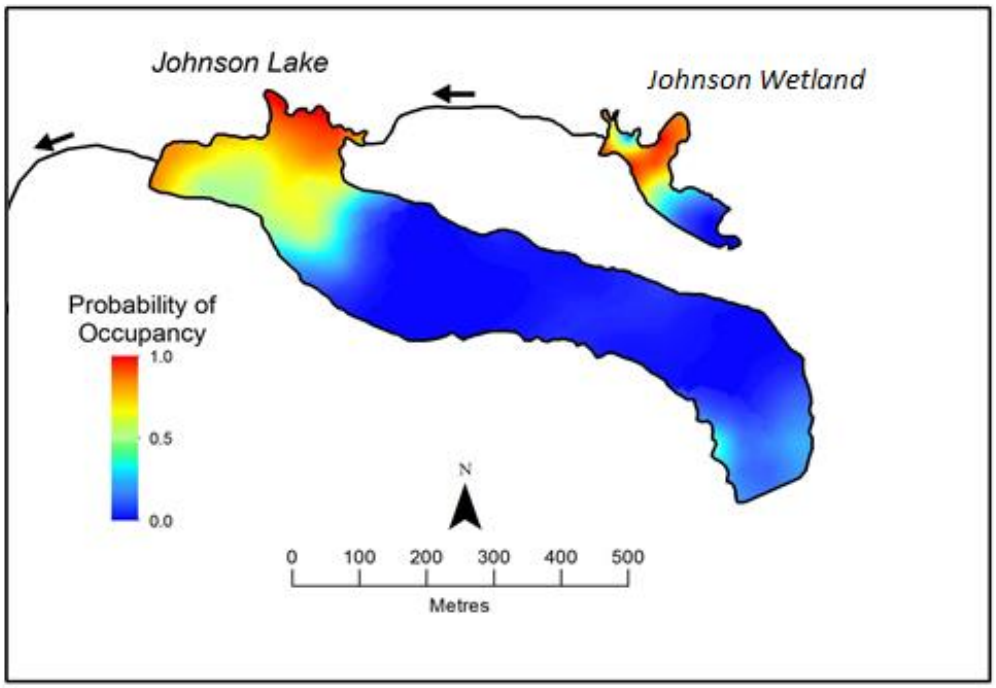

Figure 6. Heat map of *T. tubifex* probability of occupancy in sediments of Johnson Lake and its associated Wetland, from sediment samples collected in winter 2016-2017. The values were calculated with the level of inorganic carbon included as a covariate.

The average estimated density of *T. tubifex* in Johnson Lake and its Wetland across all sites was 190 *T. tubifex*/m^2; with a maximum estimated density of 12,700 *T. tubifex*/m^2. Most of Johnson Lake had a density of 0 *T. tubifex*/m^2, whereas roughly half the Wetland sites had a density above 0.

The results of the genetic analyses indicated that only lineage III *T. tubifex* were present within the samples, and no *T. tubifex* individual within the study tested positive for *M. cerebralis*. Two *T. tubifex* collected for preliminary analysis in December 2016 tested positive for *M. cerebralis.*

2.5 Discussion

The results of this study revealed that *T. tubifex* have an uneven distribution within Johnson Lake and Wetland; most of the sites surveyed were unoccupied. The pattern of distribution

indicated that *T. tubifex* were concentrated in the western end of the lake, and within the Wetland. The habitat covariate analysis showed that *T. tubifex* appear to avoid IC and although less weight was devoted to OM, the combination and IC and OM still carried some weight (TABLE 1). Overall *T. tubifex* were difficult to detect in Johnson Lake, and likely occupy a larger portion of the lake than was found during sampling.

2.5.1 Imperfect Detection

Distribution studies are a resource effective method to estimate a species' presence on a landscape, but survey results can be influenced by variation in detection. Invertebrate distribution assessments are the least likely studies to incorporate imperfect detection, despite evidence that invertebrates often have patchy distributions (Kellner and Swihart 2014). The detection probability, *p*, in Johnson Lake was 0.16, which means that if *T. tubifex* were present at a site, they would only be detected 16% of the time. A detection probability this low strongly affected the estimate of occupancy; the modelled *psi* (0.19) was 58% higher than the naïve estimate (0.11). This result demonstrates that high-effort surveys are necessary to detect *T. tubifex* if they are surveyed at other locations within BNP.

A low detection rate among benthic invertebrates with uneven distributions is common (Trebitz et al. 2010, Wisniewski et al. 2013, Cortelezzi et al. 2017). Imperfect detection has been reported for several benthic species whose distribution varies with stream velocity, substrate, water temperature, stream size, sampling methods, and proportion of a site sampled (Kroll et al. 2008, Albanese et al. 2011, Shea et al. 2013). The small body size, sessile lifestyle, and sub-benthic habitat of *T. tubifex* make them particularly difficult to detect. Low-efficiency sampling methods coupled with low population density can also affect detectability (Royle and Nichols 2003). Imperfect detection is common on the fringe of a species' range as low density decreases sampling

success likelihood (Royle and Nichols 2003). All these factors can work in concert to lower the detection probability of *T. tubifex.* Imperfect detection in this study was likely due to an inability to effectively search a large enough volume of sediment to overcome *T. tubifex's* low density. Improvements to processing would allow researchers to collect and search more sediment and likely increase detection probability. Suggestions are made on this topic in the conclusion (Section 2.6 Conclusion

2.5.2 DISTRIBUTION OF *T. TUBIFEX*

2.5.2.1 *T. tubifex* Distribution and Density in Johnson Lake and Wetland

Assessment of the presence of *T. tubifex* in Johnson Lake and Wetland indicated a low proportion of the area was occupied (*psi* 0.19); however, their distribution was not uniform. The sampling grid was designed to accommodate the apparent rarity of *T. tubifex* (determined from preliminary sample collection) and utilized an extensive, equally distributed pattern throughout Johnson Lake and its associated Wetland. While *T. tubifex* were located at a number of sites, they had a skewed distribution towards the west end of the lake (Figure 4). The average density of *T. tubifex* in Johnson Lake and Wetland (190 *T. tubifex*/m^2) is low relative to values in other published studies, which can range from 5000 to 50,000 *T. tubifex*/m^2 (Zendt and Bergersen 2000, DuBey and Caldwell 2004). Past surveys have not considered imperfect detection which, if included, could further increase estimates of occupation and abundance (Mackenzie et al. 2002, Royle and Nichols 2003). A species' density typically declines at the edge of their range (Gaston et al. 2000). However, Johnson Lake appears to be at neither a latitudinal nor elevation range limit; it is south of locations in Alaska (Arsan et al. 2007b) and at a lower elevation (1426m) than Windy Gap reservoir (2384m; Google Earth 2020). The results found here do not exclude the possibility that BNP is at the limit of some combination of elevation and latitude.

2.5.2.2 *T. tubifex* Migration

The mode of migration that *T. tubifex* employ likely plays a large role in where they are found. Little is known about how they travel but it has been speculated they mostly drift, rather than purposefully migrate (DuBey and Caldwell 2004). It is likely they passively drift with the current and those that land in inhospitable locations perish, while those that land in habitable locations establish. Far more *T. tubifex* individuals were found in the Wetland than in Johnson Lake, and those that were found in Johnson Lake were near the inflow from the Wetland. This suggests that *T. tubifex* become entrained in the water flow from the Wetland to the Lake and are deposited where the inflow creek forms a sediment delta. The Wetland is richer habitat for many reasons and could provide a source to replenish the population in Johnson Lake that survives yet does not flourish. The reasons why a *T. tubifex* population may establish are discussed below but are largely related to the population processes that affect any organism, including resource competition, predation, and mating.

2.5.2.3 Population Processes of *T. tubifex*

Factors that influence the survival of many benthic invertebrates include the presence of preferred sediment, water temperature, predation, and mating opportunities. The main factor assessed in this study was sediment quality, however other factors will be discussed qualitatively. Analysis of habitat covariates within the occupancy models indicated that the most influential covariate to *T. tubifex* was the percentage of IC in the sediment. The association was negative, and once IC exceeded 35%, *T. tubifex* occupancy was reduced to zero (Figure 5). Inorganic carbon can accumulate in a system due to leaching from calcareous substrate, and usually in the form of Ca^{2+} and $CaHCO_3^+$ ions (Wetzel 1983). IC accumulation within a lake can also be influenced by the presence of species from the algal genus *Chara* (Pukacz et al. 2016). This group of charophytes grow in dense mats within Johnson Lake, their prolific photosynthesis takes up naturally occurring

bicarbonate (HCO_3^-) and leaves large amounts of unbonded carbonate ions (CO_3^{2-}) which easily bond with free Ca^{2+} and precipitate in the form of $CaCO_3$ (McConnaughey and Falk 1991). When *Chara spp*. decompose the encrustations of $CaCO_3$ on their stems are left and can accumulate in deposition zones to form sediment rich in IC (Pełechaty et al. 2013). In a controlled experiment *T. tubifex* showed no appetite for leaf litter that had been autoclaved because the bacterial colonies had been destroyed by heat (Lazim and Learner 1987). IC rich sediment may not support the bacterial colonies that *T. tubifex* consider food, and therefore avoid these areas or suffer high mortality when deposited in them.

It is also possible that *Chara* influenced OM levels and made OM a less important habitat preference for *T. tubifex* than has been previously found (Lazim and Learner 1987, Robbins et al. 1989). *Chara* grows in thick mats that can occlude the sediment below them from fresh deposition of entrained allochthonous input (Kufel and Kufel 2002), which would prevent *T. tubifex* from receiving the deposits of new sediment that they prefer and would make *Chara* patches poor *T. tubifex* habitat. During sample collection I repeatedly observed that in areas with dense *Chara* it was difficult to collect a sediment core without also collecting fragments of live *Chara*. I believe these fragments may have increased OM levels in those samples, when actually they were low in the type of OM that *T. tubifex* prefer, which is made of decomposing OM. My sample collection would not have captured the nuances of whether a sample was high in OM due to *Chara*, or due to allochthonous input.

Of the 62 *T. tubifex* specimens found, 50 were found in the Wetland while 12 were found in Johnson Lake. The Wetland appears to be preferable habitat for *T. tubifex* for several reasons. It was created by beavers that modified the landscape and flooded previous riparian areas, this decreased water velocity and increased the accumulation of fine sediment. The Wetland is rich in

decomposing leaf litter due to deciduous shrubs that shed their leaves into the water while Johnson Lake is mainly bordered by non-shedding conifers. *T. tubifex* have been demonstrated to prefer deciduous leaf matter over other habitat types (Lazim and Learner 1987), and my modelling supported that OM has some role in the determination of *T. tubifex* occupancy. Johnson Lake supports large populations of bottom feeding white suckers (*Catostomus commersonii*); however, they are not present in the wetland. In a controlled experiment, bottom feeding carp (*Cyprinus carpio L*) reduced *T. tubifex* populations by 1.7 x, which indicates the co-occurrence of a bottom feeding fish species could negatively effect the *T. tubifex* population (Riera et al. 1991). Feeding *T. tubifex* expose their anterior end to open water (Guérin and Giani 1996) which would leave them exposed to the bioturbation feeding style that white suckers employ. The Wetland is spring fed and remained unfrozen for the duration of the sampling period, whereas the Johnson Lake was fully ice covered from November through April. It has been suggested that *T. tubifex* will only grow in water that exceeds 9°C (Reynoldson 1987), which would likely occur for a more sustained period in the Wetland. Lastly *T. tubifex* have been found to prefer areas with high sedimentation rates that are often associated with creek mouths (Robbins et al. 1989). The areas within the study that had high concentrations of T. tubifex were also areas where a creek met standing water, which allowed sediment to accumulate. The likely reason for this is creek mouths provide a steady supply of sediment high in OM, which *T. tubifex* utilizes for food (Robbins et al. 1989).

When combined, the abiotic factors that drive the *T. tubifex* distribution, and the habitat factors that enable their success, indicate that there is likely a source-sink relationship between *T. tubifex* in the Wetland and in Johnson Lake. The Wetland has less IC in its sediment, lower ice cover, and less predators, which allow *T. tubifex* success. The conditions in Johnson Lake are likely less favourable, although some locations feature conditions that allow their survival. It is possible

that other factors further influence *T. tubifex* distribution within the study area and should not be discounted. They include a minimum density for effective reproduction, predation by other taxa not examined and unknown water chemistry preferences. These topics are all candidates for further studies that wish to refine our knowledge of the factors that influence *T. tubifex* success.

2.5.4 Modelling Suggestions

One difficulty modelling landscape level distribution or abundance data is how to interpret sites where no individuals were found. In this case, *T. tubifex* were only detected at 11% of the sites sampled in Johnson Lake and Wetland. There are three possible explanations for non-detection; the habitat is unsuitable (environmental), the habitat is suitable but inaccessible, and missed detections (methodological) (Lobo et al. 2010). All the habitat surveyed at Johnson Lake and Wetland was continuous and likely accessible by *T. tubifex*, therefore zeros for inaccessible habitat are unlikely. The results indicate that the non-detections are a mix of environmental and methodological zeros. Sites that had a mix of detections and non-detections among the five individual cores were indicative of presence of methodological zeros within the dataset. However, the spatial correlation of sites where *T. tubifex* were detected, and the large section of the lake with no detections indicates there are also environmental factors that dictate their distribution, and many environmental zeroes within the dataset. If a defining habitat variable can be identified then study areas can be stratified to remove areas of unsuitable habitat, and occupancy models can be effective (Guisan and Thuiller 2005). Caution should be taken with this approach to ensure the variable selected has true biological significance. For example, in this study no *T. tubifex* were found in depths greater than 2 m; however, in many other lakes their depth has exceeded 2 m (Brinkhurst 1986, Robbins et al. 1989, Nehring et al. 2003). It is likely *T. tubifex* distribution was driven by resource availability that could have been correlated with depth, but not a result of depth. Further

study is required, but a heavy presence of *Chara* could be useful for stratifying between habitat and non-habitat, in lakes where it occurs.

Strategies have also been developed to use modelling techniques to account for a high degree of non-detection and are often utilized for rare species. Although occupancy models were developed to control for imperfect detection, excessive zeros can still bias parameter estimates. Zero-Inflated Poisson and Zero-Inflated Negative Binomial models can be used to differentiate between an increase in number of individuals at a site from 0 → 1, and an increase from 1 → n (Cunningham and Lindenmayer 2005). Zero inflated models can help separate methodological zeroes (false negatives) from actual absences of the target organism. A hybrid model of zero-inflation and occupancy that combines principles from each approach is also available and can at times out perform standard linear models or occupancy independent of each other (Wenger and Freeman 2016). Failure to account for sources of zero inflation can cause bias in parameter estimates and their associated measures of uncertainty (Mackenzie et al. 2002, Wenger and Freeman 2016). Aquatic invertebrates are often monitored to detect changes in water quality, the presence of invasive species, the amount of forage for predators or the impact of human development (Reece and Richardson 1999, Hodkinson and Jackson 2005). Study objectives should be carefully assessed to determine whether a degree of imperfect detection is acceptable, and whether detection success could vary between the habitat variables to be examined. If the primary goal of the study is habitat determination, and detection success is not expected to vary among habitat types, then zero inflation models could be an effective way to gain a better understanding of habitat preferences.

2.5.5 Lineage Discoveries

The only genetic lineage of *T. tubifex* found within the study area was lineage III. This lineage is known to propagate the most TAMs per myxospore ingested, and is generally associated with the worst outbreaks of *M. cerebralis* among sympatric fish populations (Stevens et al. 2001). Homogenously lineage III *T. tubifex* populations are rare, but are found in Yellowstone National Park (Alexander et al. 2011) and the Deschutes River in Oregon (Zielinski et al. 2011). In other locations it is believed that *M. cerebralis* infection severity correlates to the proportion of lineage III *T. tubifex* within the greater population, which can change over time (Nehring et al. 2013). Changes in the dominant *T. tubifex* lineage in Johnson Lake should not be expected because the population appears homogenous. While not impossible that another lineage inhabits the study area, it is apparent from these results that lineage III makes up the dominant majority.

2.6 CONCLUSION

Occupancy models have been a useful tool for resource managers to incorporate imperfect detection into monitoring programs of organisms with cryptic life styles (Mackenzie et al. 2002, Durso et al. 2011, Wisniewski et al. 2013) and low abundance (Royle and Nichols 2003, Wisniewski et al. 2013). The recent discovery of new cases of *M. cerebralis* in southern Alberta indicate it is likely currently expanding in range. If possible future studies of *T. tubifex* should consider the bias that imperfect detection may have on results; failure to detect *T. tubifex* in a waterbody, if it is in fact present, could lead managers to place that waterbody in a lower *M. cerebralis* risk category. The occupancy survey conducted at Johnson Lake demonstrated the degree to which *T. tubifex* populations can have both an uneven distribution and be susceptible to zero-inflation. Missed detections and potential incorrect implementation of management measures put fish populations at risk. On a broader scale, assessments of benthic invertebrates are usually conducted with manual processes that may suffer from low detection rates. To avoid

mismanagement, studies should consider the effects of imperfect detection in the study design phase of the project. Different methods could provide useful results; for example, a benthic sled could filter a large amount of sediment but retain oligochaetes and might be useful in lentic waterbodies to collect a lake wide initial sample of invertebrates that could be used to determine *T. tubifex* presence. This would allow the collection of larger samples which would provide more individuals and allow increased detection and further refinement of predictions of habitat preferences.

The purpose of this study was to determine the distribution of T. tubifex in Johnson Lake. I determined that *T. tubifex* were present in numbers sufficient to cause *M. cerebralis* infection, I quantitatively measured the degree to which sediment collection can miss *T. tubifex*, I determined that the range of *T. tubifex* within Johnson Lake is likely greater than the survey results showed, and I generated habitat comparisons that incorporate such adjustments.

CHAPTER 3. THE DISTRIBUTION OF *TUBIFEX TUBIFEX* IN TWO ROCKY MOUNTAIN WATERSHEDS

3.1 ABSTRACT

Invertebrate communities are structured by environments that vary spatially and temporally. Identifying the scale that most influences the species-environment relationship is an important theme in ecology, but also has important implications for sampling. We assessed whether spatial variation in *T. tubifex* densities were best predicted by environmental characteristics measured at the reach scale (e.g. slope, fine sediment, wetted channel dimensions, and velocity) or at the landscape scale (e.g. stream segment slope and contributing area). Despite established associations between *T. tubifex* and fine sediment, the best candidate model in this study included only landscape variables, segment slope and contributing area. These results reinforce the importance of landscape level influences on microorganism habitat selection. While a mechanistic understanding of landscape effects may be difficult to discern, landscape variables are helpful to stratify ground searches for rare or patchy species.

3.2 INTRODUCTION

3.2.1 SCALE OF HABITAT VARIABLES

Habitat variables that influence the distribution of aquatic organisms in lotic environments are often scale-dependent (Frissell et al. 1986). While a species may be associated with a specific locality, that habitat may not exist without the contribution of appropriate landscape features (Frissell et al. 1986, Buendia et al. 2013). A thorough understanding of both local and landscape-

scale variables may be required to predict the occurrence or abundance of a species (Mcginnis and Kerans 2013). While local variables can be useful for a mechanistic understanding of an organism's habitat needs, managers are faced with the dilemma of monitoring species in geographically large areas. In remote or large areas comprehensive field sampling of local variables is logistically challenging.

There are various costs and benefits to assessing habitat suitability on a landscape compared to a local scale. Landscape covariates can be estimated remotely using Geographic Information Systems (GIS). These measurements can only achieve a moderate spatial resolution but are reproducible across large areas. Local habitat measured on the ground can provide more detail than landscape level measurements; however, measuring local-scale variables can also be resource intensive and more subject to collector bias. If the influence of the landscape on the presence of the target organism can be modelled, then landscape variables may help alleviate resource constraints by facilitating a stratified sample design where only the habitats possible for the organism to inhabit are sampled (Guisan and Thuiller 2005).

3.2.2 Whirling Disease

Westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) and bull trout (*Salvelinus confluentus*) in Alberta are threatened species due to the effects of climate change, habitat loss and introduced species (ASRD 2012, Fisheries and Oceans Canada 2014). One of the introduced species that threatens these indigenous trout is whirling disease, which has only recently been detected in Canada. Whirling disease has caused significant declines in trout populations within the mountainous western United States (Nehring and Walker 1996, Vincent 1996). It is caused by the myxozoan parasite *M. cerebralis*, which infects salmonids and establishes myxospore production around their spinal column (Gilbert and Granath 2003). *M. cerebralis* requires the

presence of a secondary host, *Tubifex tubifex,* (Wolf and Markiw 1984). *T. tubifex* consume myxospores which then produce triactinomyxons (TAMs) spores that infect salmonid fish (Wolf and Markiw 1984). Without the presence of *T. tubifex, M. cerebralis* cannot complete its lifecycle, and because of this pivotal role, understanding the distribution of *T. tubifex* may help predict the risk of future outbreaks (Ayre et al. 2014).

3.2.3 *TUBIFEX TUBIFEX*

T. tubifex is a cosmopolitan species found in a range of waters from pristine to polluted; however, they are not ubiquitous across all salmonid habitat in North America (Kathman and Brinkhurst 1999). At the local scale *T. tubifex* can be associated with the presence of fine sediment (Lazim and Learner 1987, Anlauf and Moffitt 2008). On a landscape scale, *T. tubifex* are often found in habitat associated with agriculture (Anlauf and Moffitt 2010), nutrient enrichment (Kaeser and Sharpe 2006) and reservoirs (Zendt and Bergersen 2000). *T. tubifex* is composed of at least six distinct genetic lineages that vary in their ability to produce TAMs when infected with *M. cerebralis* (Beauchamp et al. 2005b). Areas with a high proportion of lineage III *T. tubifex* tend to have the greatest *M. cerebralis* infection rate in fish populations (Beauchamp et al. 2005b). In mixed lineage populations *M. cerebralis* infection severity can vary over time, dependant on the proportion of lineage III *T. tubifex* within the population (Nehring et al. 2013).

3.2.4 PURPOSE OF RESEARCH

The purpose of this research was to inform management of threatened native salmonid fish species in Banff National Park (BNP) that are threatened by the invasion of *Myxobolus cerebralis*. The primary objective of this project was to determine whether *T. tubifex* were present in remote backcountry watersheds of BNP. The second objective was to quantify potential environmental covariates that could predict the distribution of *T. tubifex* at two hierarchical scales (landscape and local). Knowledge of the landscape-scale variables that can predict the abundance of *T. tubifex*

could inform management actions such as water-based activity closures (i.e. angling, watercraft). However, knowledge of the local scale could inform a more mechanistic understanding of habitat selection. I used Frissel et al. (1996)'s hierarchical classification system to define our habitat scales. I defined the landscape scale as the catchment (i.e. contributing area) and the stream segment (i.e. segment slope; slope between one stream segment and another) (Figure 7). The local scale was composed of reaches, which measured 100 m long (unlike Frissel *et al.* who define a reach as length of stream with consistent slope), and microsite.

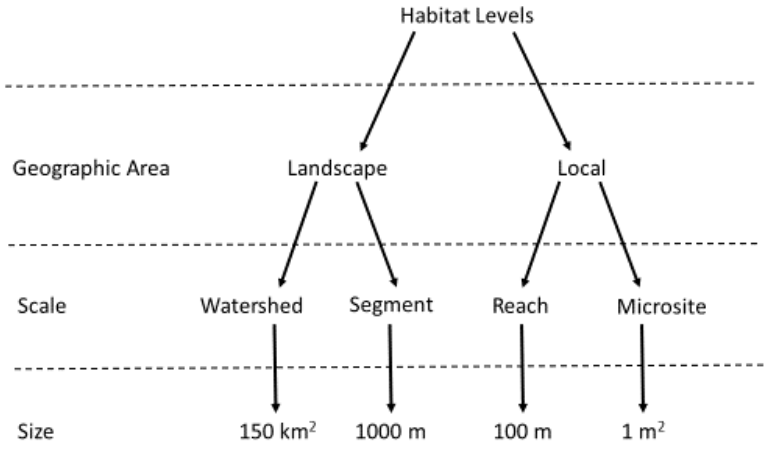

Figure 7. Definitions of habitat scales used throughout the study

Finally, I made comparisons between the two contrasting watersheds within BNP. The Cascade watershed is a higher-elevation, higher-gradient river with a natural flow regime. The Spray watershed is a low elevation, lower gradient, regulated river. I contrasted significant predictors of *T. tubifex* habitat at the within-watershed scale between these two watersheds. Cumulatively, I attempted to understand *T. tubifex* distributions at four hierarchical scales (microsite, reach, segment, and watershed), to contextualize the implications for whirling disease management and native trout recovery.

3.3 METHODS

3.3.1 STUDY LOCATIONS

Banff National Park (BNP) is located in the Rocky Mountains of Alberta, Canada. It is Canada's oldest National Park and encompasses 6,641 square kilometres of mountainous terrain, with numerous glaciers and icefields, dense coniferous forest, and alpine landscapes. Within BNP there are a diversity of stream types; stream characteristics are largely dictated by their discharge volume and the rate that they descend from their high elevation sources. Sampling was limited to 1st, 2nd, and 3rd order streams in two contrasting watersheds within BNP (Figure 1).

I studied the lower Spray watershed downstream of Canyon Dam on the Spray Reservoir, to the confluence with Goat Creek, including Goat Creek itself (Figure 9). The lower Spray River watershed is generally lower-gradient, lower-elevation and is regulated by a hydropower dam. The lower Spray River flows north and enters the Bow River as a 5th order river. The Cascade River flows south from the Bonnet Glacier at its headwaters and enters the Bow River on the north side, downstream of the Spray River's confluence with the Bow River (Figure 1). Sampling locations were limited to the Cascade River's upper reaches and tributaries; Sawback, Cuthead, Elk and Stoney Creeks, and the mainstem upstream of the Cascade-Sawback confluence (Figure 8). Both the Spray and Cascade Rivers support native bull trout and non-native brook trout (*Salvelinus fontinalis*) populations. Westslope cutthroat inhabit the Cascade watershed but have been functionally extirpated from the Spray watershed below Canyon Dam (Pers. Comm Mark Taylor).

In both watersheds, reaches were allocated at ~1 reach/stream-km and distributed randomly using random tessellation (Stevens and Olsen 2004) (Spray watershed = 45 reaches; Cascade Watershed = 86 reaches). Samples were collected in autumn of 2017 and 2018. Reaches with a gradient above 15% were eliminated because they exceeded the maximum gradient where

salmonids could reside. Furthermore, sites in areas known to be above fish barriers were also eliminated. The remaining reaches were visited, and all sampling was conducted in a single visit.

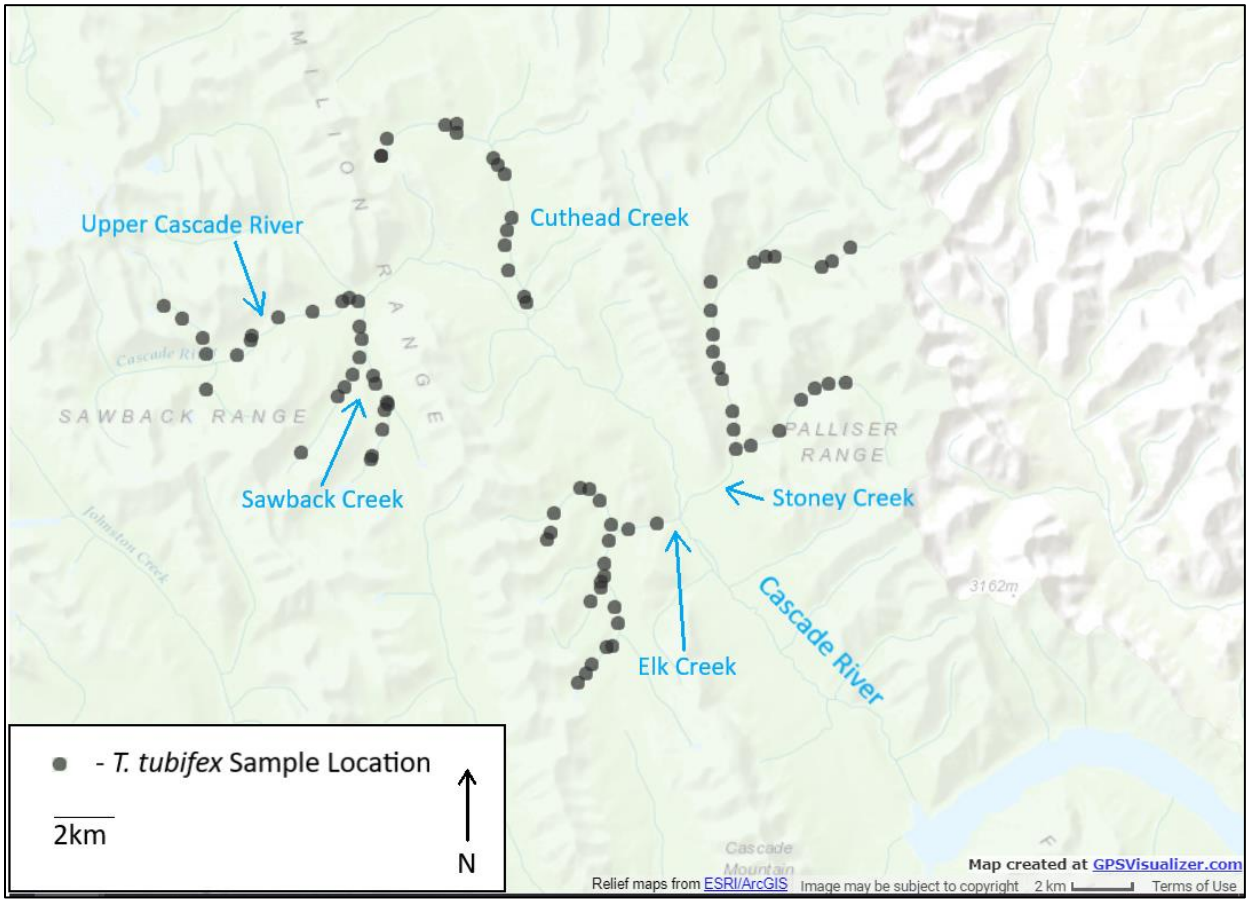

Figure 8. Locations of reaches in the Cascade watershed visited during the fall of 2017 to collect *T. tubifex* samples and habitat data

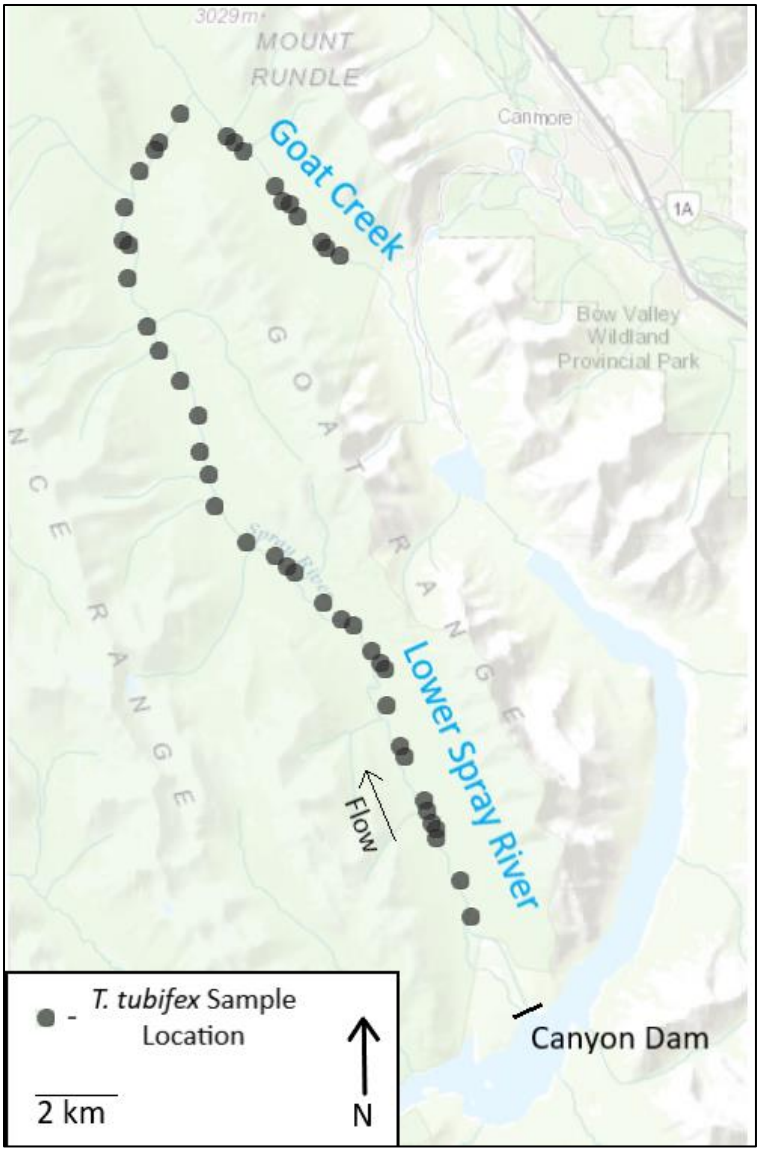

Figure 9. Locations of reaches visited during the fall of 2017 and 2018 to collect *T. tubifex* samples and habitat data.

3.3.2 Field Sampling

Three replicate samples were collected from microsites at distances of 0m, 50m, and 100m from the pre-determined UTM. Crews qualitatively determined a microsite at the lateral position along a transect that contained the finest sediment at the 0 m, 50 m and 100 m locations. An area of approximately 1 m^2 was sampled with a kick net (30 cm x 30 cm triangular opening, 400 μm

mesh) for 3 minutes. Field staff kicked consistently to standardize effort. Samples were stored in plastic bags and preserved with 70% ethanol immediately after collection.

Reach-scale habitat variables were averaged from measurements taken at 0 m, 50 m and 100 m transects in the field. For example, values for stream slope, fine sediment, wetted width, and velocity were collected at 0 m, 50 m and 100 m, but were averaged to one value per reach. To quantify slope at each transect, the distance required to drop a certain elevation was measured using a handheld surveyor's level. On steep slopes a 1m elevation drop was selected, and in low gradient sections it was reduced to 0.1 m. After these measurements were made, a rise over run calculation was completed to determine percent slope. Values were then averaged among the three transects to determine an overall reach average.

At each replicate transect, an underwater camera (*Olympus TG-5, 4000x3000 pixels*) was used to capture images of the stream bottom, which were captured at 2 m intervals across the width of the stream following the protocol in Turley et al. (2016). Photos were imported into Photoshop (*Adobe Photoshop Ver. 19.0*) and overlaid with a grid of 100 squares. Squares where the majority of sediment was under 2 mm were counted using a standardized approach outlined in Turley et al. (2016).

Stream velocity was measured at 40% of total stream depth along three lateral positions at each transect (*Hach FH950*). Nine velocity measurements from 3 lateral positions (i.e. left, centre and right) at 3 transects (i.e. 0m, 50m, 100m) were averaged and used as reach-scale velocity. A measuring tape was used to determine the wetted width of the stream at the three transects which was also averaged to a reach-scale stream width.

3.3.3 Landscape predictor variables

Segment slope and contributing area were landscape variables calculated from a hydroline network generated from a 30m digital elevation model (DEM). A segment was measured from stream node to stream node. I excluded ephemeral streams by removing 1st order hydrolines that were tributaries of 3rd order and higher streams. Historically, these streams are dry in late summer (pers. comm. M. Taylor). To generate segment slopes, point values for each stream segment were extracted from the DEM raster to generate an elevation drop over a segment. These were compared with the length of each segment using the ArcGIS tool 3D analyst, to generate a segment slope value.

Contributing area was generated for each reach using ArcGIS with the Accumulate Values Downstream tool from the STARS toolset (Peterson and Ver Hoef 2014). Nodes were generated at each stream junction and accumulated area was calculated at each node based on hydroline and DEM models. The most downstream node that was still above each sample point was taken as the value for watershed contributing area. The contributing area of the Spray River was set at a relative mark of zero for both nodes immediately below the Canyon Dam and the Goat Creek reservoir assuming that upstream surface runoff was captured by the reservoirs rather than the downstream lotic environment.

3.3.4 Lab Processing

Kick net samples were filtered with 400um sieves to remove ethanol and very fine sediment. Larger rocks and debris were rinsed over the sieve and then removed. The retained material was then distributed onto white specimen trays and thoroughly examined for any specimen that resembled an oligochaete. Each specimen was examined for chaetal hairs and bifid chaetae, which can distinguish *T. tubifex* from other species (Brinkhurst 1986). Suspected *T. tubifex* were preserved individually in vials with 70% ethanol and sent to the University of Alberta

Molecular Biology Service Unit for lineage confirmation by genetic analyses. Samples were analyzed using qPCR and followed techniques described in Nehring et al. (2013). Each potential *T. tubifex* was tested to confirm their species, and their lineage using sequences developed by Beauchamp et al. (2002). *T. tubifex* were also tested to determine if they were infected with *M. cerebralis* using a HSP70 sequence, and a protocol adapted from Cavender et al. (2004). Only those specimens that tested both positive as *T. tubifex*, and had a lineage determined, were included in the analyses.

3.3.5 Analyses

Initially, my intent was to utilize occupancy modelling to analyze data from the Spray and Cascade; however, the high variation in habitat between replicates violated the assumption that all sampling locations (and replicate locations) must be occupiable. Therefore, I utilized general linear models (GLM), which are a common tool for ecologists to measure associations between a dependent variable such as abundance counts and an independent variable such as habitat measures. The mean density of *T. tubifex* (*T. tubifex*/m^2) from each reach was modelled as a Poisson distribution and as a response to either local or landscape predictor variables. While occupancy models were used in previous analyses, too many non-detections prevented accurate occupancy estimates. For this analysis, individual *T. tubifex* counts were averaged for the reach (3 microsites/reach), which reduced the probability of a false absence, but also reduced the resolution of the habitat analysis, because microsites values from a reach were averaged rather than considered individually.

Data exploration was conducted following Zuur *et al*. (2009). Collinearity of predictors was determined for all habitat covariates. If two predictors were > 0.60 correlated the predictor that was less relevant, based on past literature, was dropped from the analysis. Wetted width was

dropped from the analysis because it was inversely correlated with segment slope. Reaches with a segment slope > 4% were removed from the analysis as they were determined to likely be outside the range of inhabitable *T. tubifex* slope. These points were considered to be outliers as no occupied site had a segment slope greater than 2 % and past study has confirmed *T. tubifex* preference for low gradient areas in other locations (Anlauf and Moffitt 2008). Spatial autocorrelation of *T. tubifex* positive sites was tested with a Moran's I test; no spatial correlation existed. Model selection was completed using the drop1 function where insignificant variables are dropped from the analysis sequentially, to give the best fitting model (Zuur et al. 2009). In this case the variables for percent fine sediment and mean velocity were dropped which left the significant variables of segment slope and contributing area.

T. tubifex densities were analyzed using several models to assess habitat associations at reach and segment scales. To calculate the dispersion parameter ϕ, the residual deviance was divided by the degrees of freedom. The over-dispersion parameter was 1.59. Because significant variables achieved a level of significance <0.003, this level of dispersion was deemed acceptable (Zuur et al. 2009).

Response and deviance residuals were plotted against predicted values and examined for patterns. No major patterns were observed, and the model was judged to have a good fit. Residuals were plotted against significant predictor variables to test for fit and the results were deemed acceptable (Hardin and Hilbe 2001).

3.4 RESULTS

Of 86 reaches sampled in the Cascade watershed, none were occupied by *T. tubifex*. Of the 45 reaches sampled in the Spray watershed, 13 (29 %) were occupied by *T. tubifex*. The most *T.*

tubifex found in a single reach was 15 individuals. No reach contained *T. tubifex* at all three replicate microsites and only one reach contained specimens in two replicate microsites. Genetic assessment indicated that all specimens evaluated were lineage III, the lineage most susceptible to *M. cerebralis* (Beauchamp et al. 2005). No other lineages were present in the Spray watershed. No *T. tubifex* tested positive for *M. cerebralis*.

3.4.1 Reach and segment-scale habitat variables

The absence of *T. tubifex* in the Cascade watershed excluded it from consideration for habitat models. For the Spray watershed, there was a significant negative relationship between segment slope and densities of *T. tubifex* ($p < 0.001$; Table 2,

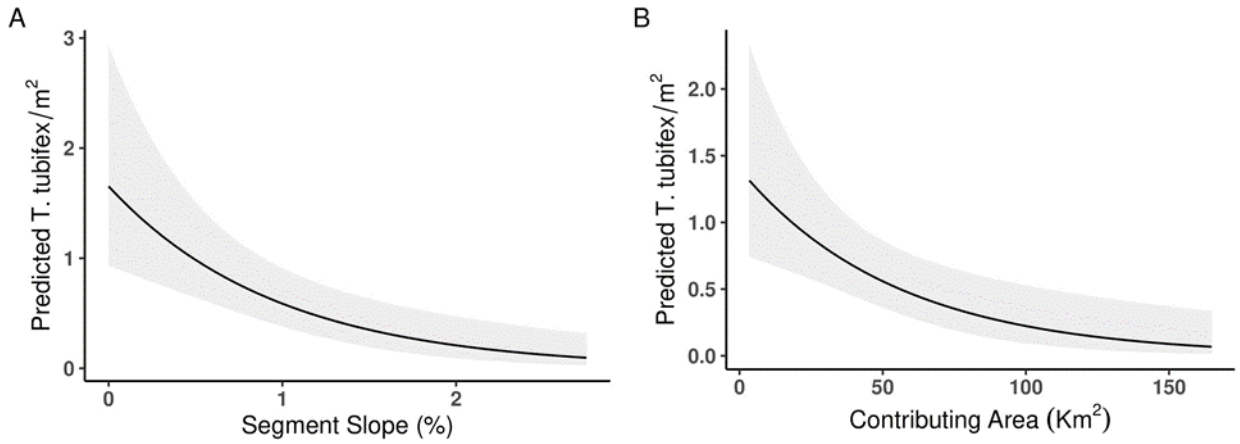

FIGURE 10) where density decreased to zero as segment slope exceeded 2.5%. *T. tubifex* densities were also negatively associated with contributing area ($p<0.01$; Table 2), where density decreased to zero as contributing area exceeded 150km^2.

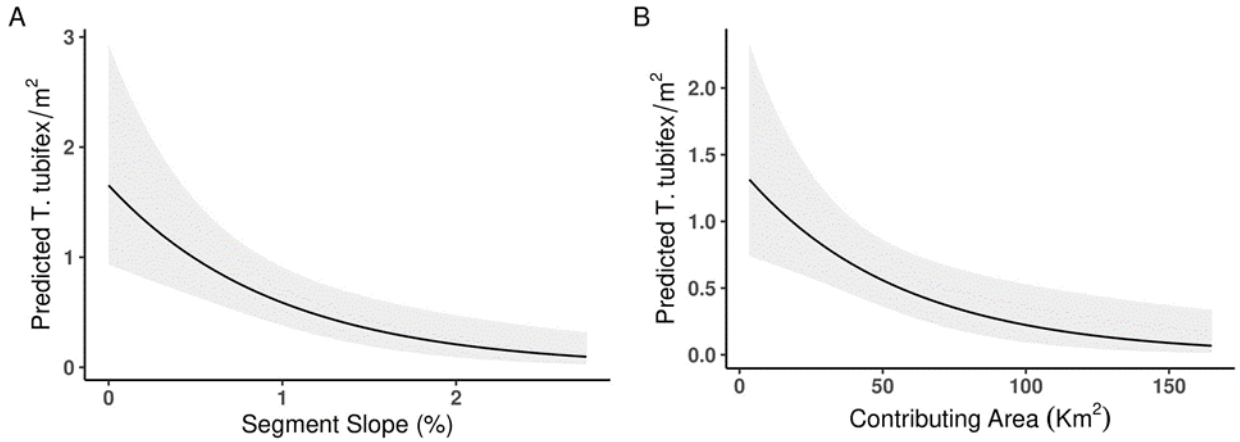

Figure 10The final model predicted 22 % of the variation in *T. tubifex* between reaches. Reach scale measures of stream velocity, percent fine sediment and wetted width were not significant variables in *T. tubifex* density models.

Table 2. Results of the Poisson GLM model on *T. tubifex* density that incorporated segment slope and contributing area. Insignificant covariates were removed from the model during the model selection phase.

	Estimate	**Standard Error**	**Z Value**	**p Value**
Intercept	1.635	0.474	3.448	0.000565
Segment Slope	-1.035	0.281	-3.675	0.000238
Contributing Area	-0.0184	0.006	-2.980	0.002881

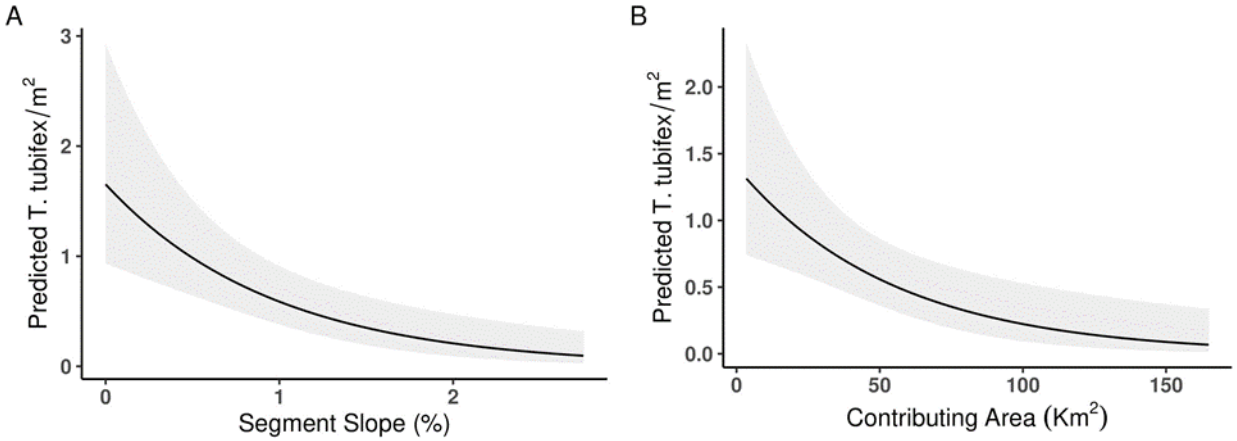

Figure 10. The effect of (A) river segment slope and (B) river contributing area on *T. tubifex*

abundance estimated by Poisson GLM In both cases, *n* = 45 reaches, fitted values are plotted with the solid line and 95% confidence intervals are the grey shaded areas).

3.4.2 WATERSHED COMPARISON

At the reach scale, the Cascade River was generally steep (mean reach slope = 5.2 %, SD = 4.5) with narrow (mean reach width = 4.3 m, SD = 2.7), high velocity reaches (mean reach velocity = 0.36 m/s, SD = 0.19) and little fine sediment (mean reach fines = 5 %, SD = 10). The average segment slope was 5.43 % (SD = 3.27) and contributing area ranged between 1.05 and 57.33 km^2, respectively (FIGURE 11, Table 3).

At the reach scale, the Spray river was less steep (mean reach slope = 0.80 %, SD = 0.5) and wider (mean reach width = 10.1 m, SD = 3.0) than the Cascade River. Velocity in the Spray watershed was slightly higher (mean reach velocity = 0.45 m/s, SD = 0.24), and the Spray watershed contained more fine sediment (mean reach fines = 19%, SD = 14) than the Cascade watershed (mean reach velocity = 0.36 m/s, SD = 0.19, mean reach fines = 5%, SD = 10). Segment slopes and contributing areas were 1.26 % (SD = 0.80) and 164.87 km^2 (range = 3.43 km^2 to 164.87 km^2) respectively.

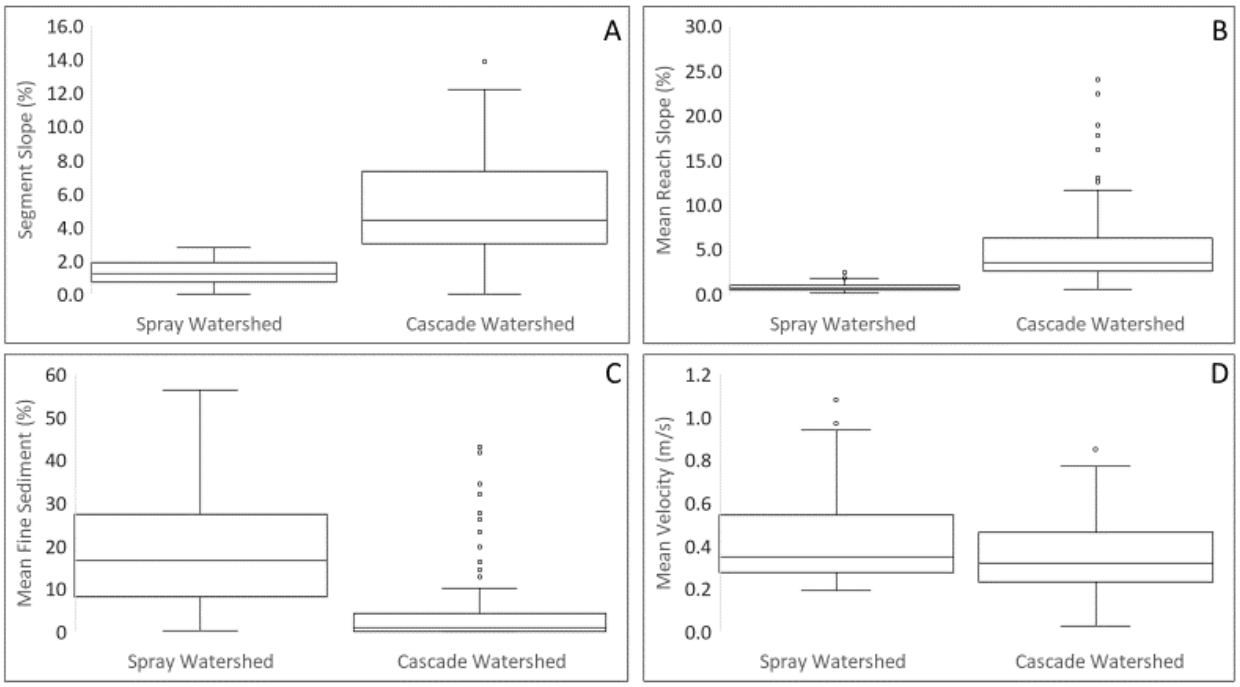

Figure 11. Summary boxplot comparison of habitat covariates in the Spray and Cascade watersheds: A) segment slope, B) reach slope, C) fine sediment D) velocity. The line within the box represents the median value for each watershed.

Table 3. Summaries of habitat metrics collected during *T. tubifex* sample collection during autumn, 2017 and 2018 in the Spray and Cascade watersheds in Banff National Park. The study area in the Cascade watershed was made up of smaller tributaries which resulted in a smaller Max Contributing Area.

Habitat Variable	Spray Watershed		Cascade Watershed	
	Mean	SD	Mean	SD
Segment Slope (%)	1.26	0.80	5.43	3.27
Reach Slope (%)	0.8	0.5	5.2	4.5
Velocity (m/s)	0.45	0.24	0.36	0.19
Fine Sediment (%)	19	14	5	10
Wetted Width (m)	10.1	3.0	4.3	2.7
Max Contributing Area	164.9		57.33	

3.5 Discussion

3.5.1 Summary of landscape and local effects

There were no *T. tubifex* collected from the 86 reaches in the Cascade watershed. Without *T. tubifex*, *M. cerebralis* would be unable to complete its lifecycle (Wolf and Markiw 1984). However, *T. tubifex* were found in 29% of sampled reaches within the Spray watershed. All *T. tubifex* collected were genetically identified as lineage III, the lineage that generates the highest production of TAMs when infected (Beauchamp et al. 2005b). Waterbodies that contain solely lineage III *T. tubifex* are rare, but not unknown (Alexander et al. 2011, Zielinski et al. 2011). This finding is a concern for managers because lineage III propagates the most severe *M. cerebralis* infection among trout populations (Beauchamp et al. 2005b, Barry Nehring et al. 2014). While both watersheds have populations of susceptible fish, only those in the Spray watershed should be at risk of *M. cerebralis* infection; the Cascade watershed appears to lacks the presence of the required *T. tubifex* intermediate host. Indeed, 67% of brook trout and bull trout from the Spray watershed tested positive for *M. cerebralis* with two separate assays (pepsin digest and PCR) in 2016 (Pers. Comm. M. Taylor). However, 785 westslope cutthroat, bull trout and brook trout collected in 2016 all tested negative for *M. cerebralis* in the Cascade watershed using the same methods (Pers. Comm. M. Taylor).

3.5.2 Biological explanation for the importance of landscape variables

In this study *T. tubifex* density was found to be most significantly associated with segment slope, rather than percent fine sediment. Fine sediment has explained *T. tubifex* distributions at the local (microsite) scale in past studies (Anlauf and Moffitt 2008, 2010). Although it is assumed that landscape scale segment slope is predictive of fine sediment, these variables were poorly correlated in the two BNP watersheds studied (r=0.04). The reason why segment slope was more predictive of *T. tubifex* densities is not well understood. It is possible that segment slope captures both the ability of *T. tubifex* to inhabit an area, and the ability to colonize an area. Oligochaetes have been

found to migrate using upstream drift (Williams and Hynes 1976). However, there are likely limits on the gradient where that is a probable mechanism for colonisation. It is possible that many patches of fine sediment would be suitable for *T. tubifex* if they were able to colonise them, but high velocity bars their access.

3.5.3 *T. TUBIFEX* HABITAT PREFERENCES

A second explanation for the association between *T. tubifex* and segment slope, rather than percent fine sediment, may be related to our sampling protocol. We photographed the river substrate and used image analysis to quantify surface fine sediment (Turley et al. 2016); however, the ratio of particulate organic matter to sand was not quantified. *T. tubifex* has been shown to prefer areas rich in organic matter (Robbins et al. 1989) and low gradient sections of streams tend to accumulate organic matter and *T. tubifex* (DuBey and Caldwell 2004). It is possible that some sites in the Spray watershed produce a sandy, nutrient poor fine sediment that is inappropriate for *T. tubifex* habitat, and that this nuance was not captured by our fine sediment assessment. Furthermore, our protocol to measure fine sediment did not consider sub-surface fines. Sutherland et al. (2010) found that in the Saint John River basin, New Brunswick, Canada, sub-surface fine sediments were a better predictor of land use compared to surface sediment metrics.

T. tubifex were sampled at the micro-habitat scale (< 1 m) at three transects within a 100m reach. The sampling was designed to account for imperfect detection using an occupancy model; however, too many zeros prevented the quantification of an accurate occupancy estimate and the three replicates counts were subsequently pooled into one value per reach. Patches of fine sediment were smaller than expected and it is possible that they required more sampling at smaller spatial scales to effectively detect *T. tubifex*. By collapsing the data per reach, I removed variation at the micro-habitat scale, which may be the scale at which fine sediment is most relevant. For example,

one particular micro-site with a large amount of fine sediment may have had a high density of *T. tubifex*, but if its two other microsite counterparts did not, the modelled response at the reach scale would have reflected an average amount of fine sediment from the three microsites. In this case the effect of fine sediment may have been lost in the averaging process. Additionally, the microsites could not be modelled independently because the three microsites within a reach were not truly independent relative to microsites at other reaches.

The density of *T. tubifex* was also negatively associated with contributing area in the Spray watershed. This was after the elimination of high gradient sites from tributaries, which indicates *T. tubifex* preferred the conditions in the upper mainstem of the Spray. This finding contrasts with past research that indicate *T. tubifex* prefer lower elevation streams (Schisler and Bergersen 2002). The combination of peak flow elimination and diversion of water into hydroelectric infrastructure reduces the Spray River's ability to flush fine sediment (Eaton 2000; Wilcock et al. 1996). Presumably that effect is strongest starting at the top of the watershed with the smallest contributing area and diminishes downstream as channel velocities are adequate to entrain fine sediment. It is worth consideration that in this watershed the proximity to the dam may be a better measure of the sediment effect. Indeed, when measured the contributing area covariate was highly correlated with distance from the dam (r=0.98), which suggests the dam could be affecting *T. tubifex* densities downstream.

3.5.5 Watershed Differences

This study found the Spray watershed supported *T. tubifex* while the Cascade watershed did not. The two watersheds are geographically close together and both watersheds join the Bow River within ca. 10 km kilometres of each other. They are generally cold, nutrient poor systems with headwaters that arise from a mix of springs, headwater lakes and a reservoir. Snowmelt and

reservoir draw contribute to flow in the Spray River, but the Cascade watershed is unregulated, and dominated by snowmelt and glacial melt from the Bonnet glacier. This means the Spray River may be warmer, less prone to flushing flows, more prone to sediment accumulation, all of which have been suggested to be linked to *T. tubifex* success in past studies (Reynoldson 1987, Zendt and Bergersen 2000, Anlauf and Moffitt 2008).

The within-watershed analysis suggested that segment slope and contributing area were important variables that predict *T. tubifex* density. Average segment slopes were lower in the Spray watershed which may explain watershed scale differences. However, contributing area may have been a proxy for river regulation, given the location and nature of the effect was close in proximity to Canyon Dam. This suggests that a major difference in the two watersheds, with respect to *T. tubifex*, is the presence of an impoundment on the Spray River.

3.6 CONCLUSION

Invasive aquatic species are a major threat to native species and early detection of invasion is pivotal to preventing their establishment (Gallardo et al. 2016). Understanding the distribution of a parasite's hosts could help create and implement activities to prevent additional range expansion, like angling closures or boating regulations. The ability to examine habitat variables on a landscape scale provides a framework for stratifying entire watersheds into likely and unlikely habitat based on GIS variables. The lack of *T. tubifex* presence in the Cascade Watershed is a positive sign for the conservation of the threatened trout that live there. while knowledge of *T. tubifex* presence in the Spray River allows managers to learn what habitat features they prefer in a river setting.

CHAPTER 4. CONCLUSION

4.1 SUMMARY

My results indicate that *T. tubifex* had a patchy distribution at all scales examined in Banff National Park, and they were present, at low abundances, in two of the three waterbodies assessed. Within the Spray watershed and Johnson Lake, *T. tubifex* distribution was not uniform, and seemed to be driven in part by the presence of appropriate habitat. It is possible that migration routes, source-sink processes or other habitat variables not assessed have additional roles in the distribution of *T. tubifex*.

4.2 STUDY DESIGN RECOMMENDATIONS

The non-ubiquitous nature of *T. tubifex* presence within the areas studied suggests that it will be difficult to make assumptions about their presence in other waterbodies within the region. The study of the Cascade watershed demonstrates that even when seemingly appropriate habitat is available it may not be utilized or colonized by *T. tubifex*. These results suggest that a high level of effort should be maintained in future studies to avoid accidental classification of a waterbody as *T. tubifex* free. The absence of a species on a landscape can be difficult to determine with certainty. Uncertainty could be reduced by running a power analysis, which can make a recommendation on the amount of effort required to determine species presence. Alternatively an additional course of sampling could be conducted with different methods to reduce the possibility of methodological zeroes. Suggestions for additional methods include detection with eDNA, using a core sampler in soft sediment areas, or using invertebrate drift nets to capture entrained *T. tubifex*.

Further study will be required to gain a comprehensive understanding of why T. tubifex preferred the locations they were found; however, it provides an important lesson for the strategies that should be implemented for further *T. tubifex* study in the region. Firstly, researchers must decide whether the goal of the study is simply to determine whether *T. tubifex* are present, or whether they desire to learn more about why they inhabit certain areas. The best approach to determine presence should involve an initial desktop survey of all the low slope areas within the region. Based on the results of the Spray watershed and Johnson Lake studies, there are likely extensive areas of waterbodies that are uninhabited by *T. tubifex* simply because of unfavourable environmental conditions. Waterbodies should be stratified into categories, where the area with the lowest segment slope and the least inorganic carbon and most organic carbon in the sediment are deemed most likely to contain *T. tubifex*. Additionally, in the Spray watershed and Johnson Lake there were no sites that contained *T. tubifex* within all replicate samples from a site, which indicates a single replicate may be insufficient. The habitat covariates considered in this study were non-exhaustive and were selected as suitable candidates for landscape scale studies. Laboratory experiments would also be useful for more precise studies on the exact habitat preferences of *T. tubifex*.

4.3 Management Recommendations

The management of *Myxobolus cerebralis* has several approaches that could be taken to reduce the chance of its spread throughout BNP. One of the most important actions is to focus on prevention, which will be more effective than any strategy to control *M. cerebralis* once it has established in a waterbody. Prevension strategies include education regarding invasive species spread, identifying where *M. cerebralis* has already established and where it hasn't, and closures of infected areas deemed a high risk to prevent accidental spread. Identification of areas where *T.*

tubifex are present will identify what fish populations are at risk of contracting *M. cerebralis.* I recommend that a map of the park be constructed with likely and unlikely *T. tubifex* habitat. If the resources are available ground surveys should also be conducted, starting with the areas most likely to contain *T. tubifex*. Based on the studies in Johnson Lake and the Spray Cascade River areas with low segment slope (<3%) , low inorganic carbon (30%) and high organic matter content would be the best locations to survey first. Kicknetting and coring were effective at collecting *T. tubifex*, however if resources were available multiple methods could be employed. Potentially eDNA could assist with detection / non-detection surveys. Lastly, I recommend further study on the habitat preferences of *T. tubifex* in the BNP region. More detailed knowledge of the conditions *T. tubifex* require to persist would improve the resolution of any attempt to predict where *T. tubifex* will be found.

The scenario encountered at Johnson Lake demonstrate that small populations of *T. tubifex* can create a high infection rate amongst fish in the same waterbody. Resource managers must balance a conservative approach of determining presence/absence at many locations or conducting in-depth habitat assessments in few locations. With either method, lessons can be learned from this study, and previous literature. Areas that feature low segment slope and low inorganic content, and high organic matter are the most likely to be inhabited by *T. tubifex.* If these areas are uninhabited it is unlikely that marginal habitat would be inhabited; however, a thorough search would also consider marginal and poor habitat because its occupation is not impossible. Lastly, detection probability was low in BNP, and this should be taken into account for future studies in this region.

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