Investigation of Western Pearlshell Mussel (*Margaritifera falcata*) Mortality in Bear Creek, King County, Washington: A Disease Ecology Approach

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This is to certify that I have examined this copy of a master's thesis by

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Abstract

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The western pearlshell mussel, *Margaritifera falcata*, was historically the most common freshwater mussel species in streams in forested watersheds in the Pacific Northwest. Upper Bear Creek in King County, Washington supported an apparently healthy and stable population of freshwater mussels as recently as 10 years ago. Within the last 5 years, observations suggesting general decline became more serious when mussel beds became dominated by empty shells, rather than living mussels. Field surveys, a caged mussel relocation experiment, and lake toxicity screening was used to determine: 1) How much *M. falcata* mortality has occurred and what is the spatial extent of die-offs along Bear Creek? 2) Do healthy *M. falcata* become diseased when relocated to Bear Creek for an extended period of time? 3) If healthy freshwater mussels become diseased when relocated to Bear Creek, what is the timeline for the onset of symptoms and is there a pathological sequence of events that lead to mortality? 4) Could an algal toxin be contributing to *M. falcata* mortality? Results from this study indicate that heightened *M. falcata* mortality is occurring downstream, but not upstream, of Paradise Lake and that early fall is a critical time for this mortality. Two lines of evidence support these statements. The caged mussel relocation experiment supported observations made during the survey that suggested that non-predation related mortality occurred downstream, but not upstream of Paradise Lake. Mortality observed in both the caged mussel relocation and the fathead minnow toxicity testing suggest that a toxic substance or stressor is

occurring in Paradise Lake and the Bear Creek – downstream mussel relocation site starting in late August and early September. While there is an indirect spatial and temporal-link between patterns of *M. falcata* mortality along Bear Creek and Paradise Lake toxicity, the link between conditions at Paradise Lake and the downstream relocation site has not been explicitly tested. Further studies are needed to determined what is causing mortality, or if the cause of this mortality is part of a natural process (e.g., a freshwater algal toxin) or anthropogenic (e.g., pollution).

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Introduction

North America supports 302 species of freshwater mollusks, making it the epicenter of freshwater bivalve diversity (Williams et al. 1992, Graf and Cummings 2007). Freshwater mussels provide important ecosystem functions in aquatic habitats, coupling pelagic–benthic material cycling through the removal of algae from the water column, the biodeposition of silt and detritus, the conversion of digested particles to dissolved nutrients, and bioturbation (Jørgenson 1990, Vaughn and Hakenkamp 2001, Vaughn et al. 2004). These processes, in turn, affect benthic productivity and food-web dynamics. Freshwater mussels are also known for their strong association with freshwater fish. Their life history includes a larval stage which is dependent on a freshwater fish host, enabling freshwater mussels to disperse upstream in a unidirectional flow environment (Dillon 2000).

North America's temperate freshwater ecosystems have been strongly impacted by anthropomorphic activities, and projected extinction rates suggest that freshwater species loss is occurring at a similar rate to species loss in tropical forests (Ricciardi and Rasmussen 1999). Both globally and in the United States, freshwater mussels have been particularly susceptible and are the most imperiled taxa (Lydeard et al. 2004, Strayer et al. 2004).

Although freshwater mussel diversity is relatively low west of the Rocky Mountains, the western states support at least 8 species, 6 of which are endemic (Box et al. 2006). Despite freshwater mussel's imperiled conservation status globally, a clear understanding of western freshwater mussels' conservation status is hampered by a lack of information about their distribution and abundance, genetics and taxonomy, and life history and habitat requirements (Box et al. 2006). Even in regions where mussel population declines have been well documented, more experimental research is needed to identify

specific mechanisms driving decline and to understand interactions between multiple stressors (Strayer et al. 2004).

In pre-European settlement times, the western pearlshell mussel, *Margaritifera falcata* was common in western coastal streams and one of the most common species found in forested watersheds (Toy 1998). Bear Creek, a Puget Lowland stream, historically supported a large population that was harvested by Native Americans (Wong, 1993), recognized as one of the few populations of its size in the Puget Lowlands (Frest 2002), and valued by the local community. Within the last 5 years, observations suggesting general a decline became more serious when mussel beds became dominated by empty shells, rather than living mussels (Washington Trout 2002, Washington Trout 2003, Brenner 2004).

The investigation of *M. falcata* mortality described here follows other studies of the Bear Creek population that characterized basic life-history and population demographics, investigated evidence of juvenile recruitment; evaluated water and sediment quality; and tested for contaminant bioaccumulation. This research expands on the existing body of knowledge by exploring:

1. How much *M. falcata* mortality has occurred and what is the spatial extent of die-offs along Bear Creek?
2. Do healthy *M. falcata* become diseased when relocated to Bear Creek for an extended period of time?
3. If healthy freshwater mussels become diseased when relocated to Bear Creek, what is the timeline for the onset of symptoms and is there a pathological sequence of events that lead to mortality?
4. Could an algal toxin be contributing to *M. falcata* mortality?

Chapter I: Literature Review

Freshwater Mussel Conservation Status

Freshwater mussels are bivalve mollusks from the order Unionoidea that are dependent on a freshwater fish host for their larval stage, have pearly nacre, and inhabit freshwater (Nedeau et al. 2005). Depending on the species, freshwater mussels inhabit either lotic (moving water) or lentic (standing water) environments (Nedeau et al. 2005). These organisms are relatively stationary, spending most of their life partially buried in the substrate and feeding on algae, detritus, and micro-organisms filtered from the water column or scraped from the substrate (Levinton 1972, Nichols and Garling 2000, Bauer 2001, Raikow and Hamilton 2001). Biologists believe that there are about 1,000 species of freshwater mussels worldwide and that the southeastern United States is the epicenter of this group's biodiversity (Bauer 2001). Out of the 300 species recognized in the United States, 70 species are listed as being federally endangered or threatened (USFWS 2007). Twenty-one species are already known to be extinct (Williams et al. 1992). River impoundment, habitat simplification, water quality degradation, sedimentation, and the introduction of exotic fish species are often suggested as the driving factors behind population declines (Strayer et al. 2004). Despite the imperiled state of freshwater mussels, much of our understanding of the causes of decline are based on anecdotal observations and hampered by a lack of experiments capable of demonstrating the mechanisms driving loss (Strayer et al. 2004). Freshwater mussels are often impacted by long-term and cumulative effects, multiple stressors and time-lags between stressor events and population-level response, complicating the investigation of the specific causes of freshwater mussel decline (Strayer et al. 2004).

Freshwater Mussel Life History

One of the most unique and complex aspects of freshwater mussels' life history is their reproductive cycle, which is dependant on a fish host for larval development and dispersal (Dillon 2000). Reproduction occurs when males release sperm into the water and this sperm enters females through the incurrent siphon (Dillon 2000). Mature eggs residing in the marsupial gill are fertilized when water containing sperm passes over the gill. At the end of the brooding period, females release mature larvae called glochidia. There are two distinct brooding strategies among freshwater mussels. Long-term brooders (bradytictic) generally spawn in late fall, brood over the winter, and delay the release of glochidia until the spring or early summer, while short-term (tachytictic) brooders spawn in the spring and release glochidia that summer (Ortman 1911).

After glochidia are released, they must encounter a suitable fish host and become encysted on its gills or fins in order to continue develop and survive (Jacobson et al. 1997, Dillon 2000). Research has yet to uncover the exact benefits glochidia receive while attached to host fish, but aquaculture research has identified a positive relationship between specific free amino acids in fish plasma and glochidia survival (Uthaiwan et al. 2003). Despite the importance of the host fish – mussel relationship, knowledge of host specificity is limited for many North American mussel species (Haag and Warren 2003). There is evidence that not all fish are suitable hosts; in laboratory experiments with *Margaritifera margaritifera* (subsequently reclassified as *Margaritifera falcata*), coho salmon were more resistant than Chinook to glochidia infection at low exposure levels (Meyers and Millemann 1977). In addition to documented differences in fish resistance, researchers have observed differential survival rates of glochidia reared in plasma taken from different potential host species (Uthaiwan et al. 2003). Therefore, even if two different fish species accept glochidia infection, they may not be equally advantageous as hosts.

The glochidia stage typically lasts 2-4 weeks (or longer in very cool water), after which the glochidia transform into juvenile mussels and drop off into the substrate (Dillon 2000). The evolution of a freshwater fish-host requirement by these freshwater mussels is an adaptation to life in a unidirectional flow environment, enabling upstream dispersal (Dillon 2000).

Freshwater Mussel Food Web Dynamics and Ecosystem Processes

Freshwater mussels acquire food by filter feeding on suspended particles through the inhalant aperture (Nedeau et al. 2005). Potential food items for these filter feeders include plankton, dissolved organic matter, organic particles, and bacteria (Levinton 1972). Diatoms and green algae have traditionally been thought of as an important food source, since these algae are found in mussel gastrointestinal tracts and mantle cavity at higher concentrations than existed in the water column. However, stable isotope analysis indicates that bacterial carbon, and not algal carbon, is the main carbon source for studied freshwater mussels (Nichols and Garling 2000). Freshwater mussels assimilate bacteria that are associated with Fine Particulate Organic Matter (FPOM), indicating that detritus observed in the mantle and gut is intentionally ingested (Nichols and Garling 2000). Rather than being a primary carbon source, algae may contribute nutrients such as vitamins A and D, and phytosterols. While FPOM-associated bacteria are an important carbon source, FPOM is largely nutrient poor and is low in lipids, protein, and vitamins (Nichols and Garling 2000, Christian et al. 2004). Consequently, freshwater mussels must continually feed in order to survive on low quality food material (Nichols and Garling 2000).

One key ecological question is whether food availability controls the spatial distribution of mussels. In general, freshwater mussels are expected to be dietary generalists, as seasonal and fluctuating food sources would preclude the development of food specialization (Levinton 1972). Diet analysis supports this hypothesis; comparisons among different species indicate that tested species consume similar food items (Nichols

and Garling 2000, Raikow and Hamilton 2001, Christian et al. 2004). Freshwater mussels often occur downstream of lake outlets and associated wetlands, and researchers speculate that observed bivalve abundance in these locations is caused by high phytoplankton productivity in the lentic environment (Raikow and Hamilton 2001). Researchers have also proposed that a change in food quality downstream of a lake may drive observed dispersal patterns for two studied freshwater mussel species and result in niche partitioning based on food quality, which changes in relation to distance from the lake outlet (Brönmark and Malmqvist 1982). However, studies are needed to explicitly test whether food availability controls freshwater mussel occurrences.

Mussel diet and feeding behavior can significantly affect nutrient cycling through the removal of algae from the water column, the biodeposition of silt and detritus, the conversion of digested particles to dissolved nutrients and bioturbation (Jørgenson 1990, Vaughn and Hakenkamp 2001, Vaughn et al. 2004). These processes facilitate pelagic–benthic material cycling and help oxygenate stream sediments, which can increase benthic productivity and affect food-web dynamics. In detrital food webs, bacteria are viewed as an important link between inorganic nutrients and higher level consumers (Meyer 1994). Therefore, freshwater mussels may facilitate the transfer of inorganic nutrients to higher trophic levels through the consumption of bacteria. In addition to their roles as consumers in food webs, freshwater mussels are an important food source for predators such as river otters, muskrats, raccoons and skunks (Nedeau et al. 2005).

Margaritiferidae Ecology

Margaritiferidae is considered to be the most primitive and morphologically conservative freshwater mussel family (Smith 2001). Mussels from this family are often referred to as pearl mussels and have a wide distribution encompassing North America, Europe, northern Africa, the Middle East, and southern and eastern Asia. All known Margaritiferids are confined to streams and rivers and tend to be found in either neutral or

weekly acidic waters. Margaritiferids can reach large sizes (e.g., 125 mm for *M. falcata)* and have a large foot that is effective in digging and anchoring. While pearl mussels can become very large, they are slow growing, a physiologically adaptation to living in nutrient-poor, cold streams (Bauer 1998).

Margaritifera falcata – the Western Pearlshell Mussel

Although freshwater mussel diversity is relatively low west of the Rocky Mountains, the western states support at least eight species, six of which are endemic (Box et al. 2006). The western mussels are grouped into three genera: *Margaritifera*, *Gonidea*, and *Anodonta*, with the taxonomy within *Anodonta* currently under more careful consideration (Mock 2006). *Margaritifera falcata* is the only *Margaritifera* species west of the Rocky Mountains (Smith 2001). In pre-European settlement times, *M. falcata* were common in western coastal streams and were the most common species found is forested watersheds (Toy 1998). Western pearlshell mussels can live in excess of 100 years and reach sexual maturity at about nine years of age (Toy 1998).

While *M. falcata* is relatively wide spread in the western drainages and its current status is classified by the Washington State Heritage program as being apparently secure (Pacific Northwest Freshwater Mussel Workgroup pers. comm. 2007), examination of museum records indicate that *M. falcata* populations where historically much more widespread and specific populations are known to have become locally extirpated in areas such as Umatilla River in Oregon and the urbanizing Puget lowlands in Washington State (Frest 2002, Box et al. 2006, Pacific Northwest Freshwater Mussel Workgroup pers. comm. 2007). Detailed investigations of the mechanisms behind these losses are lacking, but contributing factors likely include water quality degradation, changes in hydrological regime, high sediment loading, channel and corresponding habitat simplification, dams and impoundments, reduced fertilization success due to low mussel density (Allee effect),

and changes in fish host distributions (Allee et al. 1949, Frest 2002, Box et al. 2006, Pacific Northwest Freshwater Mussel Workgroup pers. comm. 2007).

Pearl mussels are observed in non-random, aggregated patterns and controls over spatial-distribution appear to operate over more than one spatial scale (Johnson and Brown 1998, Hastie et al. 2000, Stone et al. 2004, Newton et al. 2008). It is not well understood to what extent distribution is driven by daily conditions relative to rare events. Strayer et al. (2004) distinguish between negative "censoring" mechanisms that preclude the establishment of a bed (i.e., burial, washout, suffocation, starvation, and predation) and positive mechanisms that facilitate bed establishment (i.e., habitat selection and high fecundity due to favorable habitats). Hydraulics are currently viewed as one of the key processes to determine the suitability of habitat, but assessments of habitat controls are further complicated by the role that other resources (such as host fish) may play on distribution (Newton et al. 2008). Some potential large-scale controls over freshwater mussel distribution include climate, host distribution, and historic distribution (Vaughn and Taylor 2000).

Across a 50-m reach scale, dissolved oxygen and sheer stress has been demonstrated to be an important predictor of *M. falcata* occurrence (Stone et al. 2004). At smaller scales increased wetted width, canopy cover, abundance of small gravel substrate, and distance from stream banks appear to be important factors in local distribution. Pearl mussels are commonly found in association with clean, small cobbles (Stone et al. 2004). While adults can tolerate silty or muddy conditions for unspecified periods of time, juvenile mussels are typically not found in these conditions (Hastie et al. 2000). *M. falcata* are also vulnerable to rapid aggrading stream bank conditions (Vannote and Minshall 1982). In the Salmon River Canyon, Idaho, another species of freshwater mussel, *Gonidea angulata* replaced *M. falcata* in aggrading stream reaches (Vannote and Minshall 1982). Vannote and Minshall (1982) observed that *M. falcata* mussel beds located in areas where boulders stabilized cobbles and interstitial sediment had the highest density and

oldest mussels. Research on *M. falcata* in northern California suggests that boundary sheer stress is an important driver of the western pearlshell mussel distribution and that populations tend to be located in areas where velocities during high winter flows do not displace animals or subject them to high bedload transport (Howard and Cuffey 2003).

Freshwater Mussels as Biological Indicators

In order to evaluate how biota respond to the suite of physical, chemical, and biological conditions that they encounter, it is necessary to directly measure biological endpoints (Karr and Chu 1999). In response to this need, research has been directed towards determining appropriate biological and ecological indicators. Biological monitoring has been defined as "measuring and evaluating the condition of a living system, or biota" (Karr and Chu 1999, p. 2) and ecological indicators as "measurable characteristics of the structure… composition… or function… of ecological systems" (Niemi and McDonald 2004, p. 91). In application, the range of biological and ecological indicators has been broad in scale, ranging from genetic and biochemical markers, to population and community studies, to landscape level analysis. A single species, an indicator species, can be selected for monitoring and used as a gauge for the status of other biota, under the assumption that its population status reflects conditions experienced by other species with similar ecological requirements (Niemi and McDonald 2004). In addition to determining the status of a population or ecosystem, biological monitoring can provide early-warning indicators of environmental alteration; stressor identification; trend analysis; regulatory compliance benchmarks; and forecasts of ecological change (Niemi and McDonald 2004; Van Hassel and Farris 2007b).

Disease ecology utilizes a similar concept, a sentinel organism, in the detection of diseases. Naïve animals are exposed to an environment with a suspected disease-agent to aid in pathological research (e.g. Burge et al. 2006, 2007). Disease is defined as any departure from normal condition experienced by an animal and may be caused by any combination of change in the animal itself, its environment, or any pathogens it

encounters (U.S. National Library of Medicine 2008). Sentinel animals can help answer questions about the cause and virulence of disease and serve as an indicator for the host species population.

Many times investigations using biological indicators occur at the population and community level. While this level of organization has high ecological significance, investigations at this scale are hampered by high natural variability and time-lags in response to a given stressor (Maltby 1999, Niemi and McDonald 2004). Maltby (1999) advocates studying environmental stress at the organism-level using information about how individuals physiologically-respond to stress to predict population-level effects in order to link early-detected changes at the cellular and biochemical level to ecologically relevant changes at the population and community-scale.

Since freshwater mussels are often cited as being an important aquatic indicator species, it is important to acknowledge both the benefits and challenges of using unionid mussels for assessing ecological condition. Some benefits of using freshwater mussels as an ecological indicator are that they are relatively sedentary, have a relatively large tissue mass that can be analyzed for different substances of interest, accumulate some contaminants, and provide a record of environmental conditions in their shells (Van Hassel and Farris 2007a, Van Hassel and Farris 2007b). However, other traits can make it difficult to use freshwater mussels in ecological assessments; they have slow growth rates, complex reproduction requirements, and reference populations can be difficult to find. Additionally, the belief that freshwater mussels are particularly sensitive to chemicals and provide early warning indications of contamination may be an over-simplification of their chemical tolerance (Van Hassel and Farris, 2007a). While there is verified sensitivity to some freshwater mussels for some chemicals, especially copper and un-ionized ammonia, other species have demonstrated moderate sensitivity or tolerance to a number of chemicals (e.g., chlorine and malathion) (Van Hassel and Farris 2007a).

The use of density, mortality, and physiological condition indices in freshwater mussel biomonitoring has largely been insensitive to small or moderate changes within populations and these endpoints can be affected by habitat differences (Van Hassel and Farris 2007a). These constraints support the need for development of biochemical and physiological endpoints. Physiological measures are not well-developed for freshwater mussels, but some, such as the use of cellulolytic and glycogen activity has been successful at detecting stress earlier than traditional measures. (Haag et al. 1993, Van Hassel and Farris 2007a).

Freshwater Mussel Relocations

Freshwater mussel relocations have most commonly been used for conservation and population restoration measures. A 1995 literature review identified 37 projects documenting the relocation of 90,000 mussels, with most (43%) carried out so that construction projects could comply with Endangered Species Act protections (Cope and Waller 1995). In general, relocation projects have not been intensively monitored and success has primarily been measured by short-term survivorship rates. In Cope and Waller's (1995) review, only 78% of projects reported follow-up monitoring. Of these projects, 38% were monitored for one year or less and 16% were monitored annually for five or more years.

Of monitored relocation projects, recovery of mussels and mortality varied widely. Among those studies that reported recovery and mortality rates (73%), the average recovery rate was 43% and the average morality rate was 49%, with mortality exceeding 70% in 30% of the studies (Cope and Waller 1995). Most mortality was observed within one year of relocation, but there are instances where mortality increased dramatically within the second year after relocation (Cope and Waller 1995, Newton et al. 2001). At least one study also considered growth rate between relocated and control animals, and found no difference in a study where 81% of relocation mussels were recovered alive a year later. Among studies that evaluate factors of mortality of relocated mussels, habitat

differences at the relocated site have been most commonly examined. As expected, mussels do poorly when moved from lotic environments to a lentic site (Newton et al. 2001). In the same study, sediment composition also affected survival; mussels performed best in the stream sites where mussels could dig into sediment but were not completely buried. In another study where four different freshwater mussel species were relocated to three different substrate types, survival patterns differed among species and for some species differed among habitat types (Hamilton et al. 1997). These results support the theory that habitat selection may be an important factor in relocation success, and that habitat needs and specificity differs between species. While subtle differences in substrate may affect relocation success, the most common cause of high mortality rates is attributed to drastic changes in habitat due to substrate instability (Cope and Waller 1995, Bolden and Brown 2002). Bolden and Brown (2002) recommend using the presence of existing mussels to guide site selection for *in situ* refugia, because these sites are known to be hydrologically stable enough to support freshwater mussels.

Some studies demonstrated short-term successes in survivorship, indicating that relocation may be a viable conservation strategy, particularly in the short-term (Bolden and Brown 2002). While proper handling, demonstrated transport protocols, and the selection of suitable habitat have been identified as factors that can improve survivorship among relocated mussels (Cope et al. 2003), other issues are not often not taken into consideration. The spread of disease or associated invasive species (e.g., zebra mussels) are risks associated with relocation, and the source population should be screened for pathogens and nonnative species before relocation (Villella et al. 1998). The risk of disease transmission can be particularly high in fish hatcheries rearing both bivalves and fish. Villella et al. (1998) have cautioned that genetic considerations are necessary to ensure that relocation projects meet the ultimate conservation goals of population sustainability and do not cause populations to be more vulnerable to stochastic events by reducing the effective population size, increasing homozygosity, or causing inbreeding depression.

Cope and Waller (1995) concluded that recovery and survivorship were only crude estimates of success and do not identify the long term viability of relocated mussels. They recommended the use of physiological and biochemical endpoints, in addition to more long-term (5+ years) monitoring benchmarks of survival and condition, reproduction, and recruitment. In order to help ensure successful recruitment, the verification of suitable host-fish at the destination site may be necessary, a practice that is currently not followed (Villella et al. 1998).

Mussel Decline in Bear Creek, WA

The *M. falcata* population in Bear Creek in King County, WA has been well-studied relative to other western freshwater mussel populations. These efforts have consisted of basic research aimed at determining basic life-history characteristics of *M. falcata* and applied research motivated by the observed decline. The presence of at least sixteen mussel middens close to Bear Creek indicates that a stable population of *M. falcata* was historically present at this location. The site is believed to have been used by Native Americans up to 5,000 years ago, and by the Snoqualmie tribe for fishing until 1940 (Wong 1993, W. Walsh pers. comm. 2007). In 1993, the King County Landmarks and Heritage Commission designated a 4-ha forested site around upper Bear Creek as an archaeological landmark because of its use by Duwamish and Sammamish Native Americans as an important encampment. Vannote and Minshall (1982) also observed Native American encampments in proximity to stable mussel beds dominated by old mussels, and proposed that these camp sites were selected because nearby dense, actively reproducing mussel populations could be sustainably harvested over the long-term.

Even into the early 21st Century, local biologists had noted the size and abundance of the Bear Creek population. In 2002 malacologist Dr. Terry Frest conducted an evaluation of freshwater mussel conditions is this area. While he determined that increased beaver activity may have negatively affected the mussel population through predation, dam-

building, water impoundment, and increased opening of the forest canopy, he recognized the Bear Creek population as being particularly healthy in terms of size and large numbers of young mussels in areas not affected by beavers (Frest 2002).

In 1995, the Bear Creek population was studied along with another Puget lowland population to establish basic life-history characteristics and habitat requirements for *M. falcata.* Toy's (1998) research indicated that *M. falcata* is dioecious, that hermaphrodism is rare, and that these mussels have a short term breeding strategy, brooding their eggs in the late spring to early summer and releasing glochidia shortly thereafter. Water temperature influenced both the timing of the gametogenic cycle and growth rate. Sexual maturity was size dependent and was not reached until the mussels were 9 to 12 years old. At the Bear Creek site, the population size was estimated to be 290,940 mussels along a 340 m reach with a density of 55 mussels/m^2. The age distribution was skewed toward older individuals with maximum age exceeding 90 years. This skewed distribution was consistent with the population size structure observed among highly stable populations that Vannote and Minshall (1982) hypothesized might be important for long-term recruitment to other locations. While juvenile habitat was not targeted in the survey, Toy (1998) observed a lack of fine substrate stabilized by large woody debris which characterized juvenile habitat in her other study creek, Battle Creek

In 2001, freshwater mussel beds were identified as part of a stream assessment of salmonid habitat quality (Fevold and Vanderhoof 2002). At this time biologists noted that a reach of Upper Bear Creek was dominated by shell-only beds and that the upright/undisturbed orientation of these shells suggested the mussels had died in place and not from predation. In response to concerns about increased numbers of empty *M. falcata* shells observed along Bear Creek, Wild Fish Conservancy Northwest (formally Washington Trout) conducted *M. falcata* surveys in Bear Creek during 2002 and 2003. In 2002, ten mussel beds previously identified during the 2001 King County stream assessment (Fevold and Vanderhoof 2001) were surveyed in greater detail to obtain

baseline population data. This survey documented that live mussel densities were highest in the upper watershed and significantly lower further downstream. The average density was 14 mussels/m^2 in the four most upstream sites, versus 0.25 mussels/m^2 in the remaining six downstream sites (Washington Trout 2002). Empty shells dominated three of the study beds in the lower reaches surveyed. In 2003, Wild Fish Conservancy Northwest resurveyed five of the ten beds and conducted searches targeting juvenile mussels. While two of the five beds previously surveyed had a decrease in number of live mussel density, it could not be determined if this decrease represented a real change or was due to sampling artifact (Washington Trout 2003). In the juvenile-targeted surveys, no live mussels <30 mm length were encountered; only four mussels between 31-40 mm length were observed (Washington Trout 2003). These observations indicated a lack of mussel recruitment during the past 10 to 20 years. While freshwater mussel decline wasn't statically demonstrated between 2002 and 2003, Wild Fish Conservancy Northwest stressed that based on information provided by local residents, mussels were less abundant in Bear Creek than had been previously observed.

To investigate if contamination could be responsible for mussel decline in Bear Creek, a caged mussel bioaccumulation study was conducted in winter 2003, and King County conducted water and sediment quality sampling and analysis in Bear and Cottage Lake Creeks in 2005. In the 2003 caged bioaccumulation study, mussels from upper Bear Creek were deployed at seven locations along Bear Creek and its tributaries for a total of 65 days in late fall/early winter (Applied Biomonitoring 2004). Mussel tissue was analyzed for 8 metals and 99 organic compounds, including 27 pesticides. While a number of compounds (16) were detected in mussel tissue, levels were low and none were identified as being a significant contributor to the mussel decline. However, the role of chemical stressors could not be ruled out as a result of this study and more extensive water and sediment quality investigations were recommended.

King County collected water samples during two flow events in 2005: Base flow in September and storm flow in December (Lester 2007). Samples were analyzed for a number of metals (16) and organic compounds (167); including a number of endocrine disrupting compounds. Samples were collected from four sites in Bear Creek, and at one location in nearby Cottage Lake Creek, adjacent to an area supporting a healthy mussel population. Few contaminants were detected in this study. However, while water quality was generally good, a greater number of compounds, some at higher concentrations, were detected at the Cottage Lake Creek sampling location. Low concentrations of bacteria and other indicators of sewage contamination (e.g., caffeine, coprostanol, ethynylestradiol) suggested that leaking septic systems were not contributing contaminants to Bear Creek. Concentrations of all detected chemicals were below available water quality standards and literature based freshwater mussel toxicity thresholds. In addition, levels of contaminants associated with stormwater runoff (e.g., petroleum hydrocarbons, pesticides) detected in the samples collected during the storm event were at levels that suggest stormwater was not a significant contributor to contaminant levels in Bear Creek.

Sediment quality was also examined at 11 Bear Creek locations (samples were collected approximately every mile between Paradise Lake and its confluence at the Sammamish River) and 4 locations in Cottage Lake Creek (Lester 2007). Sediment samples were analyzed for a number of metals and organic parameters. Toxicity testing was also conducted on a subset of these samples. Two standard sediment toxicity tests were conducted using *Hyallela azteca* (survival) and *Chironomus tentans* (growth and survival); however, no toxicity was observed. When concentration data were compared to available sediment quality guidelines (Smith et al. 1996), five metals in Cottage Lake Creek and one metal in Bear Creek were above sediment guidelines. The effects of these concentrations to benthic organisms are uncertain, especially since the ratio of acid volatile sulfide to simultaneously extracted metals (AVS/SEM) generally suggested that

these metals were not bioavailable and therefore not likely to cause adverse effects to aquatic life.

In 2004 King County conducted freshwater mussel surveys in Bear, Covington and Stossel Creeks (Brenner 2005). Mussel density data in Bear Creek suggested a significant population decline when compared to data from Toy (1998) and previous assessments conducted by Washington Trout (Washington Trout 2002, Brenner 2005). However, different sampling techniques used by each study complicated density comparisons among the three studies. In 2002, Washington Trout surveyed individual beds, targeting observed aggregations of mussels rather than surveying a continuous length of stream, as was done by Toy (1998) and Brenner (2005). Washington Trout (2002) recorded densities of 110, 35, and 51mussels/m^2in Upper Bear Creek. Toy (1998) surveyed 340 m of stream length through the placement of a 0.25 m^2 sampling frame randomly placed along 34 transects and estimated a density of 55 mussels/m^2 , while Brenner (2005) surveyed 100 m of stream, sampling 0.25 m wide transects placed at 5 m intervals and estimated a density of 11 mussels/m^2.

In 2004, King County also estimated sex ratios through the use of gamete smears. While sex ratios at Covington and Stossel Creeks were approximately 1:1 male to female, ratios from Bear Creek were dominated by males at a 4:1 ratio (Brenner 2005); however, sample size for this assessment was relatively small (n=25). In September, 2005 King County staff returned to the Bear Creek site to recapture 25 mussels that had been marked a year before. Of the original 25 marked mussels, only 15 were located, and of those 14 were empty shells (B. Brenner, unpubl. data). During this site visit in late September 2005, King County staff also observed hundreds of empty shells at the Bear Creek location.

As part of the 2004 study by King County, a small number of mussels from Bear and Stossel Creeks were collected for histological examination (Brenner 2005). Results

indicated that the Bear Creek mussels were severely diseased. The epithelium height in the digestive gland was low, the gills were lacking cilia and had chitinous lesions, and bacterial colonies were noticed in association with the gills (Elston 2005). The histological assessment of Stossel Creek mussels suggested they were healthy.

Several traits of freshwater mussels, such as limited movement and ability to avoid stressors, long life-spans and late maturation age make freshwater mussels vulnerable to changes in the environment. Additionally, aquatic environments (and freshwater mussels inhabiting these environments) are vulnerable to anthropogenic impacts, both from changes to the aquatic environment itself (i.e., habitat simplification, riparian-loss, and direct pollutant discharges) and from changes within the contributing basin that affect instream conditions (i.e., hydrology, non-point source pollution). Though the Bear Creek *M. falcata* population is relatively well-studied relative to other freshwater mussel populations west of the Rocky Mountains, research to date has not identified a likely cause for the sudden decline that is illustrated by hundreds of empty shells occupying sites once dominated by living mussels. Research to date does not suggest that freshwater mussel decline in Bear Creek is an example of the general freshwater mussel population loss that accompanies anthropogenic environmental change. Effective impervious surface area in the Bear Creek subbasin is estimated to be lower than the 10% level commonly cited to degrade aquatic conditions in western Washington (Booth and Jackson 1997) and water quality monitoring and bioaccumulation studies to date have drawn no relationship between components typically associated with stormwater pollution and mussel mortality in Bear Creek (Salazar 2004, Lester 2007). Aquatic degradation can occur at development levels resulting in less than 10% effective impervious surface area, but this degradation is not quantified nor understood well (Booth and Jackson 1997). While biological impacts have been observed at very low levels of contamination (e.g., behavioral responses of juvenile coho salmon to low levels of copper (2 µg/L), (Sandahl et al. 2007)) and shifts in the species community composition have been observed along a forested to urban gradient (Morley and Karr

2002), specific die-offs of aquatic species caused by changes associated with low density development have not been well documented.

Notes to Chapter I

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Chapter II: Western pearlshell mussel bed resurveys in Upper Bear Creek, King County, WA

Summary

Upper Bear Creek, a Puget lowland Creek in the Lake Washington drainage, historically supported a large *Margaritifera falcata* population. In 2007, surveys were conducted in 10 *Margaritifera falcata* mussel beds located along approximately 5 km of Bear Creek, in King County, Washington, for which there were baseline data from 2002. For these surveys, transect-based sub-sampling was employed in large beds (> 50 mussels) and whole-bed counts were used in small beds (< 50 mussels). Although the baseline surveys did not examine beds further upstream than Paradise Lake, the 2007 survey added 3 new sites in this upstream area that were not part of the 2002 survey. Between 2002 and 2007, the number of live *M. falcata* observed in Bear Creek significantly declined in 4 of 5 sub-sampled beds. While empty shells were found in the beds surveyed through whole-bed searches, no live mussels were observed in 4 of the 5 beds. Baseline monitoring in 2002 documented the highest mussel densities in the most upstream beds surveyed; 30.0 – 110.5 mussels per m2. By 2007, densities at these sites were significantly lower and ranged from 0.6 – 1.1 m2. Due to the large number of empty shells observed at the resurveyed sites, the decline in the number of live animals encountered is hypothesized to be driven by mortality. Given that the timing of mortality for these individuals can not be determined, it is not unexpected that a decrease in live mussels would not be closely matched by empty shells at all sites. The loss of shells could be caused by fluvial transport, streambed aggradation, and physical or chemical degradation. The size distribution of empty shells suggests that all size classes of mussels experienced mortality and the sudden abundance of empty shells did not reflect natural senescence among similarly aged individuals. At sites where surveys were initiated in 2007, both live mussels and empty shells were observed, but only one site was dominated by empty shells. Scratch marks on the shells and shell piles on the stream banks at this site

dominated by empty shells indicated that most of these mussels died from predation. These signs of predation were not apparent at the resurveyed sites located further downstream in the watershed, suggesting different causes of mortality at the resurveyed sites and at the newly established sites. Results from this survey suggest that *M. falcata* is nearly extirpated from the mainstem of Bear Creek downstream of Paradise Lake and highlights the need to search for causative factors for this decline.

Introduction

Given freshwater mussels' (Order Unionoida) imperiled conservation status in many locations **(**Strayer et al. 2004, USFWS 2007), their important role in some stream ecosystems (Jørgenson 1990, Vaughn and Hakenkamp 2001, Vaughn et al. 2004), and potential role as *in situ* bioindicators (Applied Biomonitoring 2004, Van Hassel and Farris 2007a, Van Hassel and Farris 2007b), freshwater mussel surveys are an important tool for understanding natural mussel distribution and potential anthropogenic effects on mussel occurrences. Surveys can assess population status by answering questions related to presence/absence, extent, and density; identify associated trends over time; assess the influence of anthropogenic and natural impacts; and provide understanding about freshwater mussel biology and ecology (Strayer 2003). Survey sampling designs range from complete coverage of a sample area, to probability-based sampling, to informal surveys. These survey designs differ based on survey goals, the target population, study site characteristics, resource availability, and *a priori* knowledge of the mussel population of interest (Strayer 2003). Complete coverage designs are resource intensive and often not feasible to implement on a larger scale, while informal surveys are often biased and cannot be used to draw inferences about an entire mussel population (Strayer 2003). Although freshwater mussels are relatively sedentary, there are other characteristics (such as patchy distributions over small and large spatial scales) that influence the selection of probability based sampling schemes (Strayer 2003, Stone et al. 2004).

Margaritifera falcata, the western pearlshell mussel, was historically common in western coastal streams and is the most common species found in forested watersheds (Toy 1998). While *M. falcata* have declined in the Puget Lowland Trough area, a population in Bear Creek, King County, WA (Figure 2.1), was still relatively intact in terms of mussel density, bed size, and presence of young mussels as recently as 2002 (Frest 2002). The first formal survey of this population was conducted in 1995. At this time, the population was estimated to be 290,940 mussels along a 340 m reach in Upper Bear Creek, and the average mussel density was 55 mussels/m^2.

Due to freshwater mussels' potential to reflect water quality and salmonid host population conditions, and out of the concern that the mussel population might be declining, Wild Fish Conservancy Northwest (formerly Washington Trout) initiated baseline monitoring of *M. falcata* populations in Bear Creek in 2002 (Washington Trout 2002). This baseline survey established bed size, mussel density, physical channel characteristics, and population age structure for 10 *M. falcata* beds located along approximately 5 km of Bear Creek (Washington Trout 2002). The Wild Fish Conservancy Northwest survey indicated that, in 2002, live mussel densities were highest in the upper watershed. The four most upstream beds averaged 57 mussels/m^2, while the lower 6 beds only had an average density of 0.24 mussels/m^2. In 2003, Wild Fish Conservancy Northwest resurveyed 5 of the ten original beds. While 2 of the 5 beds experienced a decrease in number of live mussels/m^2, the number of live mussels in the other 3 beds increased. However, it could not be determined if the observed decrease represented a real population change or if it was due to a sampling artifact (Washington Trout 2003).

In response to concerns that *M. falcata* appeared to be declining in Upper Bear Creek, the King County Water and Land Resources Division (WLRD) conducted mussel surveys in Upper Bear Creek and 2 other mussel-bearing creeks in King County in 2004 (Brenner 2005). Mussel density data from the 2004 Bear Creek survey suggested population

decline when compared to data from Toy (1998) and previous assessments conducted by Washington Trout (Washington Trout 2002, 2003, Brenner 2005). Different sampling techniques were used in each survey, complicating comparisons among studies. In 2002, Washington Trout surveyed individual beds and recorded densities of 110.52, 34.71, and 51.43 mussels/m^2. Toy (1998) and Brenner (2005) each conducted surveys along a stream reach in Upper Bear Creek. Since *M. falcata* distribution is often patchy (Strayer 2003, Stone et al. 2004), it is likely that these surveys covered areas with mussel beds and areas without mussels. Toy (1998) surveyed 340 m of stream length through the placement of a 0.25 m^2 sampling frame randomly placed along 34 transects and estimated a density of 55 mussels/m^2, while Brenner (2005) surveyed 100 m of stream, sampling 0.25 m wide transects placed at 5 m intervals and estimated a much lower density of 11 mussels/m^2

In addition to surveys, King County performed a limited mark-recapture study (n = 25) in Bear, Stossel, and Covington Creeks (Brenner, unpublished data). Mussels were deployed in 2004 and recaptured one year later. At Bear Creek, 15 of the 25 tagged mussels were recovered; 14 of which were empty shells. During this field visit in late September 2005, hundreds of empty shells were also observed, some of which still contained soft flesh, suggesting recent mortality. In September 2005, scientists from King County visited the survey site in Upper Bear Creek and observed that the site was dominated by hundreds of empty shells and few living mussels.

Although it was clear that a large number of *M. falcata* mussels have died at the Upper Bear Creek, the ratio of live to dead mussels has not been quantified. Additionally, it was unknown if *M. falcata* beds up- or downstream of this site are currently dominated by empty shells. The purpose of this survey is to compare existing conditions in Bear Creek to the baseline data collected by Wild Fish Conservancy Northwest in 2002 (Washington Trout 2003) to establish the spatial extent of heightened mortality along Bear Creek.

Since the individual fate of animals was not tracked over the 5 year period, comparisons of both live mussels and empty shells observed in each bed were used to infer mortality.

Methods

Study Location

Bear Creek is located about 32 km northeast of Seattle, WA, and drains 8,646 ha (Figure 2.1). Its headwaters originate at a maximum elevation of 192m and originate in a wetland complex. Bear Creek drains to the Sammamish River, near sea level. This subbasin is part of the Greater Lake Washington watershed. The Sammimish River drains to Lake Washington, and then to Puget Sound via locks that were constructed for boat passage.

Located on the urban-wild interface, the area around Bear Creek is experiencing some development as the nearby cities of Woodinville and Redmond grow. Currently characterized by primarily rural, low-density development, total impervious area has increased from 3.6% to 4.5% between 1991 and 2001 (Simmonds et al. 2004). The Bear Creek subbasin still has relatively intact aquatic habitat, however, fish passage barriers, loss of channel complexity and flow, increased sedimentation and poor water quality have degraded habitat conditions in some parts of the basin (WRIA 8 Steering Committee 2002).

Survey Methods

In July and September 2007, 10 freshwater mussel beds in Bear Creek that were previously identified in 2002 were resurveyed following the protocol developed by Wild Fish Conservancy Northwest (Washington Trout 2002). These beds cover approximately 5 km of Bear Creek (Figure 2.1). Beds were relocated by the use of both Global Position System (GPS) coordinates and through site reconnaissance with a member of the original survey team. In the original protocol established by the Wild Fish Conservancy and implemented in 2002, each mussel bed was identified by the presence of either live

mussels or empty shells. The length and average wetted width of the stream reach where each mussel bed was located was also measured. In 2002, the sampling protocol differed between sites based on whether or not a total count of all visible mussels in the bed was feasible. Sites with greater than 50 live mussel or empty shells employed transect-based sub-sampling, while whole-bed visual surveys were used at sites with fewer than 50 live mussels or empty shells. In the 2007 survey, bed size was determined by measuring the average wetted width of each site, and calculating the length necessary to replicate the previously monitored area as closely as possible.

The 2002 protocol surveyed sites with greater than 50 live mussels or empty shells were surveyed along 3 evenly spaced transects placed perpendicular to the stream flow. The spacing between transects depended on the length of the mussel bed. Mussels were enumerated along these transects using 1/16 m^2 (0.25m by 0.25m) quadrats. Transects were surveyed across the wetted-width of the stream channel from left-bank to right-bank. In each quadrat all live mussels and pairs of empty shells were counted and live mussels were measured along the longest axis to the nearest mm using vernier calipers. All live mussels and empty shells were then returned to their original location. The 2007 survey methods matched the 2002 protocol, except that length along the longest axis was determined for both live mussels and empty shells. In instances were some of the shell had eroded, the total length was estimated. Also, water depth measurements were taken at set intervals along the transects in 2002, but in 2007 no water depth measurements were made. At least 50 live mussels or empty shells had been present in beds 1,2,3,4, and 8 (Figure 2.1) in 2002. Transect-based sub-sampling was employed at these sites again in 2007.

At sites with fewer than 50 mussels, the entire stream bed was scanned for visible live mussels and pairs of empty shells using a viewing box (a plastic box or bucket with a plexiglass bottom. In 2002, the location of live and dead mussels relative to the midline of the bed was recorded. In 2007 a map showing mussel locations was sketched instead.

In 2002, water depth was characterized by measuring the water depth at set intervals along a measuring tape that was extended across a representative area of the bed, but no depth measurements were made in 2007. As in the transect-based protocol, total length measurements were only made for live mussels in 2002, but were along made for empty shells in 2007. In 2002, less than 50 live mussels or empty shells were observed in beds 5,6,7,9, and 10 (Figure 2.1) and whole-bed surveys were conducted at these sites in 2007.

In 2007 three additional survey sites upstream of Paradise Lake were established. A more comprehensive survey of the area upstream of Paradise Lake was not initiated because of land-access, lack of suitable habitat, and time constraints. The three additional sites lacked established baseline data, however surveying these sites enabled analysis of the distribution of mortality in an area upstream of Paradise Lake and the reach of upper Bear Creek where significant mortality had been observed (Figure 2.1). At these 3 newly established sites located within the Paradise Valley Natural Area (PVNA), a visual scan of the area indicated that there were less than 50 mussels present. Therefore, the whole-bed visual search protocol was used. Much of the aquatic area within the PVNA consists of a stream-wetland complex with multiple beaver dams. Depositional areas were not included in the survey, thereby limiting the total area surveyed to 114 m^2. The three survey sites in the PVNA were relatively close to each other: PV1, PV2 and PV3 (Figure 2.1). A large pool, road and culvert separated site PV1 from PV2. Sites PV2 and PV3 were contiguous, but site PV3 consisted of a deeper run downstream of a beaver dam where siltation was beginning to occur. PV3 was dominated by empty shells that appeared to have been transported by a predator, and therefore was considered separately from site PV2 since mussel mortality appeared to be caused by different processes.

Statistical Analysis

At sites that employed quadrat-based sampling, differences between mussel densities in 2002 and 2007 were tested using the Wilcoxon Rank non-parametric two-sample tests. A

non-parametric test was used because mussel densities per quadrant were not normally distributed. The Kolmogorov-Smirnov Test of Composite Normality was used to test for a normal distribution of shell lengths for live mussels and empty shells. These statistical tests were conducted with S-Plus 8.0 statistical software (TIBCO Software Inc, Palo Alto, CA).

Results

While the area of each identified *M. falcata* bed was similar in size in 2002 and 2007, more quadrats were surveyed in 2007, suggesting that wetted width had increased since 2002 at many locations and resulted in a larger search area in 2007 for quadrat-based surveys (Table 2.1). There was less variation at sites where the entire bed was surveyed than at sites that employed transect based sampling.

Four of the 5 survey sites where quadrat-based sampling was employed experienced a significant decrease in mussel density between 2002 and 2007 ($p<0.001$) (Table 2). The magnitude of change was statistically significant in the 4 most upstream beds which had the highest mussel densities in 2002 (< 30mussels per m^2). By 2007, densities at these sites were less than 1.2 m^2 (Table 2.2). The only sub-sampled site with no significant change in mussel density was the most downstream bed, site 8, where no mussels were detected in 2002 and 2 were found in 2007.

At sites where whole-bed surveys were conducted in 2007, no live mussels were observed in 4 of the 5 sites (Table 2.3). Two of these 4 sites had very low numbers of live mussels in 2002. The only whole-bed survey site with live *M. falcata* was the most downstream bed at site 10, which changes from 1 live mussel in 2002 to 10 live mussels in 2007

The 6 most upstream sites that had the most live mussels in 2002 were dominated by empty shells in 2007 (Figure 2.2). Between 2002 and 2007, the decline in live mussels

was generally matched by an increase in pairs of empty shells (Figure 2.3). The site with the largest discrepancy in total (live + empty shells) mussels observed at each bed between 2002 and 2007 was site 3. At this site, the increase in empty shells only accounted for approximately half the decrease in the number of live mussels observed between survey years.

In the beds located upstream of Paradise Lake that were within close proximity, there was great variation among sites in the ratio of live mussels to pairs of empty shells (Figure 2.4). The most downstream bed (PV3) was dominated by live animals, the most upstream bed (PV1) was dominated by empty shells, and site PV2 was composed of 60% live mussels and 40% empty shells. At site PV1, the majority of empty shells of the mussel shells were lying in piles on the stream bank or had scratch marks, suggesting mortality was caused by predation (Table 2.4).

The size range of mussels was similar for live organisms and empty shells, both up- and downstream of Paradise Lake (Figures 2.5 and 2.6). The distribution of size classes of empty shells observed downstream of Paradise Lake was normally distributed (ks = 0.0446, p = 0.0001). Live mussels, and all mussels upstream of the lake are subject to low sample size issues, therefore their size class distribution was not clear (ks = 0.0835, p = 0.5). The smallest and largest empty shells encountered were located downstream of Paradise Lake, while the smallest and largest live mussels were encountered upstream of Paradise Lake. The smallest *M. falcata* empty shell was 19.4 mm long and the largest shell was 123.4 mm long. The smallest live *M falcata* individual was 29 mm long and the largest live individual was 121.7 mm long.

On December 6th, 2007 PV3 was visited in association with a caged mussel relocation experiment at this site (see Chapter III). This site visit occurred 3 days after a large storm event that delivered approximately 3.5 inches of rain to the area in 24 hours (King County WLRD 2008). This was particularly intense precipitation for the region. Clean

cobbles of approximately the same size had been transported to the site (the bed had visibly aggraded and cages on site (see Chapter III) had been buried. Many *M. falcata* occupied this area of cobble-deposition. A quick count enumerated 104 mussels within a ~4.9m^2 area (21.3 mussels/m^2).

Discussion

Between 2002 and 2007 the *M. falcata* population in Bear Creek experienced a significant decline in the area downstream of Paradise Lake. The 2007 survey indicated the maximum *M. falcata* density was 1.2 mussels/m^2, and only 32 live *M. falcata* mussels were observed. All sites that previously supported a relatively healthy mussel population in 2002 were dominated by empty-shells in 2007. Statistically significant decline was observed in 4 of the 5 sites which employed sub-sampling methods. At the remaining 5 sites, statistical comparisons were not made because the areas were not sub-sampled, but decreases were observed in 4 of the 5 beds. The only sites where a decline was not observed were in the lower section of the creek where mussel densities were low in 2002; at sites 8 and 10.

While the fate of individual mussels was not tracked between 2002 and 2007, the shift in composition in beds from live mussels to empty shells suggests high mortality at these sites. Mortality is inferred, but is not directly demonstrated, since mussels had not been individually marked in 2002, nor had the decrease in live mussels been precisely matched by an increase in empty shells. The change in total number (live mussels plus pairs of shell halves) of mussels encountered between 2002 and 2007 was lower at sites which employed transect based sampling than those sites with whole bed surveyed. In general, mussel counts were lower in 2007 than 2002. Given that the timing of apparent mortality for these individuals is unknown, and could have occurred several years ago, it is not unexpected that a decrease in live mussels would not be matched by an increase in the number of empty shells. Fluvial transport, streambed aggradation, and physical or chemical degradation could partially explain the discrepancies in total mussel counts

between 2002 and 2007. In 2003, half of the sites surveyed in 2002 were resurveyed. Comparisons revealed that the range of change observed in mussel density among transect-based surveys was -26.3 to 41.1 for live mussels /m^2 and 6.1 - 17.7 for shells/m^2 (Washington Trout 2003). These ranges indicate a magnitude of error plausible for transect based sampling employed in this survey. Between 2002 and 2007, changes in both live mussels and empty shells were well above this range, proving another line of evidence that changes detected represented mortality, but highlighting that the exact degree of mortality can not be quantified given the variability of the survey technique. Mark-recapture survey techniques are the best method to estimate survival for the population as a whole because this survey technique documents the fate of individual mussels (Strayer 2003). However, results from this study illustrate the fixed-bed surveys in which live mussels and empty shells are enumerated can be useful in detecting mortality, particularly if probability based sub-sampling is employed.

Downstream of Paradise Lake, empty shells were found in the stream bed, not on the stream banks. Some of these shells were partially buried and oriented upright, and even appeared alive before close examination. This observation of shell location and orientation is consistent with earlier observations of empty shells in Bear Creek (Fevold and Vanderhoof 2001) and suggested that the animals may have died in place and not as a result of predation. Previous research has also failed to demonstrate that predation was the primary cause of mortality. Limited predation reconnaissance conducted in 2003 at site 2 through the use of a Trail Master© infrared trail monitor linked to a camera did not document predation (Washington Trout 2003).

The greatest number of live mussels were found upstream of Paradise Lake. While one bed upstream of Paradise Lake was dominated by *M. falcata* shells, many of these shells were piled onto the bank and/or had scratch-marks. As such, predation is hypothesized to be the cause of mortality. Using a bed-wide visual search, only 41 mussels were encountered at all three of the sites upstream of Paradise Lake. However, this count

likely under represented the number of mussels at this location as revealed by the opportunistic count of 104 mussels in December 2007. The discrepancy between the summer 2007 survey and casual observations in December 2007 highlights the limitations of whole bed, visual surveys. Consistent search effort is more difficult to ensure in whole bed surveys, especially relative to quadrats which help ensure that that the entire sampling area is completely and consistently searched (Strayer 2003). Additionally, a portion of a freshwater mussel population is often buried, requiring sediment excavation to achieve an exhaustive accounting of the population (Strayer 2003). Visual mussel estimates often constitute the target population for freshwater mussel surveys, but the ratio of buried to exposed mussels will vary over time and space and should be considered when comparing different areas or the same area at different times. Another weakness of whole bed surveys is that there is no sampling error to estimate, despite the reality that the whole bed count is imprecise because not all mussels will be encountered (Strayer 2003). In whole bed surveys, estimate ranges cannot be calculated to inform the likely range of population size, because the proportion of missed mussels in unknown. The only sampling design recommended for population density or detecting an impact is probability-based sampling in which the sampling units (i.e.: quadrats) are excavated (Strayer 2003). The results of this study, especially the surveys in Paradise Valley, highlight the limits in whole bed visual surveys- sites that employed transect-based sampling tended to have less variation in the total number of individuals (live mussels or pairs of dead shells) encountered.

Size distributions for both live mussels and empty shells provided no indication of an association between mortality and size. The size distribution of empty shells was similar to the previously reported distribution of live mussels. (Toy 1998, Washington Trout 2002). In addition, results from the survey revealed the presence of empty *M. falcata* shells of sexually immature mussels based on field measured shell lengths and a size to sexual-maturity relationship previously established for this population (Toy 1998). It is clear from this survey that mussels across all sizes were subject to mortality. From the

size distributions of empty shells, it can be inferred that the sudden abundance of empty shells does not reflect natural senescence among similarly aged individuals.

Conclusion

Between 2002 and 2007 the *M. falcata* population in Bear Creek experienced a significant population decline downstream of Paradise Lake. The 2007 survey indicated that the maximum *M. falcata* density was estimated to be 1.2 mussels per m^2, and only 32 live *M. falcata* mussels were observed. All sites where previously high mussel density had been observed in 2002 were dominated by empty-shells in 2007, suggesting mortality. The size distribution of shells suggested that all mussel size classes experienced mortality and that the sudden abundance of empty shells does not reflect natural senescence among similarly aged individuals. In the area above Paradise Lake where surveying was initiated in 2007, both live organisms and empty shells were observed. Scratch marks on the shells and shell piles on the stream banks indicated that many of these mussels in the area upstream of Paradise Lake likely were subject to predation. The location and orientation of upstream shells differed from those downstream of Paradise Lake, where many mussels appeared to have died in place in the stream bed. The casual observation of more than 100 *M. falcata* at one of the survey sites, when about 20 mussels were encountered during the earlier survey, illustrates that whole-bed surveys conducted on only one occasion may have high associated error. While population conditions upstream of Paradise Lake are less certain, results from transect-based surveys conducted downstream of Paradise Lake indicate a significant decline in the *M. falcata* population with mortality of both sexually mature and immature animals. These results call attention to the need to investigate the cause of mortality of these animals.

Tables and Figures

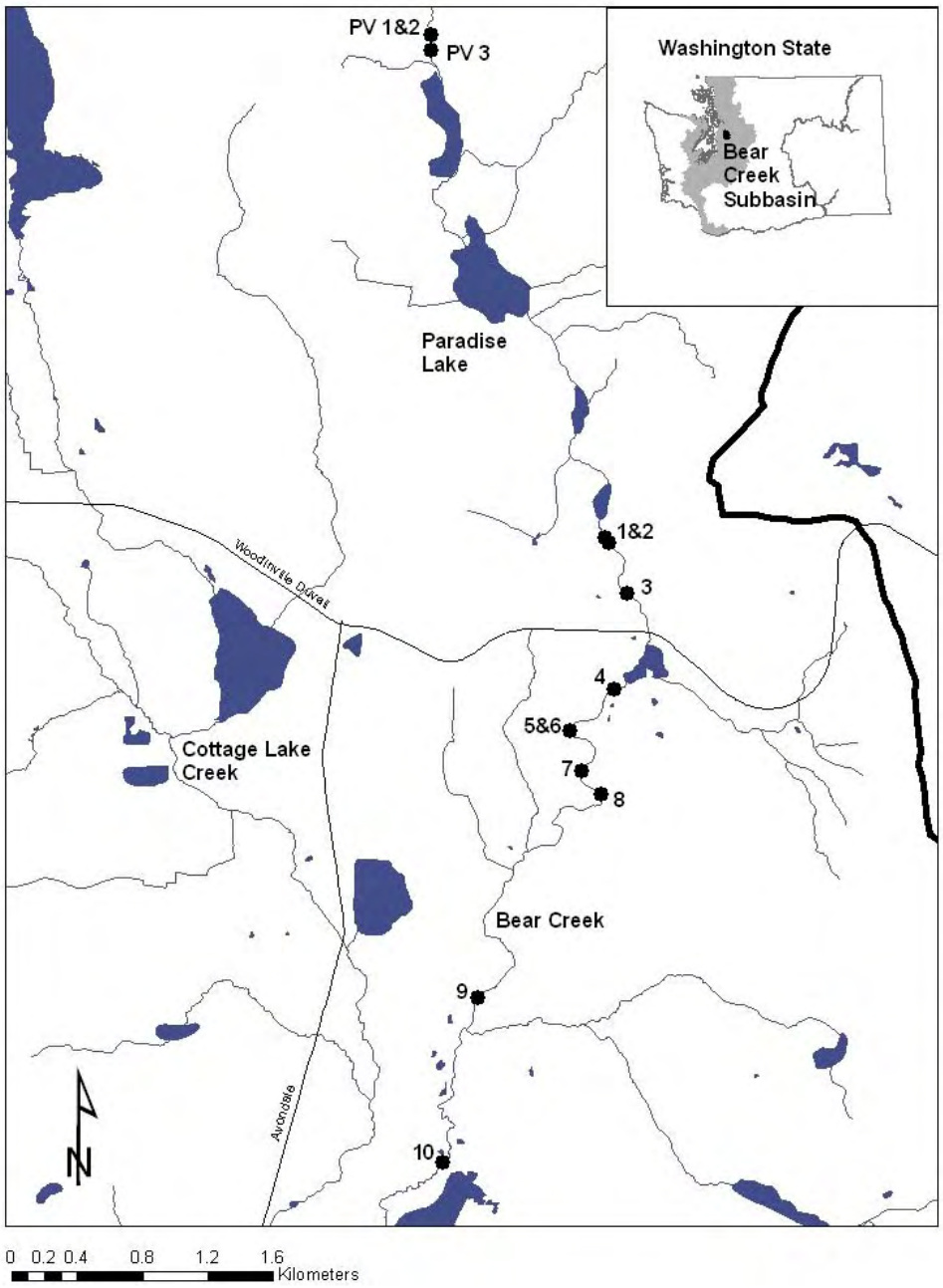


Figure 2.1 Location of *M. falcata* mussel beds along Bear Creek surveyed in 2007 with the NW border of the subbasin illustrated with a thick-black outline. The general location of the Puget Trough is displayed in the inset map.

Table 2.1 Area sampled at each bed site for 2002 and 2007 *M. falcata* survey

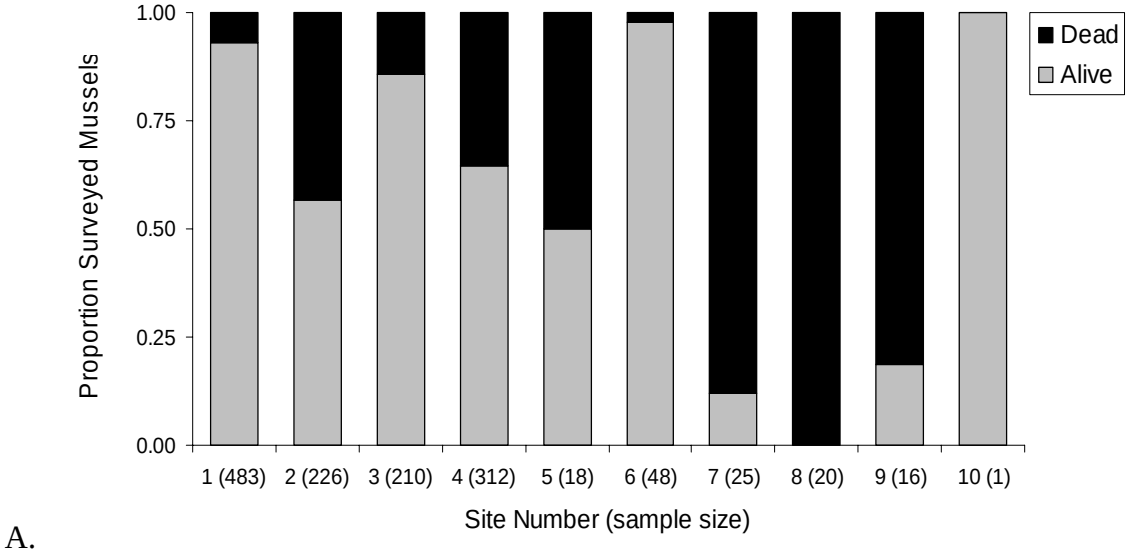
Bed #	Bed Habitat Area in 2002 (m^2)	No. of Quadrats sampled in 2002	Area sampled 2002 (m^2)	Bed Habitat Area in 2007 (m^2)	No. of Quadrats sampled in 2007	Area sampled 2007 (m^2)
1	69	65	4.1	78	98	6.1
2	68	59	3.7	71	81	5.1
3	73	56	3.5	60	65	4.1
4	743	107	6.7	624	111	6.9
5	100	n/a	100	105	n/a	105
6	37	n/a	37	37	n/a	37
7	49	n/a	49	44	n/a	44
8	80	78	4.9	89	99	6.2
9	181	n/a	181	218	n/a	218
10	134	n/a	134	171	n/a	171
PV1				26	n/a	25.7
PV2				15	n/a	14.8
PV3				73	n/a	72.8

Table 2.2 Comparison of 2002 (Washington Trout 2002) and 2007 *M. falcata* quadrat-based surveys and Wilcoxon Rank non-parametric two-sample test results

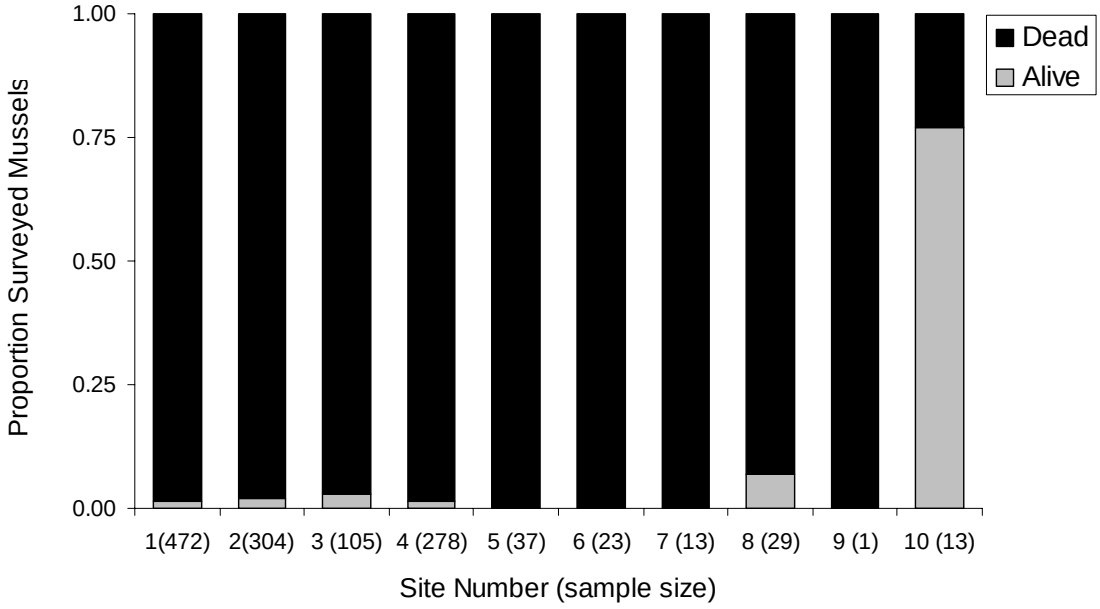
Bed	Total Live 2002	Total Live 2007	2002 Average Quadrat Density (mussels per m^2)	2007 Average Quadrat Density (mussels per m^2)	Z-value	p value
1	449	7	110.5	1.2	9.83	0
2	128	6	34.7	1.2	6.1997	0
3	180	3	51.4	0.7	6.1894	0
4	201	4	30.1	0.6	8.114	0
8	0	2	0.00	0.3	-1.2508	0.211

Table 2.3 Comparison of 2002 (Washington Trout 2002) and 2007 *M. falcata* whole-bed visual surveys

Bed	Total Live 2002	Total Live 2007	2002 Bed Density (mussels/m2)	2007 Bed Density (mussels/m2)
5	9	0	0.09	0.00
6	47	0	1.27	0.00
7	3	0	0.06	0.00
9	1	0	0.01	0.00
10	1	10	0.01	0.06



A.



B

Figure 2.2 Proportion of live and dead (represented by empty shell pairs) *M. falcata* detected by A) 2002 surveys and B) 2007 surveys.

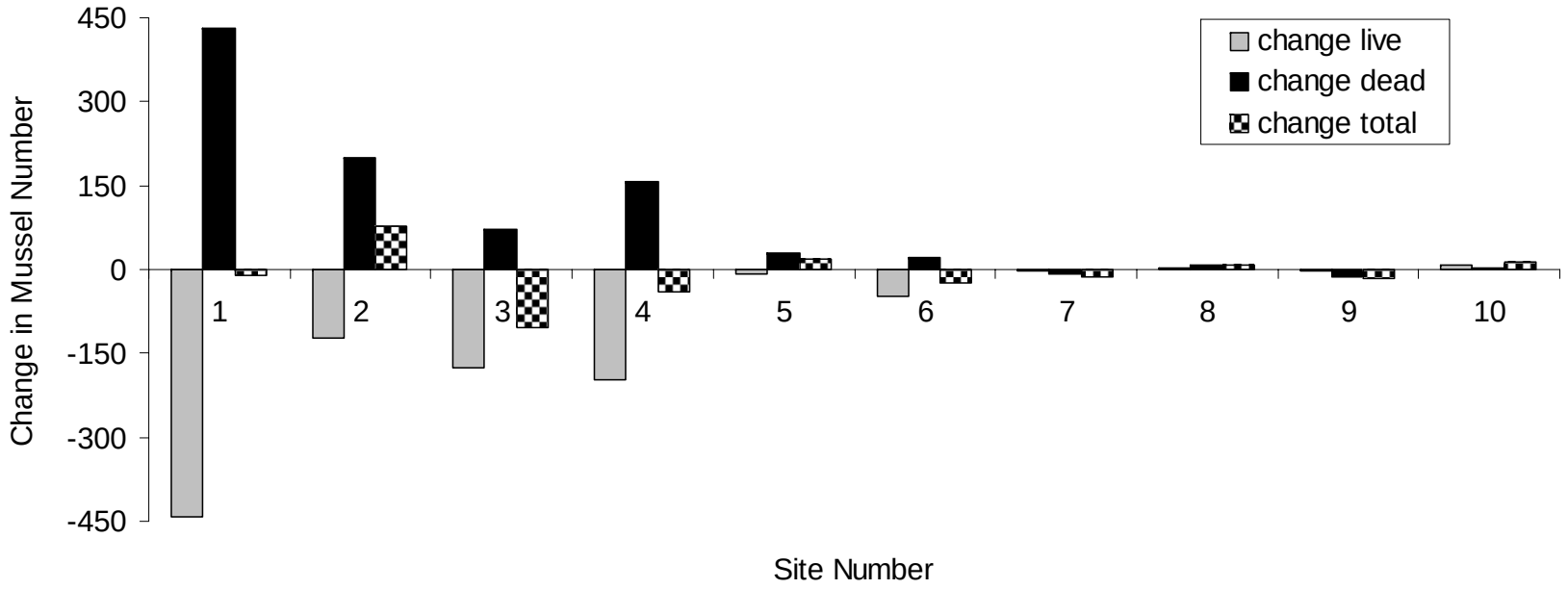


Figure 2.3 Change in live mussels and dead mussels (represented by empty shell pairs), and total number of mussels (live mussels plus pairs of shells) detected at each *M. falcata* site between 2002 and 2007 surveys.

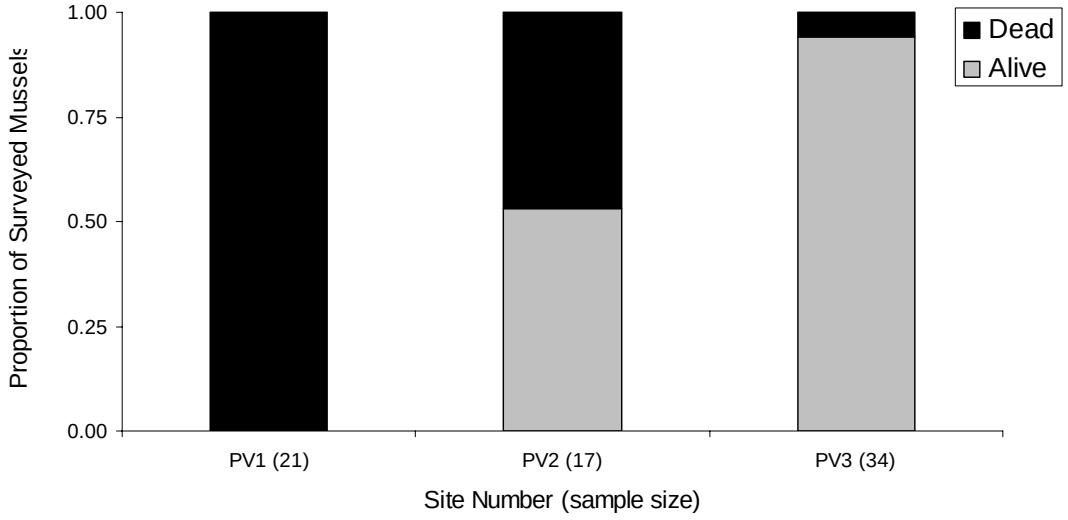
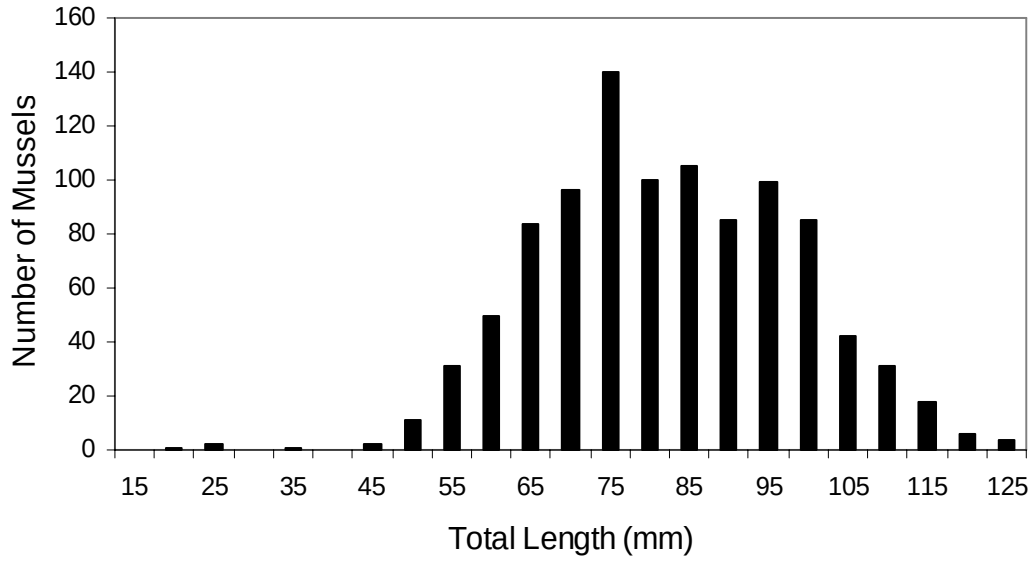


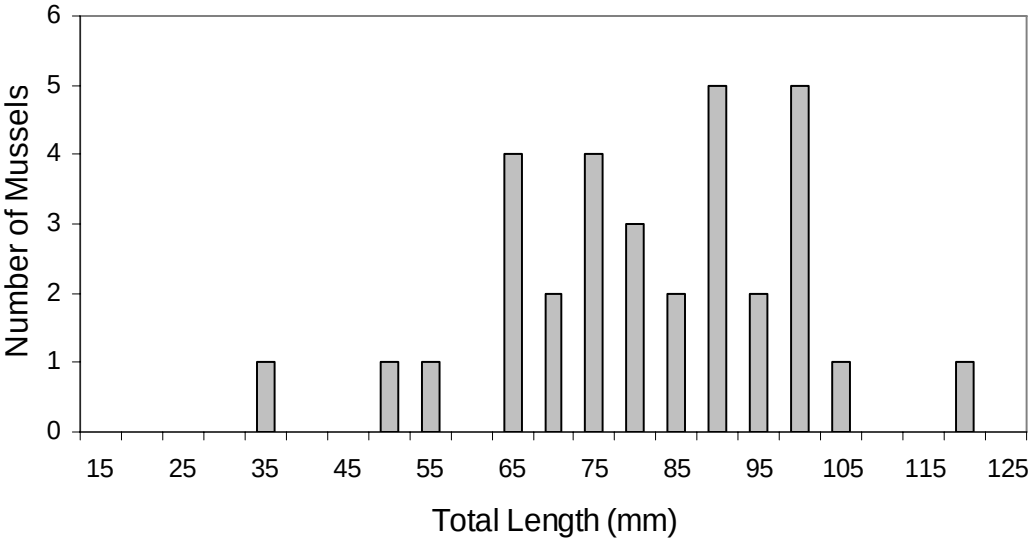
Figure 2.4 Proportion of live and dead (represented by empty shell pairs)*M. falcata* detected at three sites upstream of Paradise Lake in 2007.

Table 2.4 Percent of *M. falcata* shell pairs that had indications of predation such as scratch marks at surveyed beds located upstream of Paradise Lake.

Site	Dead (#)	Indication of Predation (%)
PV1 (21)	21	61.9
PV2 (17)	8	25.0
PV3 (34)	2	50.0



A.



B.

Figure 2.5 Size distribution of A) all dead (pairs of empty shells) *M. falcata* (ks = 0.0446, p = 0.0001) and B) live *M. falcata* (ks = 0.0835, p = 0.5) detected during mussels surveys at the 10 sites downstream of Paradise Lake.

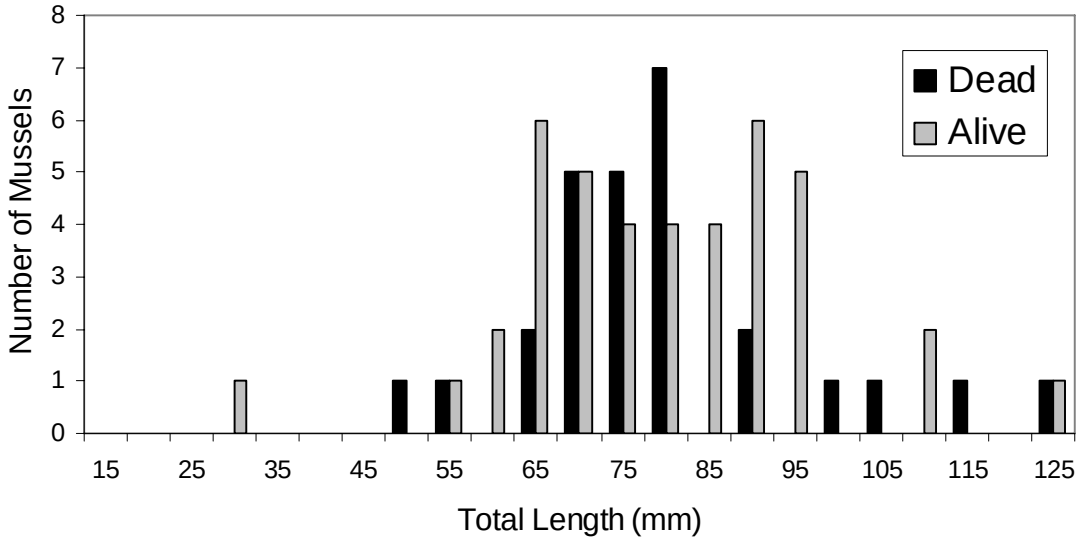


Figure 2.6 Size distribution of all dead (pairs of empty shells) (ks = 0.2197, p = 0.0017) and live (ks = 0.0813, p = 0.5) *M. falcata* detected during mussels surveys at the 3 sites upstream of Paradise Lake.

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Chapter III: A caged mussel relocation experiment to investigate *Margaritifera falcata* mortality in a Puget Lowland stream.

Summary

The western pearlshell mussel, *Margaritifera falcata*, was historically the most common freshwater mussel species in streams in forested watersheds in the Pacific Northwest. Upper Bear Creek in King County, Washington supported an apparently healthy and stable population of freshwater mussels as recently as 10 years ago. However, multiple surveys indicate that the mussel population at the site experienced a marked decline. An *in situ* caged mussel sentinel transplant study was initiated at 2 Bear Creek sites. One site was located downstream of a lake along Bear Creek, Paradise Lake and the other site was located upstream of Paradise Lake. A third site located on Cottage Lake Creek served as a control site. Mussels were moved to the downstream site along Bear Creek and to the control site in both winter, 2006 and spring, 2007, but were only transplanted to the upstream site along Bear Creek in spring, 2007. Relocated mussels were routinely checked for mortality, and were examined using histology, the study of tissue morphology, and glycogen levels to evaluate their condition. Additionally, Paradise Lake was screened for toxicity using a fathead minnow laboratory test, and water used for this testing was examined for algae known to be toxic. Results showed that *M. falcata* relocated to the site on Bear Creek downstream of Paradise Lake experienced approximately 40% mortality by early December 2007, with the onset of mortality in early September, 2007. Both sets of mussel cages that had been relocated to the site in late November/early December 2006 or in early May 2007 experienced mortality. Mortality was not observed among mussels relocated to the upstream site on Bear Creek (above Paradise Lake), nor at the control site on Cottage Lake Creek. Toxicity was detected in the fathead minnow tests for Paradise Lake water at generally the same time as the initial onset of *M. falcata* mortality at the downstream site. No algae commonly known to be toxic were observed. While there is an indirect spatial and temporal-link

between patterns *M. falcata* mortality along Bear Creek and Paradise Lake toxicity, the link between conditions at Paradise Lake and the downstream relocation site has not been explicitly tested. Lower glycogen levels were observed in *M. falcata* individuals with visibly weakened adductor muscles, and differences in the digestive gland were noticed in dead mussels collected downstream of Paradise Lake, but neither monitoring glycogen nor tissue morphology provided an early-warning indication that the BC-DS relocated mussels were stressed. The apparently good condition of the tissue morphology of some mussels collected after death, especially among the digestive gland epithelial cells, suggested that mortality was relatively rapid in mussels collected in September and October and there was not a prolonged pathological sequence of events leading to mortality. Additional investigation of the surviving mussels may provide additional information about the unknown stressor and the mechanism of mortality.

Introduction

Freshwater mussels are among the most imperiled taxa in the United States; out of the 300 species recognized in the United States, 70 are listed as federally endangered or threatened (USFWS 2007a). Despite this imperiled conservation status, much of our understanding of the causes of mussel decline are based on anecdotal observations and hindered by a lack of experiments targeting the mechanisms driving decline (Strayer et al. 2004).

The western pearlshell mussel, *Margaritifera falcata,* was historically the most common freshwater mussel species in streams in forested watersheds in the Pacific Northwest (Toy 1998). Locally they have become extirpated in many areas, including streams in the urbanizing Puget lowlands (Figure 3.1) (Frest 2002, Pacific Northwest Freshwater Mussel Workgroup pers. comm. 2007). A host of factors are thought to have contributed to the general decline of freshwater mussel populations and include water quality degradation, changes in hydrological regime, high sediment loading, channel and

corresponding habitat simplification, dams and impoundments, Allee effects in reproduction, and changes in fish host distributions (Allee et al. 1949, Frest 2002, Strayer 2003, Box et al. 2006, Pacific Northwest Freshwater Mussel Workgroup pers. comm. 2007).

Upper Bear Creek in King County, Washington historically supported an apparently healthy and stable population of freshwater mussels as recently as 10 years ago (Toy 1998, Frest 2002). Additionally, freshwater mussel middens in this area provide archeological evidence that this site historically supported a stable *M. falcata* population (Wong 1993, Vannote and Minshall 1982). However, by 2004 the mussel density at this site (11 mussels / m^2) had declined, and in early fall, 2005, hundreds of empty shells, some still containing soft flesh were observed (Brenner 2005, B. Brenner pers. comm. 2007). In 2006, Hastie and Toy (2008) resurveyed upper Bear Creek and estimated that mussel density declined from 56.0 to 6.9 mussels / m^2 over the 11 year period.

As discussed previously in chapter II, in 2007, surveys were conducted in 10 *M. falcata* mussel beds located along approximately 5 km of Bear Creek, in King County, Washington, for which there were baseline data from 2002. Three additional sites were added to the 2007 survey, because the baseline surveys did not examine beds upstream of Paradise Lake (Figure 3.1). Results from this survey indicated that between 2002 and 2007, the *M. falcata* population experienced a drastic decline in mainstem Bear Creek downstream of Paradise Lake. All sites that initially had the highest mussel density in 2002 (.0 – 110.5 mussels per m^2) were dominated by empty-shells in 2007. The size distribution of empty shells suggested that all size classes of mussels experienced mortality and that the sudden abundance of empty shells did not reflect natural senescence among similarly aged individuals. In the area above Paradise Lake where surveying was initiated in 2007, both live mussels and empty shells were observed. Scratch marks on the shells and shell piles on the banks indicated that many of the mussels in the area upstream of Paradise Lake were subject to predation. These

observations of shell location and orientation differed from conditions downstream of Paradise Lake, where many mussels appeared to have died in place in the stream bed. Results from these surveys indicated that there was a need to investigate the cause of mortality of these animals and to test whether the cause of mortality did indeed differ upstream and downstream of Paradise Lake.

As part of the assessment conducted by King County (Brenner 2005) in November 2004, 4 live, but apparently sick mussels and 4 dead mussels were collected and submitted for histological analysis. The histology assessment revealed that the Bear Creek animals had distinctly different tissue morphology than animals collected from a different Puget Lowland creek, Stossel Creek. *M. falcata* collected from Bear Creek were characterized by sloughed gill epithelium, low to very low digestive gland epithelium height, and bacterial colonies in association with the gills (Elston 2005). It was recommended that examination of healthier mussels be conducted in order to determine a sequence of pathological events that may lead to mortality.

While the specific stressors associated with urbanization that influence mussel decline are not well-understood, preliminary investigations at Bear Creek do not link basin development to the sudden and severe *M. falcata* observed mortality. Water and sediment quality monitoring, in addition to bioaccumulation studies have failed to detect any contaminant at a level known to be harmful (Applied Biomonitoring 2004, Lester 2007). While biological impacts have been observed at very low levels of contamination (i.e., behavioral responses of juvenile coho salmon to low levels of copper (2 µg/L)), (Sandahl et al. 2007) and shifts in the species community composition have been observed along a forested to urban gradient (Morley and Karr 2002), specific die-offs of aquatic species caused by changes associated with low density development have not been well documented. Riparian and instream habitat do not appear to have changed between 1995 and 2006 (Hastie and Toy 2008).

Since it is hypothesized that mortality is likely driven by different phenomenon in *M. falcata* beds located downstream versus upstream of Paradise Lake, conditions at Paradise Lake warrant a closer look. Paradise Lake is a 7.3 ha eutrophic lake that drains 975 ha and generally has good water quality (King County Small Lakes Program 2008). This lake is routinely monitored by King County as part of the King County Small Lakes Program. Cyanobacteria, the harmful algal group most common in freshwater systems, is typically only present at low densities in this lake. Routine phytoplankton monitoring of Paradise Lake in 2005 indicated that an unknown Chrysophyte, not previously identified in other small lakes monitored by King County was the dominant algal species in the lake (S. Abella pers. comm. 2006). While toxicity among freshwater microalgae other than cyanobacteria is rarely observed, it is not unreasonable to expect that other freshwater microalgae may be toxic, especially those taxa with toxic marine counterparts (World Health Organization 2003). *Chrysochromulina parva* was implicated in a fish kill in a small Danish Lake with sewage contamination and high pH (Hansen et al. 1994) and *Euglena sanguinea* in the mortality of catfish in an aquaculture facility (Zimba 2004).

In situ caged mussel bioassays are often used for biomonitoring because mussels bio-accumulate chemicals and reflect integrated and cumulative exposures to contamination, and can be deployed in the field to target site-specific conditions (ASTM 2001). Traditional endpoints in toxicity testing including mortality, growth, and reproductive success, but growth metrics can be difficult to assess in some species of freshwater mussels with naturally slow growth rates. Given that changing environmental conditions in general can affect performance, growth, or reproductive output (Dahlhoff 2004), endpoints similar to those employed in toxicity studies can be used to evaluate stress on a more general level. While traditional measurements of growth are generally not sensitive in Unionid mussels, glycogen monitoring has the potential to provide a growth related, sub-lethal stress indicator that can be used as a tool to monitor general animal health. Glycogen is the primary energy store in bivalves and often considered to be an indicator of condition (Naimo and Monroe 1999, Patterson et al. 1999).

In order to better understand the cause of mortality, an *in situ* caged-mussel sentinel study was initiated in which healthy *M. falcata* from a nearby stream were relocated to Bear Creek and monitored for signs of disease and mortality. Given evidence that mortality is driven by different phenomena in *M. falcata* beds located downstream versus upstream of Paradise Lake, Fathead minnows were used to screen for a potential algal toxin. The mussel relocation experiment and lake toxicity screening aimed to answer the following questions:

1. Do healthy *M. falcata* mussels become diseased when relocated to Bear Creek?
2. If healthy mussels become diseased after relocation, do mussels placed both up- and downstream of Paradise Lake show similar symptoms?
3. If healthy mussels become diseased after relocation, what is the timeline for the onset of disease and is there a pathological sequence of events that leads to mortality?
4. If disease is observed, what is the pathological agent causing disease?
5. Could an algal toxin originating in Paradise Lake could be contributing to *M. falcata* mortality downstream?

Methods and Materials

Study Location

Bear Creek is located within the Puget Sound Lowlands, about 32 km northeast of Seattle, WA, and drains 8,646 ha. Its headwaters originate at a maximum elevation of 192 m and originate in a wetland complex. Bear Creek flows for over 19 km before draining to the Sammamish River, near sea level (Lee 2008). Cottage Lake Creek, within the Bear Creek subbasin, flows for just under 11 km before joining Bear Creek. The Bear Creek subbasin is part of the Greater Lake Washington watershed. The Sammamish River drains to Lake Washington, and then to the Puget Sound via locks that were constructed for boat passage.

Puget lowland topography is strongly influenced by erosion and deposition associated glacier advance and retreat during the Vashon stade of the Frasier glaciation about 18-15,000 years ago (Booth et al. 2003). Soils in the study area are dominated by Alderwood gravelly-sandy loam and underlain by relatively impermeable till (NRCS 2008). The predominant vegetation types are Douglas-fir (*Pseudotsuga menziesii*) / western hemlock (*Tsuga heterophylla*) / western red cedar (*Thuja plicata*) forest and scrub-shrub and Sitka spruce (*Picea sitchensis*) forested wetland areas.

Located on the urban-wild interface, the area around Bear Creek is experiencing development as populations in the nearby cities of Woodinville and Redmond increase. The Bear Creek subbasin still has relatively intact aquatic habitat, however, fish passage barriers, loss of channel complexity and flow, increased sedimentation and poor water quality have degraded habitat conditions in parts of the drainage (WRIA 8 Steering Committee 2002).

Experimental Approach

A caged-mussel sentinel study was employed, where naïve mussels (mussels from a currently healthy population) were relocated to Bear Creek. First a visual survey and health reconnaissance established whether mussels from Cottage Lake Creek could serve as a source population. Once mussels were relocated to treatment sites along Bear Creek, and a control site on Cottage Lake Creek, they were monitored for evidence of disease, including; changes in tissue morphology (histology), glycogen level, and mortality.

For histological analysis, each mussel was removed from its shell and a cross-section of tissue that contained foot, digestive gland, mantle, and gill was placed in Invertebrate Davidson's Solution (Shaw and Battle 1957) for 24 hours and processed for routine paraffin histology (Luna 1968). De-paraffinized 4 µm sections were stained with hematoxylin and eosin and viewed by bright field microscopy.

In addition to monitoring the condition of the relocated mussels, environmental conditions, including water temperature, pH, conductivity, and alkalinity were monitored at each relocation site. Paradise Lake was screened for evidence of an algal toxin through a lab-based screening level fathead minnow toxicity test.

Cottage Lake Creek Reconnaissance and Health Screening

In September, 2006 a visual survey of *M. falcata* was conducted along a reach of Cottage Lake Creek to estimate the population size and determine the number of animals that could be removed for the mussel relocation study without negatively affecting the existing population. This survey targeted stream reaches that supported previously identified mussel beds based on observations by biologists conducting habitat assessment surveys and was restricted to areas where property access could be obtained.

Surveyors walked the streams and counted mussels visible on the stream bottom. In deep areas or in water with low visibility due to turbidity or surface glare, a snorkel mask was used to observe mussels. Stream length was measured with a cloth meter tape. Additionally, surveyed stream reach lengths were geo-referenced with a Garmin GPS unit (UTM, NAD1983). Mussel survey effort is expressed as meters of stream surveyed per unit hour. The total stream area surveyed was 647.1 m**.** This area was not continuous, but contained 4 distinct sections.

Fifty-nine individuals were collected from the Cottage Lake Creek population to screen for infectious disease and insure no risk of infection to Bear Creek fauna through the relocation process. Histology slides were examined by trained pathologists for evidence of disease.

M. falcata Relocation

The *M. falcata* relocation experiment was conducted in 2 phases (Table 3.1). Phase 1 was initiated in November and December 2006 when mussels were relocated from their source site in Cottage Lake Creek, to a treatment site 5.5 km downstream of Paradise Lake in Bear Creek, "BC-DS" (Figure 3.1) where significant mortality had been previously observed. Mussels were also relocated from the Cottage Lake Creek source site about 80 m upstream and represented the study control location, "CL-C" (Figure 3.1). No native *M. falcata* were observed at this location, but the habitat appeared suitable, as substrate, flow, and riparian cover was similar to a downstream reach were *M. falcata* were present. Phase 2 began in early May, 2007. For Phase 2, additional mussels were relocated to BC-DS and CL-C, and another site was established in Bear Creek, upstream of Paradise Lake, BC-US (Figure 3.1). BC-US was located approximately 1.4 km upstream of Paradise Lake in the Paradise Valley Natural Area. This site was chosen because *M. falcata* had previously been observed in this area and overall habitat conditions were of good quality and similar to those at the relocation site downstream of Paradise Lake. The catchment area upstream of the Cottage Lake Creek control site is more than twice that draining to the BC-DS site; 2900 ha versus 1241.2 ha, respectively. The BC-US site was located about 6.9 km from the BC-DS site and drains 381.4 ha.

M. falcata were placed in cages at both the control and treatment sites to facilitate routine monitoring and collection, and to guard against predation. For the Phase 1 relocation, 3 cages, approximately 30 cm x 45 cm x 20 cm in size and constructed of coated hardware fabric were placed at each site and held in place with 4 ft x ¼ in rebar. Fewer mussels were placed in cages for Phase 2, and as such, the cage size was scaled down to 25 cm x 30 cm x 15 cm. Zip-ties were used to close the cages and could be removed and replaced, enabling researchers to remove and check mussels. The cages were buried into the sediment as best as possible, although the sediment at both sites was coarse and only a few inches deep covering a hard, compacted layer. Each cage was secured to the stream bottom by driving 4 ft x ¼ in rebar stakes though the cage and into the sediment.

The rebar stakes were driven approximately 1 m down into the sediment with a sledgehammer. Cage placement was primarily chosen based on the absence of salmon redds and target areas within the base-flow wetted width.

For the Phase 1 relocation, mussels were deployed on two separate days, approximately a week apart. On November 25, 2006, groups with 45 mussels each were moved from the source population to the control in Cottage Lake Creek and the treatment site downstream of Paradise Lake in Bear Creek. For both phases, mussels were collected by hand and stored in a large cooler filled with water until being measured, tagged and moved. Each mussel was dried off with a rag, a Floy® Shellfish Tag with a unique number was affixed to the shell with Cyanoacrylate adhesive and the mussels were left to air dry for 30 minutes before placing them in the cooler with water once again. The water in the cooler was replaced approximately every 2 hours while the mussels were held for processing. On December 2, 2006 45 additional mussels were moved from the source population to each of the control and treatment sites. After the December 2nd relocation event, a total of 180 mussels (2 sites x 3 cages/site x 30 mussels/cage) had been moved to CL-C and BC-DS. Mussels were not collected for histological analysis at the time of the mussel relocation. Instead, mussels from the Cottage Lake Creek source population and surviving mussels native to Bear Creek were collected on December 12th, 2006 to establish the baseline histological condition of mussels from these locations.

The Phase 2 relocation was initiated to assess whether conditions in Paradise Lake could be related to the mussel die-off downstream of the lake. On May 2nd, 135 mussels (3 sites x 3 cages/site x 15 mussels/cage) were relocated to CL-C, BC-DS and BC-US (Figure 3.1). Six mussels were collected for histological analysis on May 2nd to determine baseline conditions at the time of relocation

Survivorship

Cages at all sites were visually checked at least weekly for mortality from December 2nd, 2006 through December 6th, 2007. Following the first observation of mortality, observations were increased to twice per week and tactile searches were used to insure that no dead mussels were present. Semiweekly mortality checks occurred from September 6th, 2007 to November 1st, 2007.

Histology Analysis

Mussels were collected from each cage on a monthly basis for routine histological analysis; any dead mussels identified at this time were also collected for analysis. During the last routine sample event on November 6th, 2007, two mussels from each cage were collected. Dead mussels were collected from September 6, 2007 to December 11, 2007. Histological interpretation included classification of: reproductive stage (from Toy 1998, Table 3.2, Appendix A); % gill intact; and ranked as high, medium, or low for digestive gland vacuolization, epithelial dilation, and cell necrosis/sloughing (Appendix B). Intact gill was also classified as high (> 70% intact), medium (31-69% intact, low (1-30% intact) and 0 (no intact gill) (Appendix B).

Glycogen Assay

M. falcata glycogen levels in the foot tissue were determined for Phase 1 individuals. The glycogen assay was based on the indirect amyloglucosidase method outline by (Burton et al. 1997). In this method, glycogen is first converted to glucose with amyloglucosidase, and a colorimetric reaction determines glucose concentration. A standard calibration curve was run with each assay to determine the concentration of known glycogen concentrations. Standards were prepared for each assay at the following concentrations: 0.1, 0.25, 0.5, 0.75, and 1.0 mg/ml. A tissue/standard blank was prepared to correct for the absorbance of the tissue homogenate / glucose standards, and an enzyme blank was prepared to correct for the glucose present in the amyloglucosidase.

Glycogen levels for each animal were independently analyzed. At the time of dissection, foot tissue was retained and stored at -80ºC until analysis could be conducted. 0.1 - 0.2 gms of foot tissue was excised from each mussel and finely minced before homogenization to a fine slurry with 2 ml of 0.1 M trisodium citrate buffer, pH 5 in a 7 ml Tenbroek homogenizer. The homogenate was rinsed into a test tube with the remaining trisodium citrate buffer, pH 5 at a ratio of 5 mL buffer **:** 0.15 g foot tissue**.** The resulting homogenate was heated in a boiling water bath for 5 minutes to inactivate endogenous glycosidases and then cooled to room temperature. The glucose standards were prepared with glycogen from *Mytilus edulis* (Sigma-Alridge) in sodium citrate buffer pH 5. The enzyme blank was prepared with 1 part enzyme to 10 parts buffer.

To convert the glycogen to glucose, each tissue homogenate, standard, and enzyme blank was incubated overnight at room temperature with 0.5% amyloglucosidase (Sigma-Aldridge**)** in citrate buffer at a ratio of 10 parts homogenate to 1 part enzyme solution. The tissue blank was prepared using tissue for each animal included in the assay, but no amyloglucosidase was added to the standard. After the incubation period, all samples were centrifuged at 1300g for 30 minutes, and the glucose concentration was determined using the Trinder Reagent (Diagnostic Chemicals). Manufacture's directions were followed for the Trinder Reagent, except that absorbance was measured at 492 nm. Unknown absorbances were regressed against the standard curve.

The final glycogen concentrations were determined using the following equation:

[Adjusted abs. glycogen] = [Abs. glycogen] – [abs. tissue blank] – [abs. enzyme blank] + [abs. sodium citrate buffer]

The glycogen concentration was then corrected for the dilution of the sodium citrate buffer, and reported as mg/ml.

Paradise Lake Toxicological Screening

A total of 19-L of water were collected and composited from 1.0, 1.5, 2.0, and 5.0-m depths at the approximate middle of Paradise Lake. This water was then filtered through a 25 µm sieve and diluted to 3-L with filtered lake water. The water sample was filtered to generally increase the abundance of rare taxa and to concentrate the algae concentration. Samples were collected at approximately weekly intervals (n= 9) between July 30th 2007 and September 17th 2007.

Water samples were transported to the King County Environmental Lab (KCEL) and used to conduct a screening level 96 hour fathead minnow toxicity test. Each toxicity test consisted of well water control and a treatment that used Paradise Lake water (concentrated algal water sample). For both the control and the treatment, each fathead minnow test was replicated in 4 beakers; each beaker containing 10 fish. Seven to twelve day-old fathead minnows were used in all testing. At 48 hours the water in each beaker was renewed with the respective water source. Mortality was assessed every 24 hours.

An aliquot of water from each 3-L lake water sample was also collected for phytoplankton analysis. 50-ml was preserved in 1% Lugol's iodine and 50-ml was preserved with 1% glutaraldehyde and archived. Fifteen-ml was preserved lightly in Lugol's iodine and sent to WATER Environmental Services, Inc. for phytoplankton identification and composition analysis. Phytoplankton density, number, and volume calculations were not accurate because the use of the sieve skewed the sampling towards larger phytoplankton.

Stream Environmental Parameters

Recording flow and temperature gages are located on both Cottage Lake and Bear Creeks. The Bear Creek stream gage at the Woodinville-Duvall Road is within 1 km downstream of the BC-DS and the Cottage Lake Creek gage at NE 132nd ST is located within 1.5 km downstream of the CL-C site. The gage at the Woodinville-Duvall Road is

the most upstream gage in Bear Creek. To provide additional hydrologic information, stream velocity was measured using a Marsh-McBirney Model 2000 Flo-Mate flow meter at each site on September 27th, 2007. Velocity was recorded at 0.6 depth from the water's surface and at 0.5-m intervals across the creek cross-section at all sites and just upstream, downstream, and toward the channel center of each cage.

Temperature was recorded hourly from December 21, 2006 until November 6, 2007 using calibrated Onset HOBO® temperature logger that was secured to the most upstream cage at each site. Temperature measurement of the BC – PV site was initiated on June 9th**,** 2007. From May 12th to September 13th, water levels were sufficiently low at the BC-DS site that the temperature logger was periodically exposed to the air. Therefore, temperature measurements from the downstream recording temperature gage were used to construct the temperature record between June 5th and August 13th. Due to a technical problem temperature data were not recorded at this gage from May 12th until June 5th, and so a temperature profile could not be constructed for this period.

Conductivity was measured approximately weekly from November 9th, 2006 to November 9, 2007 (Oakton Instruments, Vernon Hills, Il); dissolved oxygen was measured weekly with an YSI meter (YSI-85, YSI Incorporated, Yellow Springs, OH) from November 9, 2006 to March 5, 2007. Alkalinity and pH were determined with an aquarium test kit and monitored approximately weekly from January 19th, 2007 to September 26th, 2007.

Statistical Analysis

All statistical tests were conducted using S-Plus 8.0 statistical software (TIBCO Software Inc, Palo Alto, CA). The Kaplan-Meier method and log-rank test was used to compare survivorship within groups in the two phases of the experiment and for the BC – DS treatments in both Phase 1 and Phase 2 and to compare 96-hour survivorship curves

between fathead minnows in the experimental treatment (Paradise Lake water) and the control group. The Kaplan-Meier method is used to measure the fraction of individuals expected to live for a given duration of time, given observed mortality. This method can take into account losses that result from mussels being removed for sampling.

Since glycogen levels were not normally distributed, differences in baseline glycogen-levels, and levels measured in caged animal from December, 2006 – October, 2007 were tested using the Wilcoxon Rank non-parametric two-sample tests. Statistical differences were indicated for p-values < 0.05. In November, 2007 glycogen levels for treatment animals from BC-DS observed to be sick (n=2) were analyzed separately from animals that did not display disease behavior. Glycogen levels for BC-DS routine, BC-DS sick and CL-C were analyzed using the Kruskal-Wallis non-parametric ANOVA.

A chi-square test was used to compare the male: female: hermaphrodite ratio from mussels at the Bear Creek treatment sites (observed) against the count data from the Cottage Lake Control Site (expected).

Results

Pilot Survey and Health Screening

In Cottage Lake Creek, a total of 3,059 *M. falcata* individuals were observed over a distance of about 641 surveyed meters. Based on these data it was determined that the population was large enough to serve as a source population for the relocation experiment, as less than 12% of the observed population would be used for the mussel relocation experiment. Histopathological analysis (5% detection level with 95% confidence) found no evidence of communicable disease. Based on these findings it was concluded that the Cottage Lake Creek population could serve as the source population for the relocation experiment.

Mortality

Mortality among the relocated *M. falcata* was only observed among animals at the BC-DS site. This mortality resulted in a significant decrease in survival probability for mussels at the BC-DS site for both Phase 1 and Phase 2 relocations ($p<0.001$, $p<<0.001$, resp.; Figures 3.2 and 3.3). For both Phase 1 and Phase 2 BC-DS relocations, the onset of mortality occurred in early September 2007 and occurred at a similar rate, resulting in survivorship probability curves that could not be statistically differentiated ($p=0.52$; Figure 3.4).

Histology

Over the course of the experiment, the ratio of male, female and hermaphrodite mussels did not differ between the Cottage Lake Creek control site and the 2 Bear Creek treatment sites (Table 3.3). Hermaphrodites were observed among 2.9% of all individuals and at all sites over the course of the collection period. Mussels collected for routine sampling from each site and for each phase followed similar patterns of reproductive development timing. When sampling was initiated for the Phase 1 group in mid-December, 2006 mussels at CL-C and BC-DS sites were in the developing and early active reproductive stages (stages 1 – 2) (Figure 3.5). These stages corresponded with those observed in wild mussels collected from the Cottage Creek source site on the same day (= baseline). However, mussels native to the BC-DS were characterized as inactive and early developing (stages 0- 1) (Figure 3.7). By early/mid February, all mussels sampled at the CL-C and BC sites were ripe (stage 3) and by early May all mussels from Phase 1 and baseline mussels from the Phase 2 deployment where partially spent (stage 4) (Figure 3.6). While unfertilized eggs were commonly observed in the marsupial gill, brooding larvae were only observed in one individual collected from CL-C on June 6, 2007 (Appendix B). Spent, inactive, and early-active mussels were observed from August to November in all treatment from both Phase 1 and 2. In September, animals from the BC-DS site (both Phase 1 and 2) appeared to have delayed development in the fall of 2007 relative to the other treatment and control relocation sites. The development

stage of the Phase 2 – BC-DS treatment mussels sampled in September ranged from spent (stage 5) to early active (stage 1), while mussels in the other treatment and control were developing (stage 2) and ripe (stage 3). The developmental stages of the three Phase 1 mussels collected in November were variable; at the BC-DS location one was inactive (stage 0), one was early (stage 1), and one was ripe (stage 3), while all three mussels from the CL control were in the developing and ripe stage.

Dead animals that were collected in the fall months had greater number of individuals in the inactive (stage 0), early active (stage 1), and spent (stage 5) reproductive stages than live animals collected during the fall period (Figure 3.8). Wild mussels were collected at the BC-DS site when the study was initiated in December, 2006, and again in May 2007 and July 2007. Similar to dead animals, wild BC-DS mussels were generally characterized as inactive (stage 0), early active (stage 1), and spent (stage 5) (Figure 3.7).

No parasites were consistently observed or suspected to contribute to mortality. Ciliates were observed in tissue from dead mussels collected from the BC-DS location, but only in those mussels that were already necrotic (Appendix B). More tissue changes, including digestive gland tubule vacuolization, increased DG cell necrosis and sloughing and decreased gill condition, were observed among dead mussels collected from the BC-DS site relative to live mussels routinely sampled at other sites (Tables 3.4, 3.5, 3.7). The most striking characteristic observed among dead mussels from the BC-DS site was the presence of a highly vacuolated digestive gland relative to the healthy condition commonly observed in the digestive gland (Table 3.5). This state was observed in all dead mussels from BC-DS from the Phase 1 relocation, except those mussels whose degree of digestive gland degeneration was too pronounced to determine vacuolization. Among dead animals collected from the Phase 2 relocation to BC-DS, none had low levels of vacuolization, and half had at least medium amounts of vacuolization. The distribution of animals with low, medium, and high cellular changes to the digestive gland (cell necrosis, vacuolization, and dilation) did not differ among mussels routinely

collected from either of the 2 Bear Creek treatment sites and the Cottage Lake Creek Control Site (Tables 3.4, 3.5, 3.6). Dead animals did not have increased rates of digestive gland dilation (Table 3.6). Except for animals whose epithelial cells had almost entirely sloughed away, tubules of the digestive gland were typically characterized by an epithelium composed of columnar alpha and beta cells surrounding a small lumen, even among animals collected after death.

Sections of damaged gill tissue were observed among many mussels from all treatment groups and the control throughout the year. Associated morphological changes included squamous metaplasia, epithelial cell necrosis, and sloughing (Appendix B). However, necrosis of gill tissue from dead BC-DS animals was more extensive and advanced than from live animals collected for routine sampling, and among 72% of dead mussels collected from the Phase 1 BC-DS treatment, no intact gill was observed (Table 3.7). While only 14% of mussels from the Phase 1 BC-DS treatment had no intact gill tissue, this percentage was higher than live mussels routinely sampled, where the percent of animals with no intact gill ranged from 0-6% between treatments. Among live mussels routinely collected at all sites, including BC-DS, gill tissue was often intact and well-ciliated and there was no persistent pattern of epithelial loss (Table 3.7).

Glycogen

In general, glycogen levels were variable and clear differences were not observed among the BC-DS and CL-C groups (Figure 3.9). At the onset of mortality, glycogen levels appeared to be lower in the BC-DS group, but this difference was not significant ($p>0.05$; Table 3.8). During the last sampling event in November 2007, moribund mussels contained significantly lower glycogen levels than those in the control group.

Fathead Minnow Toxicity Screening

Toxicity was observed among fathead minnows tested with the concentrated water samples collected from Paradise Lake on August 20th and September 4th, 2007 (Table 3.9). The first occurrence resulted in 65% mortality, the second occurrence in 100% mortality. The 2 mortality events were separated by a week during which there was no observed mortality.

Following the observation of toxicity on September 4th, additional concentrated water samples were collected on September 12th and September 17th from 3 locations where mussels had been relocated, and Cottage Lake, the lake which feeds the control stream for the mussel relocation. No toxicity was observed at these additional sites (Table 3.10).

Site Conditions

During the study period, conductivity, DO, and alkalinity were higher at the Cottage Lake Creek control site than at the Bear Creek treatment sites (Figure 3.10). However, pH values were similar between the two sites. From late December to early April maximum temperatures were similar at the CL-C and BC-DS sites (Figure 3.11). From April 4th to May 12th temperatures averaged 1°C higher at the BC-DS site than at the CLC, with a maximum difference of 4.9°C higher. From June 5th to September 19th temperatures were similar at the 2 Bear Creek sites; both of which were higher than the CL-C site. The maximum temperature recorded was 25.3° C at BC-US, 24.3° C at BC-DS, and 21.2° C at CL-C. From mid-September to the end of the monitoring period in late October, temperatures were similar at the 3 sites.

Stream velocity and substrate conditions differed between sites. At the Cottage Lake Creek control site, sediment transport was common as evidenced by changes in the stream channel over the study period and the need to periodically free the cages from up to 0.6 m of sediment. At the CL-C site, sufficient sediment was deposited in 3 cages to enable mussels to partially bury themselves. At both BC sites, much less sediment

transport was observed even after high flow events. In addition, the Bear Creek substrate was compacted resulting in the need for all cages to be placed on top of the sediment. Beaver activity just downstream of the BC-US location resulted in backwater conditions at the relocation site from approximately June 6th, 2007 to approximately August 2nd, 2007, and again from October 9th, 2007 through the end of monitoring on December 6th, 2007.

Velocity and depth measurements were made when discharge was 0.37 cm/s at Bear Creek (summer base flows were approximately 0.20 cm/s) and 0.46 cm/s at Cottage Lake Creek (summer base flows were approximately 0.11 cm/s). At the CL-C site, the range of stream velocity around the cages was greater than that observed at the Bear Creek sites, and the average stream velocity was higher than the average velocity measured at either the BC-DS or BC-US site (Figure 3.12a). Maximum depth of the cages measured to the cage bottom was similar at all three sites, but cages were generally deeper at the two Bear Creek sites (approximately 7 - 10 cm), and the shallowest cage depth was at the Cottage Creek site (approximately 5 - 9 cm) (Figure 3.12b).

Velocity-depth profiles measured during fall flows at each site demonstrated that the channel at the CL-C site was of similar depth and flow. There was a distinct channel and flow rates were higher than 0.3 m/s across the entire channel, with much of the flow greater than 0.6 m/s (Figure 3.13). Both Bear Creek sites had areas of low flow in the side margin of one bank, where water was present but the velocity was too slow to measure. In the main channel area, flow was faster and deeper at the BC-DS site than at the BC-US site.

When stream-flow was normalized by contributing area, the discharge at BC-DS was higher than discharge at CL-C for both base and storm flows (Figure 3.14). The magnitude of peaks was larger at the BC-DS site, but the timing of peaks at the two sites was similar.

Summer was relatively wet in 2007 (Figure 3.15): Between June 1 and August 30th, 2007, total rain fall (17.8 cm) was in the top quartile for the period of record (1993 – 2007). Rain events coincided with both observations of fathead minnow mortality (8/18 and 9/4 rain events) and the onset of freshwater mussel mortality at BC-DS (9/4 rain event). When compared to preceding rain events the August 18th rain event was above average volume, duration, and number of antecedent dry days. It was just below average for rain intensity (Table 3.11). The August 18th rain event was preceded by 2 events with greater rain accumulation, 4 events with higher rain intensity, and 3 events with more antecedent dry days. The September 4th event was below average for rain accumulation and duration, number of antecedent dry days, and above average for average rain intensity and the number of antecedent dry days. It was preceded by 5 events with greater rain accumulation, 1 event with higher average intensity, and 3 events with more antecedent dry days.

Algal Community Composition in Paradise Lake

The phytoplankton community in Paradise Lake was dominated by Chlorophytes from late July to early August, and by Chrysophtyes from early August to mid-September, the remainder of the monitoring period (Figure 3.16). The most significant bloom appeared on September 12th; however, samples collected on July 30th, August 8th, and August 20th were all characterized by high algal cell densities relative to the other sampling times. Within the Chrysophytes, pennate diatoms were abundant in early August, *Asterionella formosa* were prevalent throughout the rest of August (Figure 3.17). The mid-September bloom was dominated by *Synura spp*. As previously indicated, the King County Small Lakes program routinely collects phytoplankton from Paradise Lake, however, whole water samples (not sieved) are collected. Comparison between data collected for the King County Small Lakes program indicated that Chlorophyta and Chrysophyta were represented at similar proportions between the two sample collection methods (Figure

3.18). Cryptophyta was detected in the raw samples at Paradise Lake, but not in the sieved samples.

Algal Community Composition at Stream Sites

Algal community composition in the samples collected on September 12th, 2007 was similar at both Bear Creek sites, however composition of the Bear and Cottage Lake Creek sites was quite different (Figure 3.19). Algae from the Chrysophyta division were most abundant at both Bear Creek sites (Figure 3.19a). Algae from the division Cyanophyta, Chlorophtya, and Chrysophtya were present in both Bear Creek sites in roughly the same proportions, but Euglenaphyta and Phyrrophyta were found only at BC-DS. Within Bear Creek, relative cell volume was higher at the downstream site. Total cell number was almost an order of magnitude higher at the Cottage Lake Creek site than either Bear Creek site (Figure 3.19). Much of this difference was driven by the Cyanophyta, which dominated the Cottage Lake Creek sample.

Discussion

M. falcata Survival

Survivorship data provided clear evidence that conditions at the BC-DS site resulted in *M. falcata* mortality, and that at the temporal scale examined, 9 months versus 4 months of relocation, time of residence to the BC-DS site did not influence *M. falcata* survivorship. Past research has demonstrated that *M. falcata* individuals in Bear Creek are long-lived; up to 100 years (Toy 1998). This life-history data, combined with 100% survival at the other treatment and control sites, suggests that a site-specific factor is causing mortality. The onset of *M. falcata* mortality for both relocation phases at BC-DS occurred within a week of one another and occurred at the same rate. The period during which mortality was monitored was not long enough to determine if mortality eventually subsided, or to pinpoint the time-period of the highest mortality rate. The timing of mortality suggests that the early fall period plays an important role in mortality.

The mortality rates observed among mussels transplanted to the CL-C and BC-US sites were lower than those reported for other relocation experiments identified in the literature. The use of cages to contain the mussels likely increased both survivorship and the ability to accurately document these trends. In Cope and Waller's review (1995), the average survival of translocated mussels was 51%, based off an average recovery of 43%. 100% recovery and survival was observed at the CL-C and BC-US sites. The use of cages enabled the mussels to be quickly located and facilitated frequent field checks in addition to guarding against predation. Substrate instability has been demonstrated as a cause of mortality for relocated mussels (Cope and Waller 1995, Bolden and Brown 2002). High stream flows from winter rains mobilized much sediment and caused rapid change in channel morphology at the CL-C site. This site location appeared particularly prone to channel changes and active winter sediment transport may thus prevent *M. falcata* from naturally colonizing the control site, even though a potential source population is in close proximity and glochidia could likely be transported to this area. Anchoring the cages to the substrate prevented the mussels from washing away, and also enabled recovery of the mussels after the cages had been buried by sediment.

Only mussels at the CL-C relocation site were able to bury themselves into the substrate; this was only possible in 2 of the 6 cages at this location. While other researchers have found substrate type and ability to burrow to be important for survivorship (Newton et al. 2001, Hamilton et al. 1997), the results of this study did not suggest that this was an important factor for *M. falcata* during the study period. It is possible that the ability to burrow may only impact *M. falcata* survivorship over a longer time period than was monitored in this study, or that sediment burrowing is not a critical factor for *M. falcata*, as long as their other habitat needs are being met.

Habitat conditions at the BC-US site appear to be the least suitable for *M. falcata*, since beaver activity resulted in backwater conditions for much of the study period. However, survivorship of the relocated mussels, and the presence of other mussels at the site,

suggested that the impact of the beaver dams were not deleterious in the short-term. Mussels in the cages at this location were frequently covered in silt, but were also observed siphoning during this period; the suspended sediment had apparently not limited the mussels' ability to feed. This site is located in the headwaters of Bear Creek and is part of a wetland-stream complex and stream conditions suggested intermittent backwater conditions over a longer duration than the study period. Sediment cobbles in the center of the channel remained clean over the entire study period, while thick silt and muck accumulations were located along the bank margins. *M. falcata* naturally inhabited the site and were also observed oriented upright and siphoning during backwater conditions. Good mussel survivorship over the study period and observations of native *M. falcata* at BC-US are consistent with our understanding of the closely related *Margaritifera margaritifera*, which persist in silty conditions for unknown periods of time (Hastie et al. 2000).

Microscopic Observations

Histological investigations did not reveal abnormalities in tissue condition before death was observed. The dilated digestive gland tubules with cuboidal epithelial cells observed in a previous examination of Bear Creek *M. falcata* mussels (Elston 2005) were generally not observed among mussels that died in the relocation experiment, though highly vacuolated digestive gland cells and high rates of cell necrosis and slough was commonly seen. One possible explanation is that digestive gland vacuolation was the more immediate cellular response and one of the first symptoms of disease, and that digestive gland dilation is a sign of stress expressed by mussels that had not died immediately after exposure to the original agent of mortality. Sparks (1972) observed that one of the first post-mortem changes in oysters occurs in the digestive tubules where the epithelium becomes necrotic and is sloughed into tubule lumina. From his observations of post-mortem changes and histological investigation, he concluded that "The digestive tubules have been found to be the most sensitive indicator of unfavorable environmental conditions, poor fixation, or post-mortem change." The highly vacuolated digestive

tubules are not dissimilar to histological changes observed in garden slugs *(Arion ater)* after ingestion of metaldehyde. The secretory area of the intestine and the digestive gland experienced necrosis and increased vacuolation (Sparks 1972). Similarly, juvenile Pacific oysters (*Crassostrea gigas*) experiencing losses due to a herpes-like virus were characterized by digestive gland necrosis and cellular sloughing, though digestive gland dilation and cubodial epithelia were also observed (Burge et al. 2006, 2007). Due to the sensitive nature of the digestive gland to changes in environmental conditions, it is not surprising that the first changes in tissue morphology may be observed in this tissue. However, changes in the digestive gland do not point to a specific cause of mortality.

At all treatment sites, most animals had moderate gill damage. Since gill tissue is especially fragile, this damage is likely an artifact from dissections. Among animals collected for routine sampling, high gill damage was rare, and very few animals had no intact gill. However, dead mussels had increased rates of animals with no intact gill. Since the adductor muscles relax and mussels lay open after death with their gills exposed to the water column and associated bacteria, this gill damage is not surprising. Therefore, the role of gill damage characteristic of dead animals is not resolved, but gill-damage was not characteristic of live mussels and is not thought to be the primary mechanism for mortality.

In general, the reproduction cycle observed in 2007 was similar to that previously documented in Bear Creek in 1996. However, Toy (1998) observed *M. falcata* in stage 3 later in the season and also progression to stage 4 and stage 5 sooner in the summer than was observed in this study. Toy correlated reproductive cycle progression with temperature by demonstrating that earlier initiation of spawning in Bear Creek relative to Battle Creek was similar to the difference in the number of degree days. In 2007, temperatures reached a higher maximum and remained higher over a longer duration than in 1996. Early spawning in 2007, relative to 1996, is therefore consistent with

reproductive-temperature relationships observed by Toy. However, it is not clear why the reproductive cycle may have been delayed after spawning in 2007, relative to 1996.

At the time-scale of this investigation, there were no persistent shifts in the timing of the reproductive cycle among mussels relocated to the BC-DS site, based on comparisons to CL-C and earlier work at the site by Toy (1998). While animals from the BC-DS site in both Phase 1 and 2 may have displayed slightly delayed development in September and October relative to control animals, by November the BC-DS mussels were observed in stages similar to those at the other relocation sites. Differences observed in September and October may have been associated with the relatively small sample size during each routine sampling, and do not suggest a drastic shift in gametogenesis among the BC-DS relocation.

Reproductive stage evaluations for a small number of *M. falcata* native to BC-DS suggests that these animals are stressed and not experiencing a normal reproductive cycle. *M. falcata* native to BC-DS were observed that are large enough to be sexually mature (Toy 1998) but lacked evidence of sexual differentiated upon microscopic examination, or were reproductively immature (stages 0 or 1) suggested that stressors experienced at the relocation site may be impacting the mussels' reproductive cycle. This hypothesis is consistent with the observation that only females in good physiological condition, with surplus energetic resources, will reproduce (Bauer 1987). However, a sex ratio dominated by males by 4:1observed by Brenner (2005) among wild Bear Creek mussels, and the observation of wild Bear Creek mussels that have a disproportionately large number of inactive or immature individuals, suggests that over a period longer than the one monitored, the reproductive cycle may be altered by stressful conditions at BC-DS.

Glycogen Analysis

While mussels from the CL-C group tended to have higher mean glycogen levels, levels were generally variable among individuals, and differences outside this range of variation did not emerge until after the onset of mortality. Statistically significant differences in glycogen levels were only observed between mussels at BC-DS with weakened adductor mussels and the control group. Glycogen variability should have been minimized through the selection of the foot tissue for analysis. Monroe and Newton (2001) found that glycogen concentrations in both undisturbed and relocated mussels were more evenly distributed in the foot tissue than in the mantle. Seasonal variation in glycogen was also lower in the foot than in the mantle, therefore changes in foot tissue glycogen is more likely to be caused by factors apart from seasonal changes or sub-sampling.

Researchers have attributed declines in freshwater mussel glycogen to a variety of causes. Monroe and Newton (2001) observed the largest decrease in glycogen in relocated *Amblema plicata* (another species of freshwater mussel) within the first 6 months of relocation. They attributed this decline largely to handing and relocation stress. In this study, however, glycogen steadily increased for the first six months after relocation and did not indicate relocation stress. Additionally, glycogen levels have been related to both differences in habitat conditions and correlated with mortality rates. Significant differences in glycogen levels were observed between *Potamilus alatus* that were relocated to deep and shallow areas of aquaculture ponds; mussels moved to deeper areas had glycogen levels that were about half the levels of mussels in the shallow treatment (Hua and Neves 2007). The low glycogen levels also corresponded with higher mortality, but glycogen samples were analyzed at the end of the study period and were not used to assess condition on an ongoing basis. In another study that compared glycogen levels between captive freshwater mussels relative to wild individuals, decreased glycogen in the captive relative to the wild mussels corresponded with increased mortality among the captive mussels (Liberty 2004). This investigation agreed with results of others (Hua and Neves 2007, Liberty 2004,) and demonstrated that a

decline in glycogen level corresponded with increased mortality. However, frequent monitoring of glycogen levels in this study failed to produce an early-warning indication that the BC-DS mussels were stressed

Environmental Factors

Temperature does not appear to be driving *M. falcata* mortality at the BC-DS sites. Temperatures were higher at BC-DS than the control site; however temperatures at BC-DS were similar to BC-US, where no mortality was observed. Studies have identified thermal stress as a cause of mortality; however, temperatures demonstrated to cause mussel mortality or physiological stress were higher than maximum temperatures recorded at BC-DS (Rajagopal et al. 2005, Hua and Neves 2007, Polhill and Dimock 1996).

While temperature did not appear to be the direct cause of mortality, temperature cannot be dismissed as a factor in mortality. Temperature requirements have not been established for *M. falcata*, but this species are observed in cold-well oxygenated water (Nedeau et al. 2005). Water temperatures at the site exceeded 20ºC, and it is possible, though not documented, that temperatures this high may be stressing mussels at BC-DS resulting in heightened vulnerability to the primary (unidentified) cause of mortality..

The initial onset of mussel mortality occurred in early fall, and mortality rates were highest in September and October. This timing is generally consistent with a “first flush” event in the Pacific Northwest, in which heavy fall rains that follow a dry summer period, flush pollutants from the surrounding watershed into the creek. During the summer of 2007, there were several significant rain events in early and mid summer, months before mortality was initially observed. Attempting to correlate rain events and mortality during the course of the study does not provide a clear understanding of the role of precipitation in observed fathead minnow and *M.* falcata mortality. Mortality did not commence with the onset of rain in the summer, neither did it appear to be a chronic response that may

have been driven by the cumulative impact of potential stormwater pollution delivered throughout the summer. Additionally, stormwater effects are expected to be more pronounced in the Cottage Lake Creek drainage than in the Bear Creek drainage, since effective impervious area is higher in the Cottage Lake Creek drainage (Simmonds et al. 2004). While the BC-DS site experienced more discharge per unit area, which suggests higher runoff, this difference is likely driven by the large lake upstream of the CL-C site which provides large storage capacity. Analysis based on 1991 and 1996 Landsat data indicate that the total impervious area in the Upper Bear Creek drainage site has increased from 3.6% to 4.5% (Simmonds et al. 2004). While more recent land-cover data are lacking, this work suggests that impervious area in the basin is still relatively low. Earlier water quality characterization also indicates that there is low stormwater-related pollution in Bear Creek. When sediment and water quality were evaluated in both Bear and Cottage Lake Creeks there were generally low concentrations of contaminants in both streams. In general, a larger number of parameters have been detected at higher concentrations in Cottage Lake Creek than in Upper Bear Creek (Lester 2007). However, the role of stormwater related mortality cannot be dismissed, and it is important to point-out that the appropriate scale for relating precipitation and mortality (and hence, the suggestion of a storm-water related mechanism for mortality) is over multiple summers and more intensive water quality sampling and analysis. In the investigation of presumed stormwater-related pre-spawn coho mortality observed in urban Puget Sound drainages, there is a relationship between the amount of pre-spawn mortality and cumulative late summer / early fall rainfall (McCarthy 2007).

Fathead minnow assays detected toxicity on two occurrences in late summer, and this indication of toxicity preceded *M. falcata* mortality in the downstream relocation experiment. Of the algal species observed in aliquots from lake samples where fathead minnow toxicity was detected, only *Euglena* has been documented to be toxic (Zimba et al. 2004). However, the *Euglena* in Paradise Lake has not been identified to species and *Euglena* is a cosmopolitan genus found in lakes and typically does not create harmful

conditions. Also, this species composed a small part of the phytoplankton community and was never a significant portion of a bloom. *Synura spp*. and *Ceratium spp*. were abundant in the lake at times and at very high concentrations could potentially cause mechanical problems that could lead to mortality (pers. com. M. Gibbons and P. Zimba 2008). It is possible that another algal species present in the lake could be releasing a toxin, in which case this event might be the first recorded documentation of toxicity for that algal taxa. Euglenaphyta and Phyrrophyta were observed within and downstream of Paradise Lake, but not upstream of Paradise Lake, and would be candidates for additional investigation. This early screening cannot reject the possibility that an algal species in Paradise Lake may produce a toxin, and the fathead minnow assay combined with downstream mussel mortality prevents us from discounting the possibility of a toxic substance in Paradise Lake, either generated through natural processes within the lake or from an anthropogenic source near the lake. While the phenomenon causing fathead minnow and *M. falcata* mortality has not been demonstratively linked, the corresponding timing is auspicious. Toxicity from the brackish-species microalgae *Prymnesium parvum* has been documented to adversely affect both fish and freshwater mussels from the Unionid family (James and De La Cruz 1989 in Watson 2001). Like much of our knowledge of non-cyanobacteria freshwater algae toxicity, the effect of potential algae toxins on freshwater mussels is largely unknown, especially in streams downstream of a lake with algae-driven toxicity. Potential lake toxicity should be studied in greater detail and linked to downstream conditions at BC-DS.

Conclusion

This study indicates that conditions at the BC-DS site result in *M. falcata* mortality. Differences in survivorship were highly significant between the BC-DS site when compared to either the BC-US site or the CL-C site. Additionally, mortality at the BC-DS site provided a strong signal that something out of the ordinary occurred at the BC-DS site that occured in early fall. Both mussels that had been relocated to the site in late November/early December 2006 (Phase 1) or in early May 2007 (Phase 2) experienced

approximately 40% mortality by early December 2007, with the onset of mortality in early fall. The environmental condition that corresponded with freshwater mussel mortality over the temporal scale monitored was toxicity observed in Paradise Lake through fathead minnow toxicity testing. While there is an indirect spatial-link between patterns of *M. falcata* mortality along Bear Creek and Paradise Lake toxicity, the link between conditions at Paradise Lake and the downstream relocation site has not been explicitly tested.

Lower glycogen levels were observed in *M. falcata* individuals with visibly weakened adductor muscles, and differences in the digestive gland were noticed in dead mussels collected from the BC-DS location, but neither monitoring glycogen nor tissue morphology provided an early-warning indication that the BC-DS relocated mussels were stressed. The apparently good condition of the tissue morphology of mussels collected after death, especially among the digestive gland epithelial cells, suggested that mortality was relatively rapid in mussels collected in September and October and there was not a prolonged pathological sequence of events leading to mortality. Additional investigation of the surviving mussels may provide additional information about the unknown stressor and the mechanism of mortality.

Tables and Figures

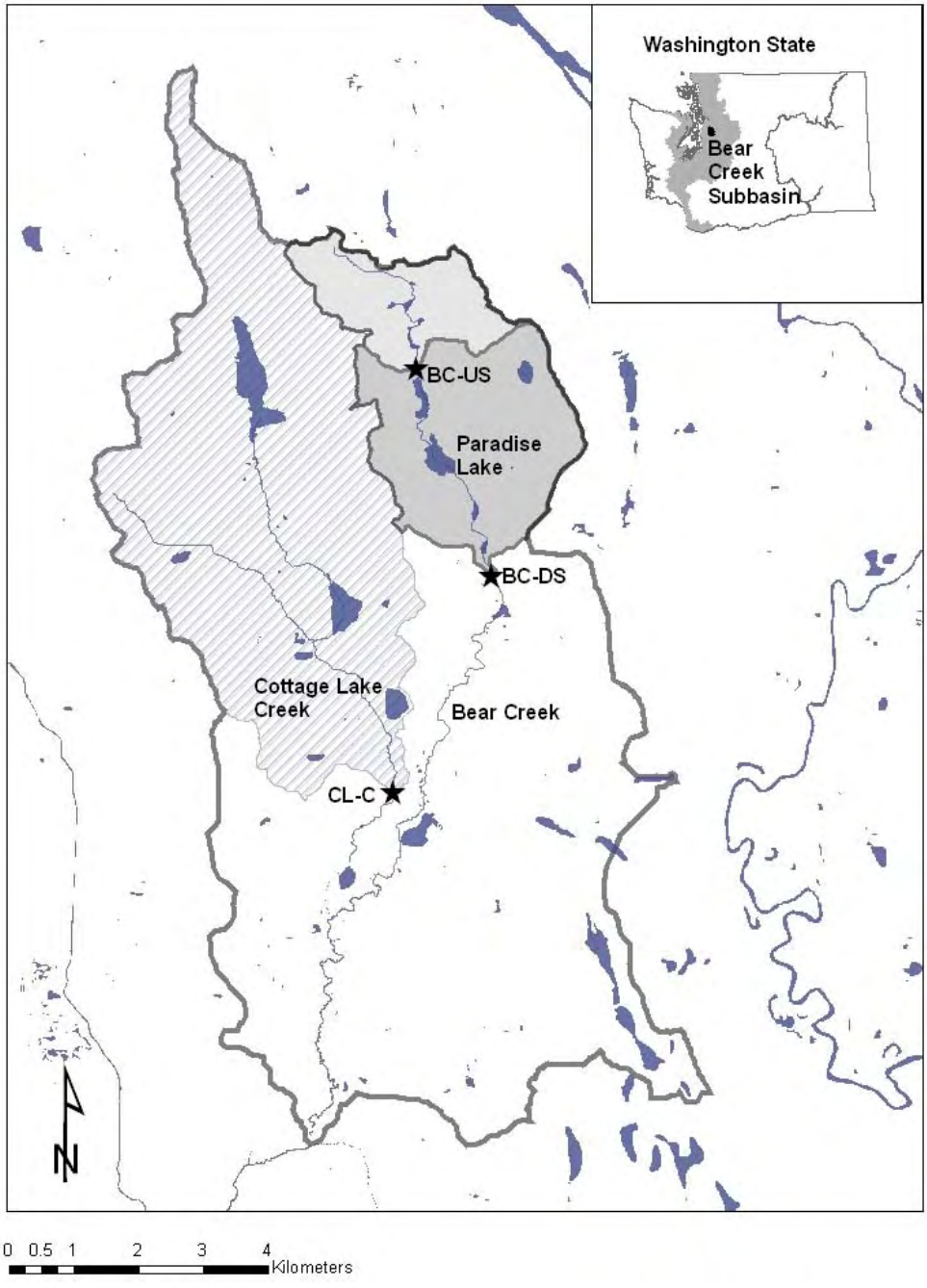


Figure 3.1 Map of relocation sites and contributing basins.

Table 3.1 Summary of caged mussel relocation experimental design

Phase 1 Study Design	
Source Population:	Cottage Lake Creek
Relocation Sites	Control - Cottage Lake, Treatment - Bear Creek downstream of Paradise Lake
Date Deployed	December 12 and 14, 2006
Size range of mussels deployed	82.1 – 116.6 mm
No. of cages per station	3
No. of mussels per cage	30
No. of mussels deployed	180 (2 sites x 3 cages x 30 mussels/cage)
No. of mussels sampled for histology	76
No. of mussels remaining for survival	104
Deployment Period	361 days (As of 12/6/2007)
Cage System	30 cm x 45 cm x 20 cm coated wire hardware cloth secured with plastic cable ties. Secured to substrate with 4, 4 foot rebar stakes
Phase 2 Study Design	
Source Population:	Cottage Lake Creek
Relocation Sites	Control - Cottage Lake, Treatment 1 - Bear Creek downstream of Paradise Lake, Treatment 2 – Bear Creek Upstream of Paradise Lake
Date Deployed	May 5, 2007
Size range of mussels deployed	88.3 – 113.3 mm
No. of cages per station	3
No. of mussels per cage	15
No. of mussels deployed	135 (3 sites x 3 cages x 15 mussels/cage)
No. of mussels sampled for histology	51
No. of mussels remaining for survival	84
Deployment Period	218 days (As of 12/6/2007)
Cage System	25 cm x 30 cm x 15 cm coated wire hardware cloth secured with plastic cable ties. Secured to substrate with 2, 4 foot rebar stakes

Table 3.2 Reproductive stages based on histological interpretation of gonadal sections (from Toy 1998).

Stage	Name	Description
0	**Inactive:**	No sexual differentiation in the visceral mass.
1	**Early:**	Some spermatagonia or oogonia and small ovocytes present.
2	**Developing**:	Developing ovocytes and sperm moving into the lumina.
3	**Ripe**:	Eggs detached from walls and individual cell wall evident. Lumen filled with mature sperm and eggs.
4	**Partially spent:**	Lumen contains < 50% of eggs and sperm, collapse of follicle walls.
5	**Spent**:	Lumen empty, female marsupia filled with eggs or glochidia.

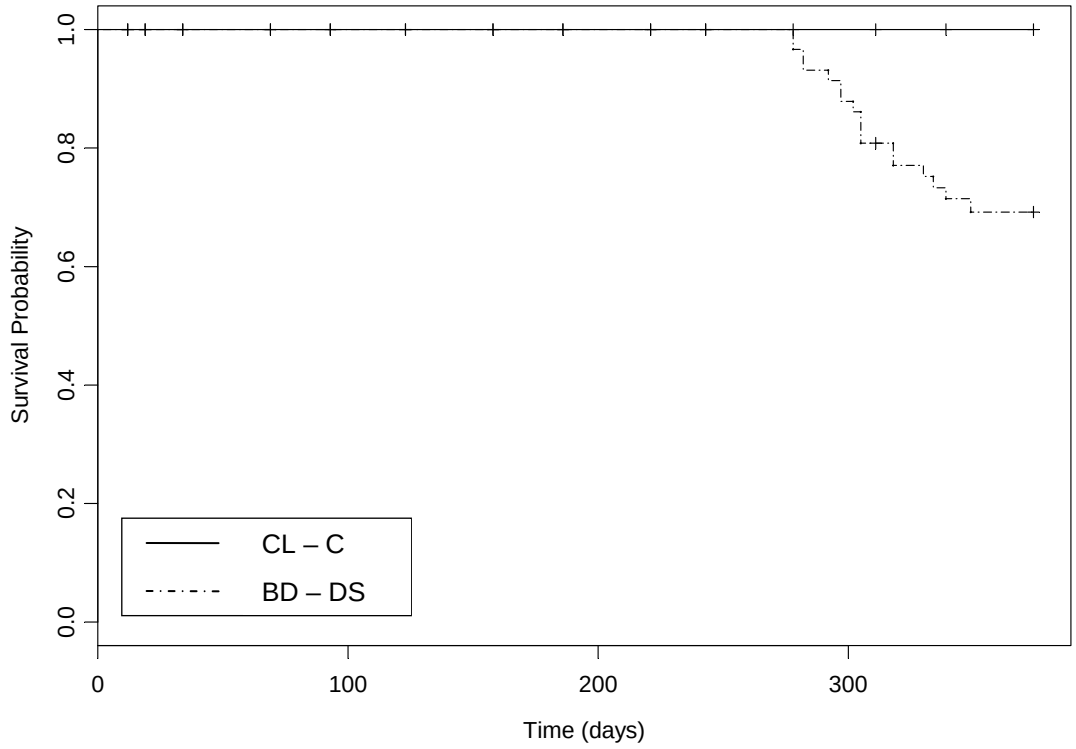


Figure 3.2 Survivorship curve for mussels relocated in December, 2006. Hatch-marks indicate routine sampling events. P = 0.0000732.

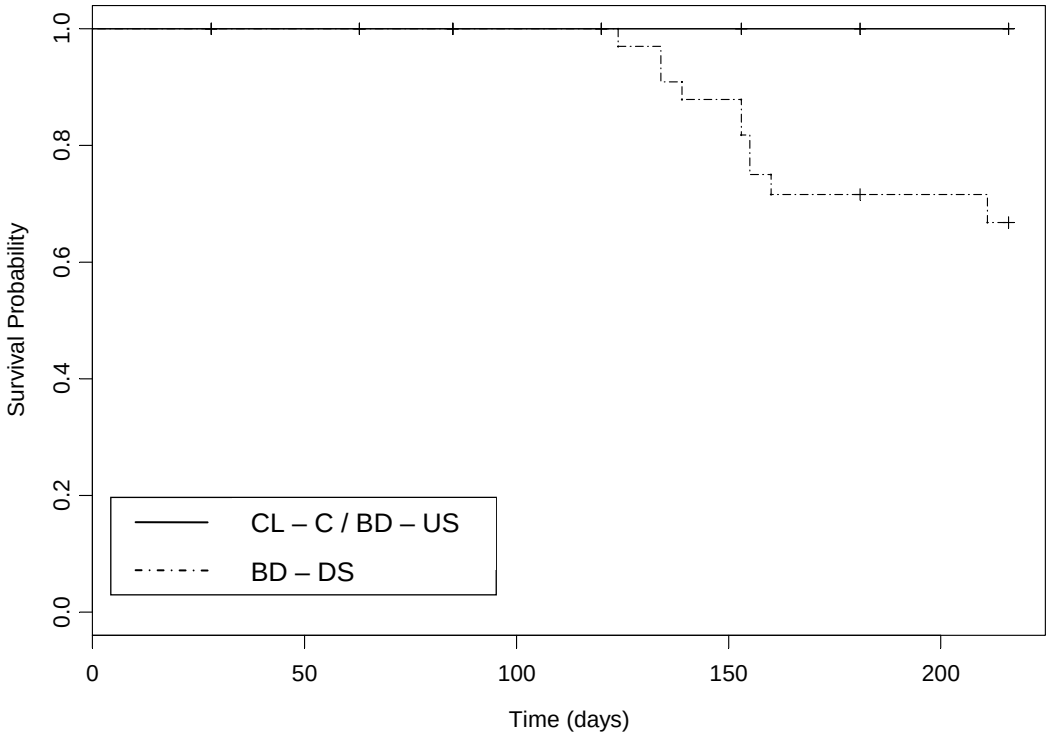


Figure 3.3 Survivorship curve for mussels relocated in May, 2007. Hatch-marks indicate routine sampling event. P = 0.0000705.

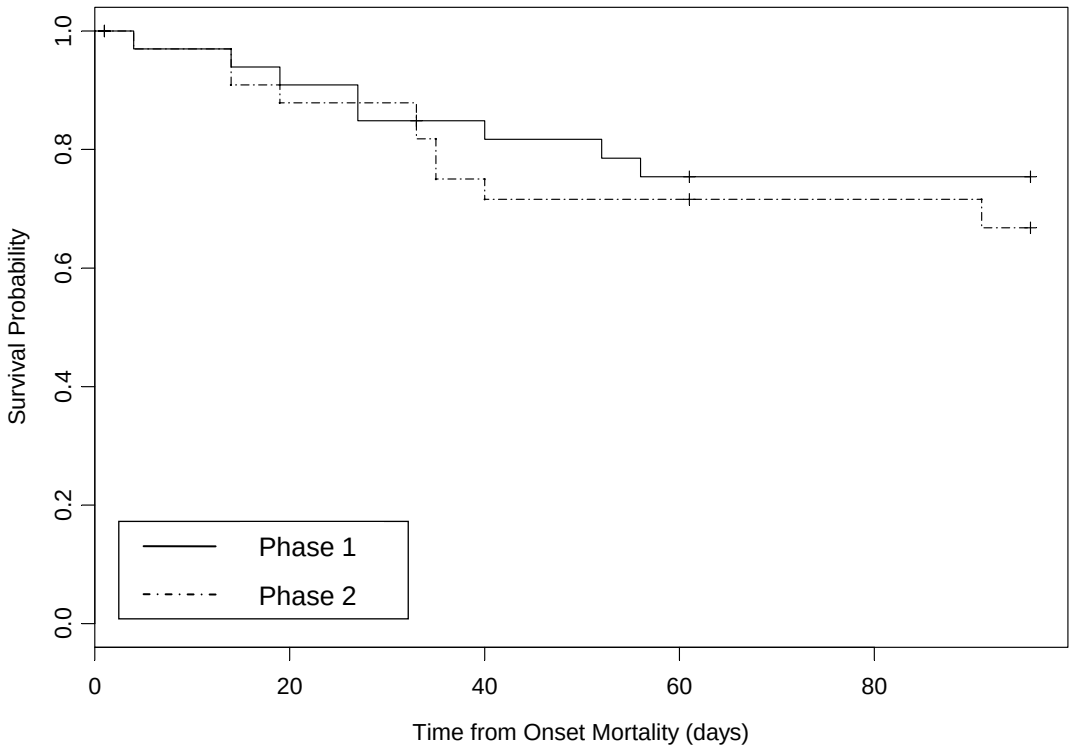


Figure 3.4 Survivorship curve for mussels at the Bear Creek – downstream treatment. Hatch-marks indicate routine sampling event. P = 0.518

Table 3.3 Counts of male and female and hermaphrodite mussels collected from relocation sites between December 12, 2006 and November 6, 2007. For mussels sampled during routine collection for Phase 1: $X^2 = 1.5309$, df = 1, p = 0.4651. For mussels sampled during routine collection for Phase 2: $X^2 = 3.6058$, df = 4, p = 0.462.

	Routine Phase 1		Routine Phase 2			Dead BC-DS	
	BC-DS	CL-C	BC-DS	BC-US	CL-C	Phase 1	Phase 2
male	23	19	11	7	11	11	3
female	16	23	8	11	9	4	3
hermaphrodite	1	1		1		2	

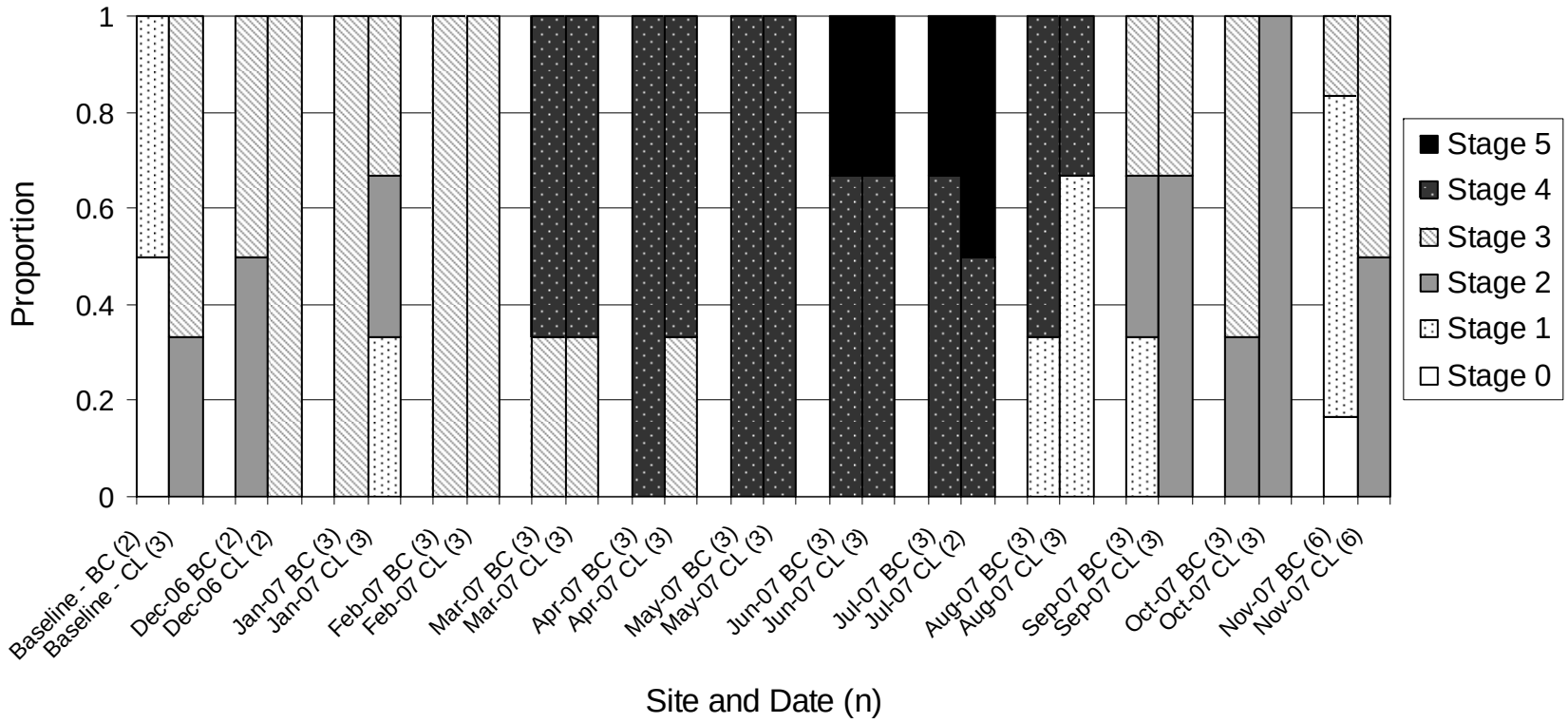


Figure 3.5 Reproductive stages for Phase 1 relocated mussels collected during routine sampling events.

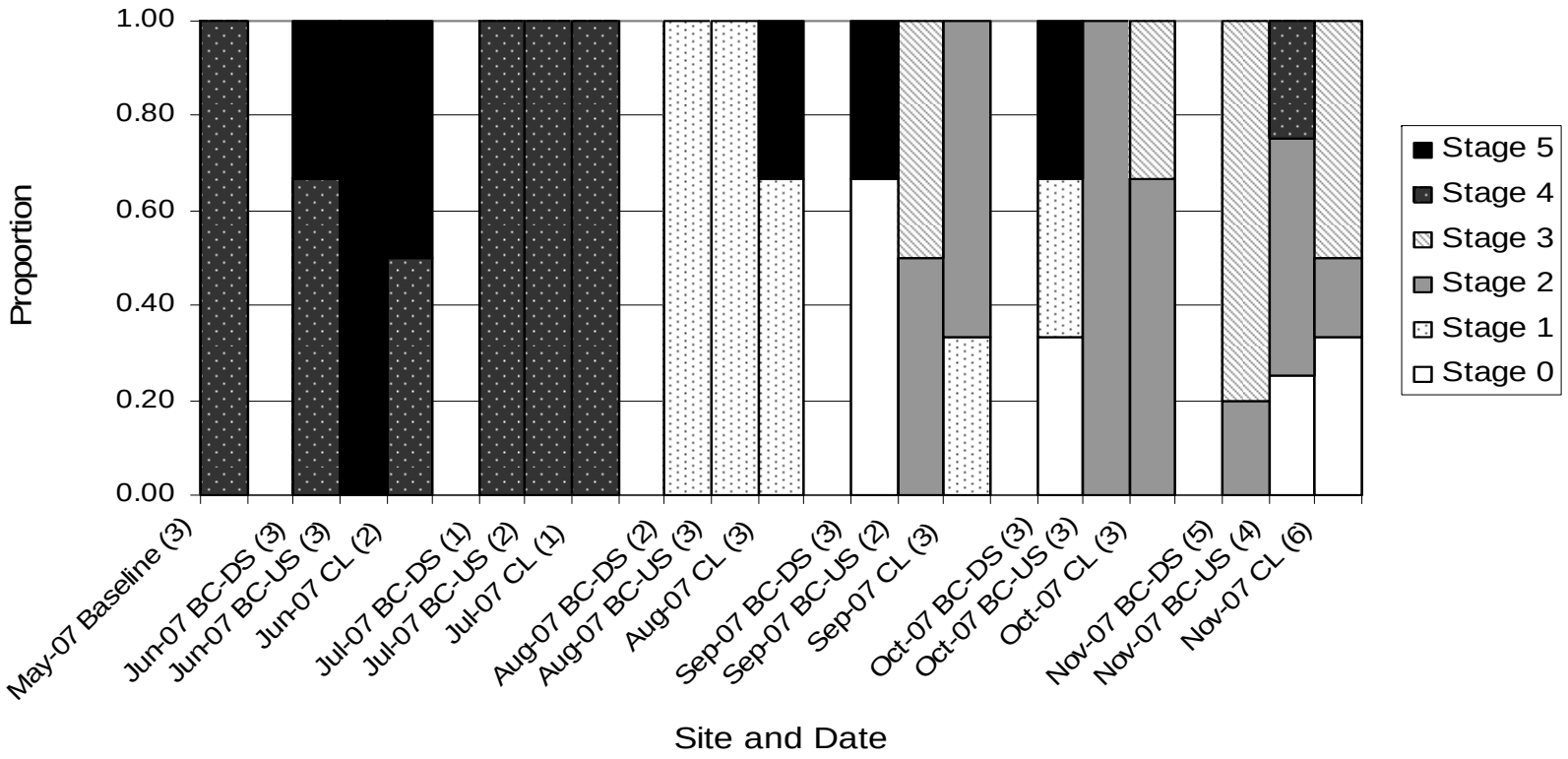


Figure 3.6 Reproductive stages for Phase 2 relocated mussels collected during routine sampling events.

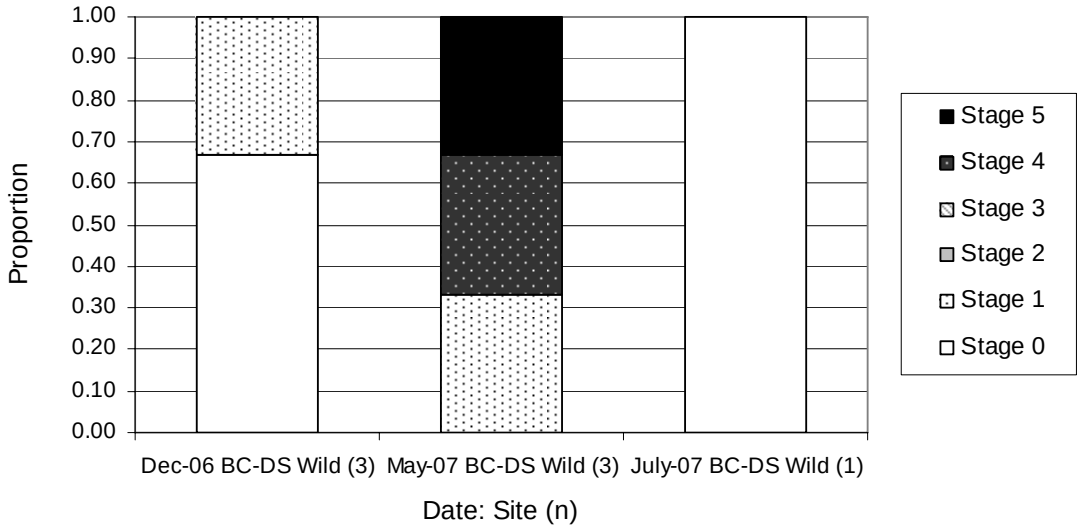


Figure 3.7 Reproductive stages for wild mussels collected from the BC-DS site during the course of the study. Light yellow bars refers to stage 0, light green to stage 1, light blue to stage 2, medium blue to stage 3, dark blue to stage 4, and black to stage 5.

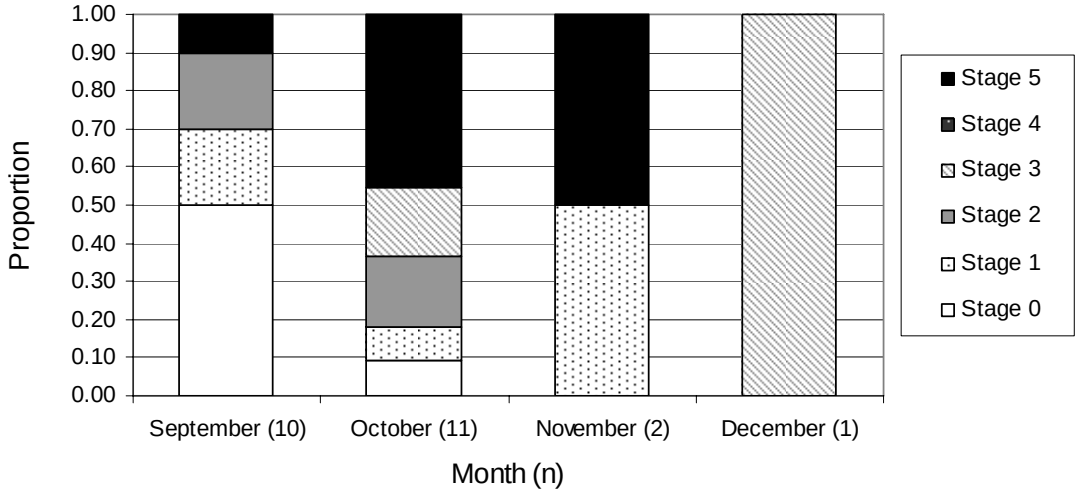


Figure 3.8 Reproductive stages for BC-DS mussels that died during the course of the study (fall 2007). Light yellow bars refers to stage 0, light green to stage 1, light blue to stage 2, medium blue to stage 3, dark blue to stage 4, and black to stage 5.

Table 3.4 Proportion of mussels with low (L), medium (M) and high (H) digestive gland cell necrosis and sloughing. Animals labeled as "sample" were collected live as part of the routine sampling, animals labeled as "mortality" were collected after they died.

Source	Phase	Sample or Mortality	n	L	M	H
CL-C	1	Sample	36	0.17	0.83	0.00
BC - DS	1	Sample	33	0.18	0.79	0.03
CL-C	2	Sample	20	0.30	0.70	0.00
BC-US	2	Sample	19	0.26	0.74	0.00
BC - DS	2	Sample	19	0.26	0.74	0.00
BC - DS	1	Mortality	15	0.00	0.73	0.27
BC - DS	2	Mortality	6	0.00	0.83	0.17

Table 3.5 Proportion of mussels with low (L), medium (M) and high (H) digestive gland vacuolization. N/A refers to tissue that is so necrotic that vacuolization couldn't be determined. Animals labeled as "sample" were collected live as part of the routine sampling, animals labeled as "mortality" were collected after they died.

Source	Phase	Sample or Mortality	n	L	M	H	N/A
CL-C	1	Sample	41	0.78	0.23	0.00	0.00
BC - DS	1	Sample	39	0.74	0.26	0.00	0.00
CL-C	2	Sample	20	0.80	0.20	0.00	0.00
BC-US	2	Sample	19	0.84	0.16	0.00	0.00
BC - DS	2	Sample	19	0.68	0.26	0.05	0.00
BC - DS	1	Mortality	15	0.00	0.00	0.73	0.27
BC - DS	2	Mortality	6	0.00	0.50	0.33	0.17

Table 3.6 Proportion of mussels with low (L), medium (M) and high (H) digestive gland dilation. N/A refers to tissue that is so necrotic that dilation couldn't be determined. Animals labeled as "sample" were collected live as part of the routine sampling, animals labeled as "mortality" were collected after they died.

Source	Phase	Sample or Mortality	n	L	M	H	N/A
CL-C	1	Sample	41	0.65	0.35	0.00	0.00
BC - DS	1	Sample	39	0.74	0.23	0.03	0.00
CL-C	2	Sample	20	0.75	0.25	0.00	0.00
BC-US	2	Sample	19	0.63	0.37	0.00	0.00
BC - DS	2	Sample	19	0.63	0.37	0.00	0.00
BC - DS	1	Mortality	14	0.33	0.33	0.00	0.33
BC - DS	2	Mortality	6	0.33	0.50	0.00	0.17

Table 3.7 Proportion of mussels with no (0), low (L), medium (M) and high (H) intact gill tissue. Animals labeled as “sample” were collected alive as part of the routine sampling, animals labeled as “mortality” were collected after they died.

Source	Phase	Sample or Mortality	n	0	L	M	H
CL-C	1	Sample	41	0.05	0.05	0.46	0.43
BC - DS	1	Sample	39	0.06	0.14	0.57	0.23
CL-C	2	Sample	20	0.00	0.11	0.58	0.32
BC-US	2	Sample	19	0.00	0.00	0.53	0.47
BC - DS	2	Sample	19	0.00	0.05	0.79	0.16
BC - DS	1	Mortality	15	0.72	0.17	0.11	0.00
BC - DS	2	Mortality	8	0.14	0.29	0.57	0.00

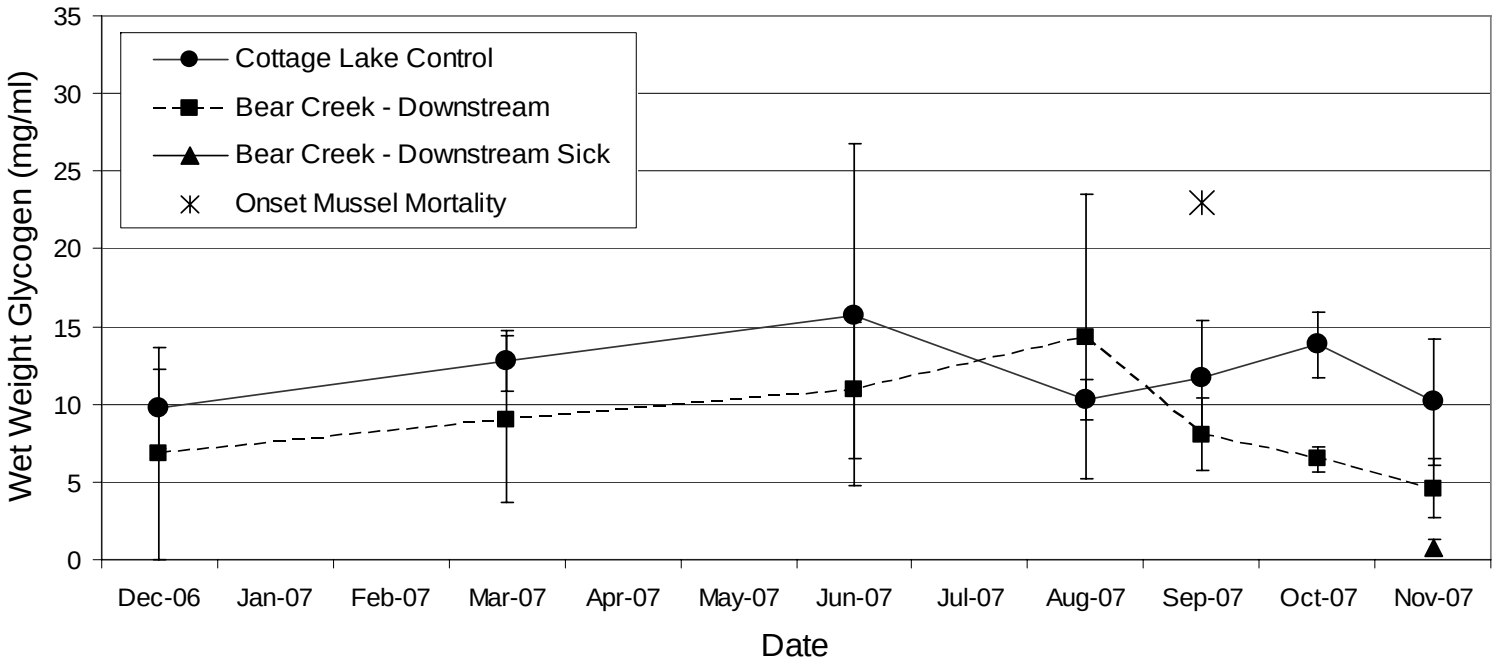


Figure 3.9 Mean glycogen concentrations (n=3) from Phase 1 relocated *M. falcata*. Error bars represent +/- 1 standard deviation

Table 3.8 Mean glycogen concentrations (mg/ml) and associated standard deviation (n=3) from Phase 1 relocated *M. falcata.* P-values from Wilcoxon Rank non-parametric two-sample tests are shown, with significant ($p<0.05$) values in bold.

Sampling Date (days deployed)	Cottage Lake Creek Control	Bear Creek Downstream	Bear Creek Downstream "sick"	P- value
Baseline (0)	5.67 +/- 1.40	5.15 +/- 4.53		0.7
December, 2006 (18)	9.7 +/- 2.56	6.83 +/- 6.83		0.4
March, 2007 (100)	21.15 +/- 13.36	9.03 +/-5.36		0.4
June, 2007 (193)	15.72 +/- 11.00	10.91 +/- 4.38		0.7
August, 2007 (257)	10.3 +/- 1.29	14.35 +/- 9.18		0.7
November, 2007 (285)	11.67 +/- 3.74	8.05 +/- 2.32		0.4
October, 2007 (318)	13.82 +/- 2.08	6.45 +/- 0.85		0.1
November, 2007 (346)	10.14 +/ 4.03	4.58 +/- 1.91	0.79 +/- 0.47	**0.04**

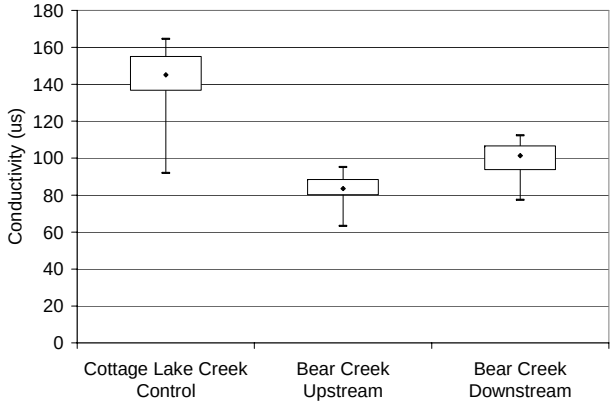
Table 3.9 Paradise Lake screening level fathead minnow 96 hour toxicity test results values and p values from long-rank tests comparing survivorship curves. Blank p-values are given for treatments with 100% survival in both the control and treatment because $X^2 = 0$ with -1 degrees of freedom.

Test Date	% Control Survival				% Treatment Survival				p value
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h	
4/17	100	100	100	100	100	100	100	100	
6/18	100	100	100	100	100	100	100	100	
7/16	97.5	97.5	97.5	97.5	100	100	100	100	0.317
7/30	100	100	100	100	100	100	100	100	
8/6/	100	100	100	100	100	100	100	100	
8/13	100	100	100	100	100	100	100	100	
8/20	100	100	100	100	100	100	77.5	35	< 0.001
8/27	100	100	100	100	100	100	100	100	
9/4	97.5	97.5	97.5	97.5	100	95	55	0	0
9/10	100	100	100	100	100	100	100	100	
9/12	100	100	100	100	100	100	100	100	
9/17	100	100	100	100	100	100	100	100	

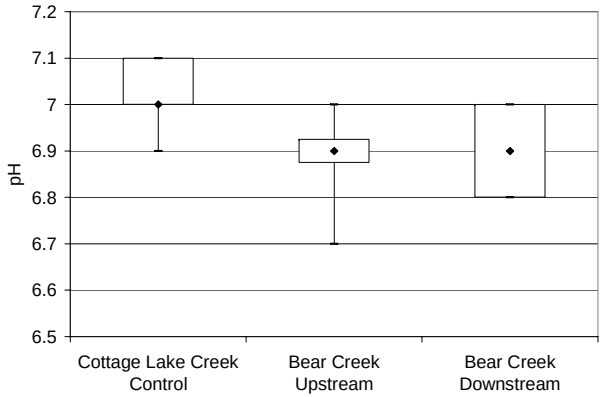
Table 3.10 96 hour screening level fathead minnow toxicity test results for sites tested in addition to Paradise Lake

	% Survival			
Test Date	Cottage Lake	Cottage Creek	Bear Creek - downstream	Bear Creek - upstream
9/12/2007	100	100	100	100
9/17/2007	100			100

A.



B



C.

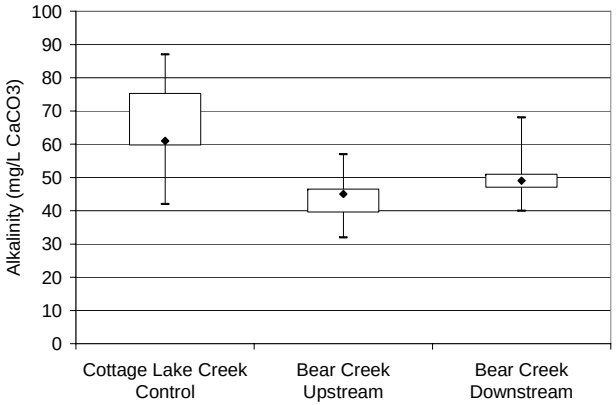


Figure 3.10 Water quality parameters collected periodically at the relocation sites: A) conductivity (n=10), B) pH (n=12), C) alkalinity (n=12). Median values are represented by a diamond, boxed values note the upper and lower quartile, and whiskers identify the minimum and maximum values recorded.

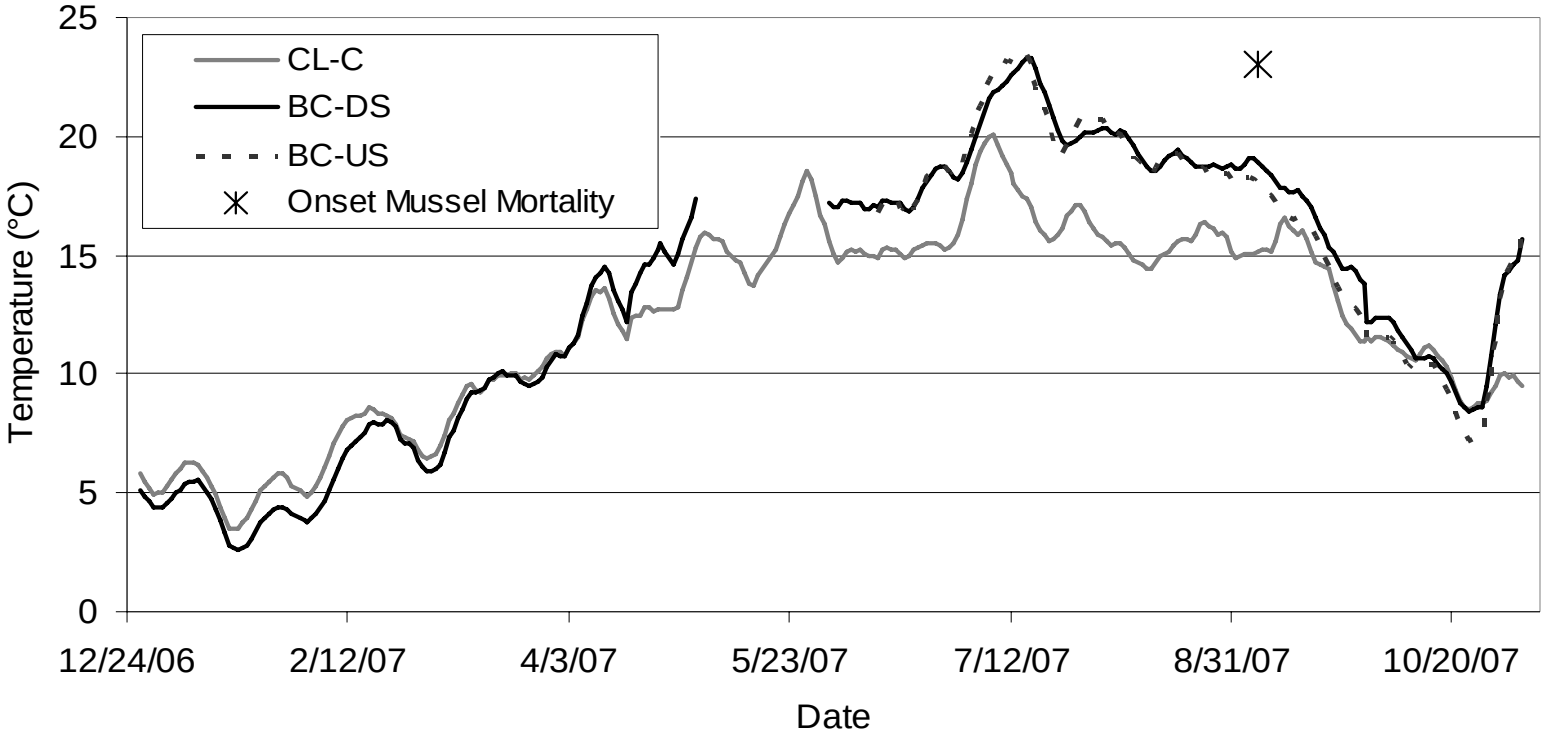
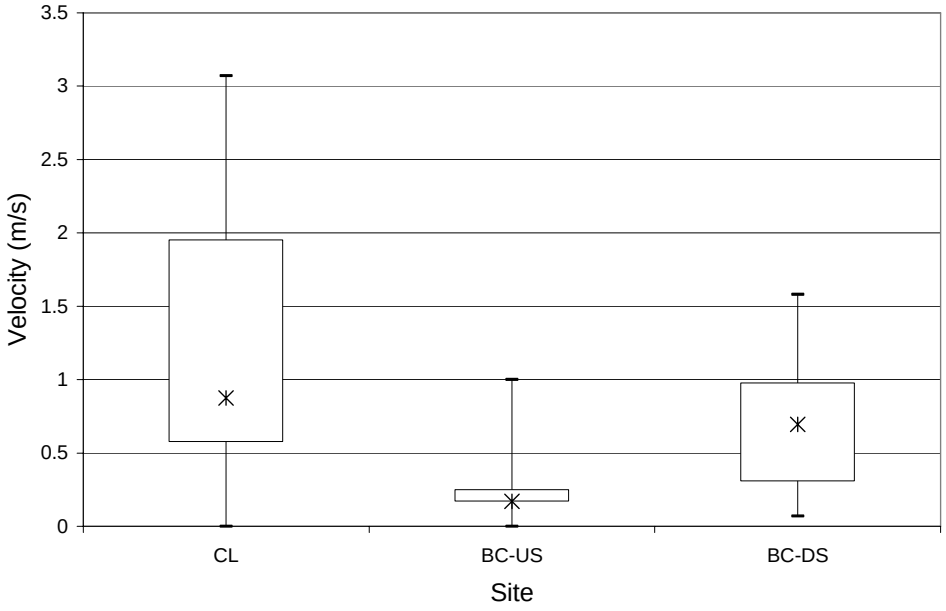
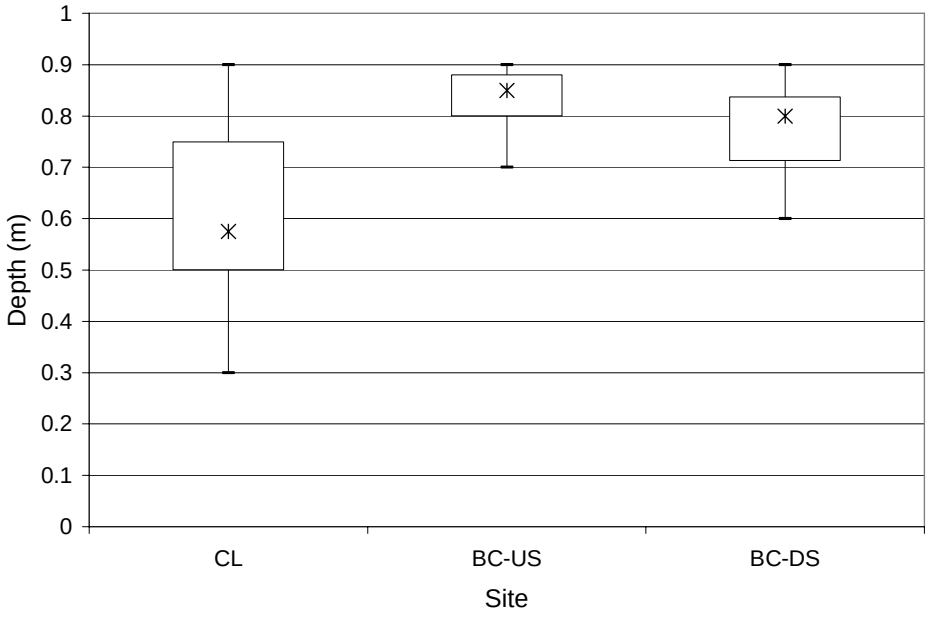


Figure 3.11 7-Day Running Max Temperature (°C). Asterisk indicates onset of *M. falcata* mortality.

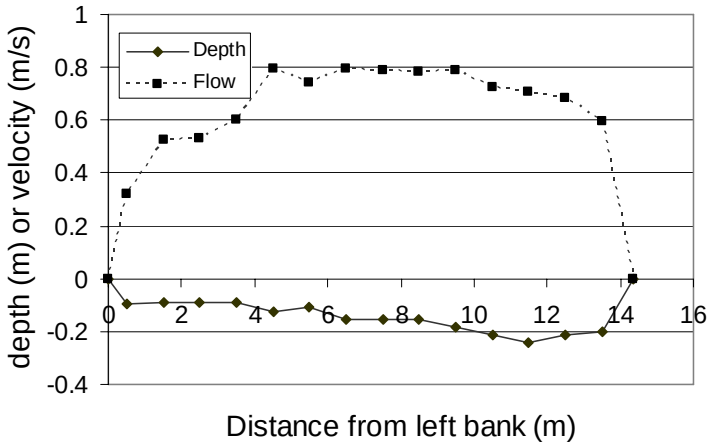


A

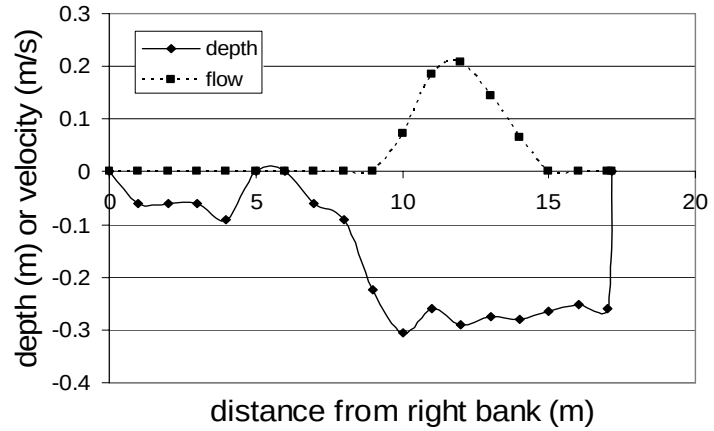


B.

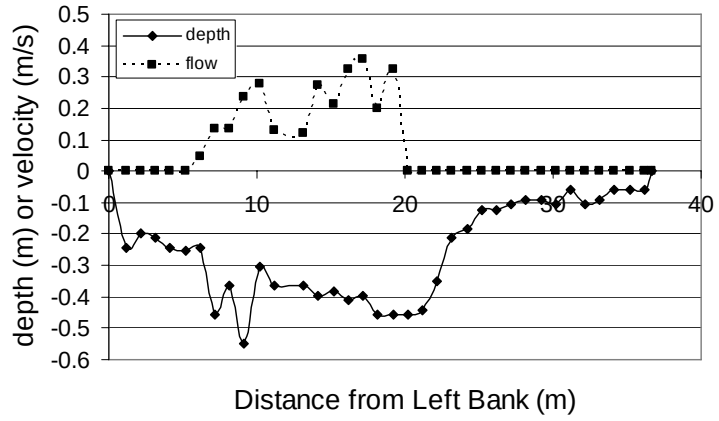
Figure 3.12 Stream velocity (A) and total depth (B) measurements surrounding *M. falcata* cages on October 3rd, 2007. Median values are represented by an asterisk, boxed values note the upper and lower quartile, and whiskers identify the minimum and maximum values recorded.



A.



B.



C.

Figure 3.13 Velocity and depth profiles recorded along cross-sections taken on October 3rd, 2007 for CL-C (A), BC-US (B) and BC-DS (C).

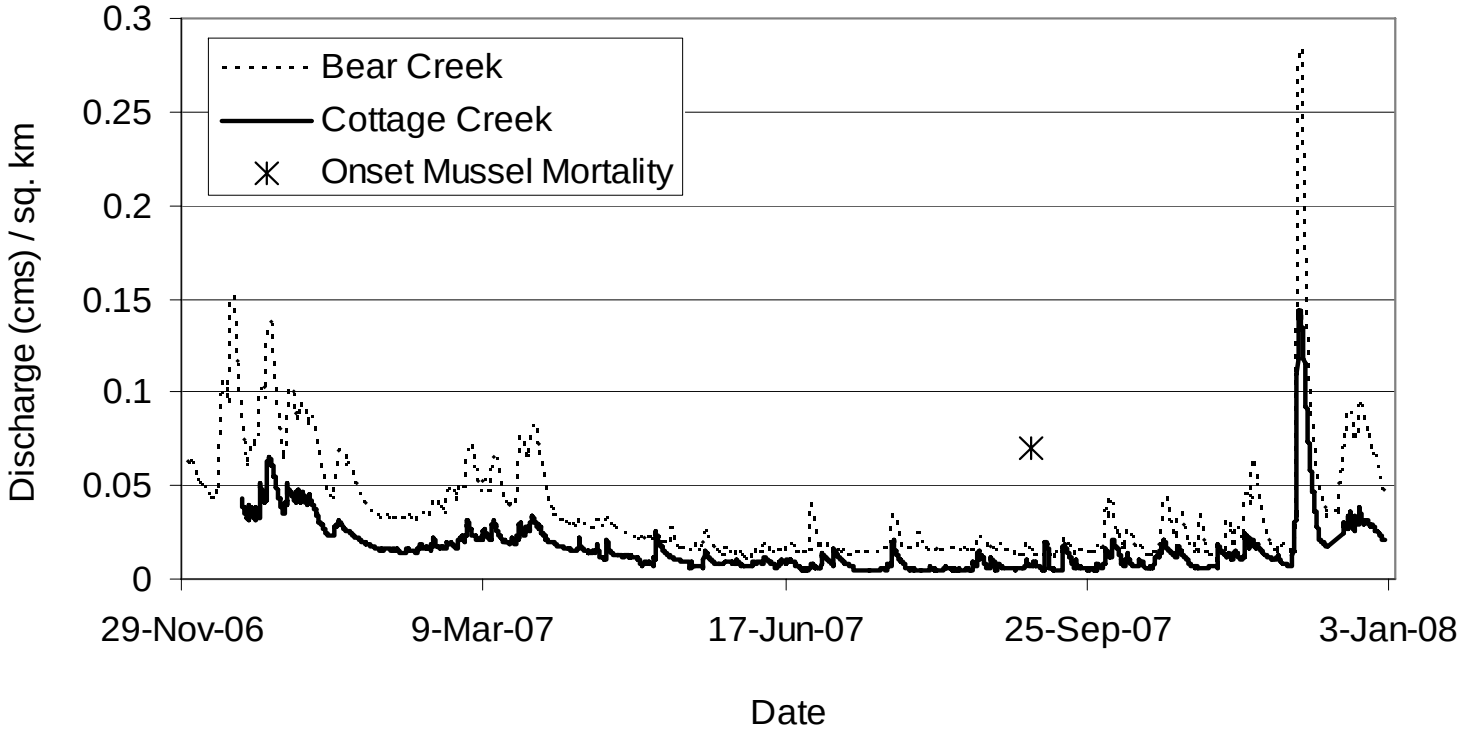


Figure 3.14 Discharge (cms) at Bear Creek (blue line) and Cottage Lake Creek (pink line) stream gages normalized by contributing area. Asterisk indicates onset of *M. falcata* mortality

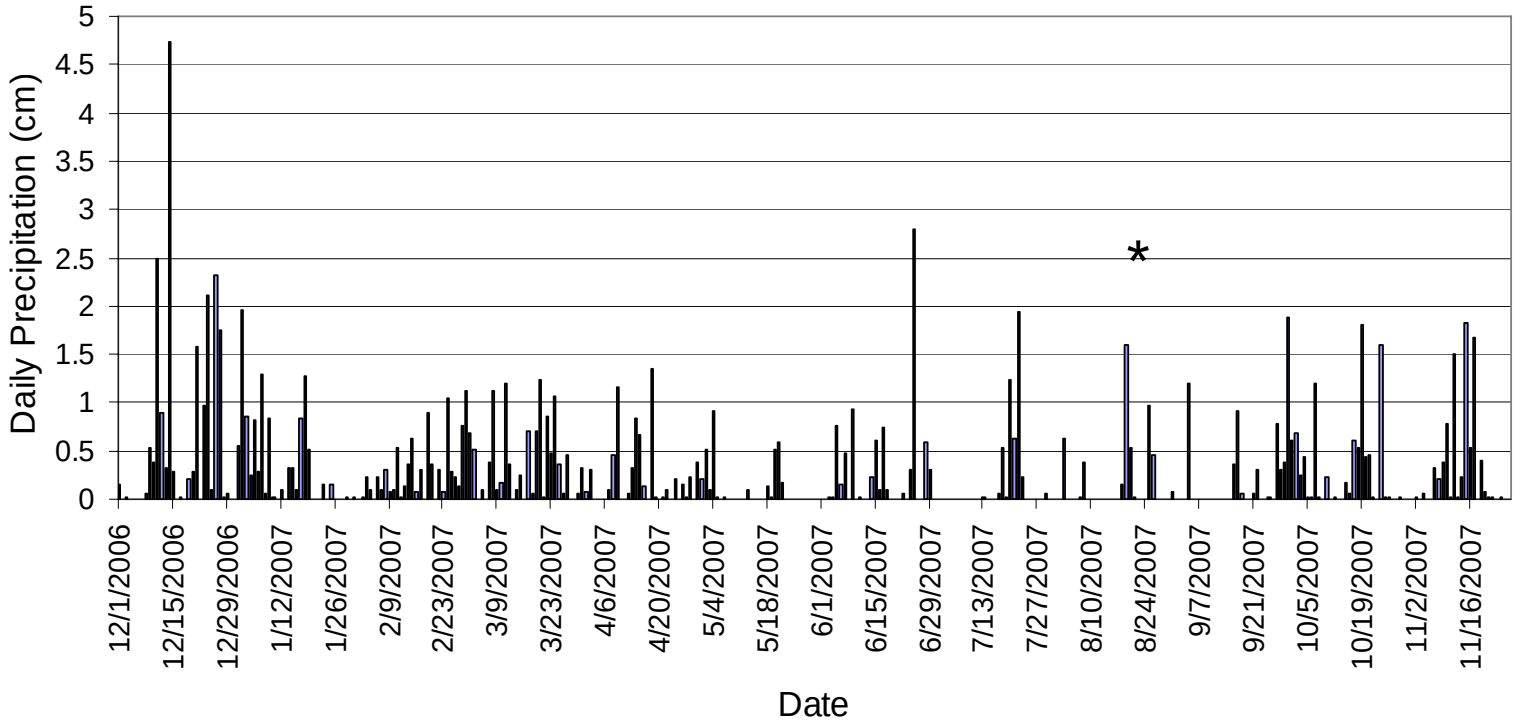


Figure 3.15 Daily precipitation (cm) at nearest rain gage to the Bear Creek subbasin from 12/1/06-12/31/07. Asterisk indicates onset of *M. falcata* mortality

Table 3.11 Characterization of rain events from early June 2007 to the onset of *M. falcata* mortality. Rain events were defined as total precipitation ≥ 0.5 cm and antecedent dry days as days with daily precipitation = 0 cm. The rain event immediately preceding observed fathead minnow toxicity (8/20 and 9/4) and *M. falcata* mortality (9/6) is highlighted in yellow.

Date of onset of rain	No. of continuous days of rain	Total rain fall (cm)	No. of antecedent dry days	Maximum rain intensity (cm/hr)
6/3/2007	5	1.45	11	0.81
6/9/2007	1	0.94	1	0.20
6/14/2007	5	1.78	2	0.51
6/24/2007	2	3.10	1	3.66
6/28/2007	2	0.89	2	0.61
7/17/2007	7	4.65	3	0.91
8/3/2007	1	0.64	4	0.51
8/7/2007	2	0.41	3	0.20
8/18/2007	4	2.31	9	0.81
8/25/2007	2	1.42	3	1.42
9/4/2007	1	1.19	3	1.32

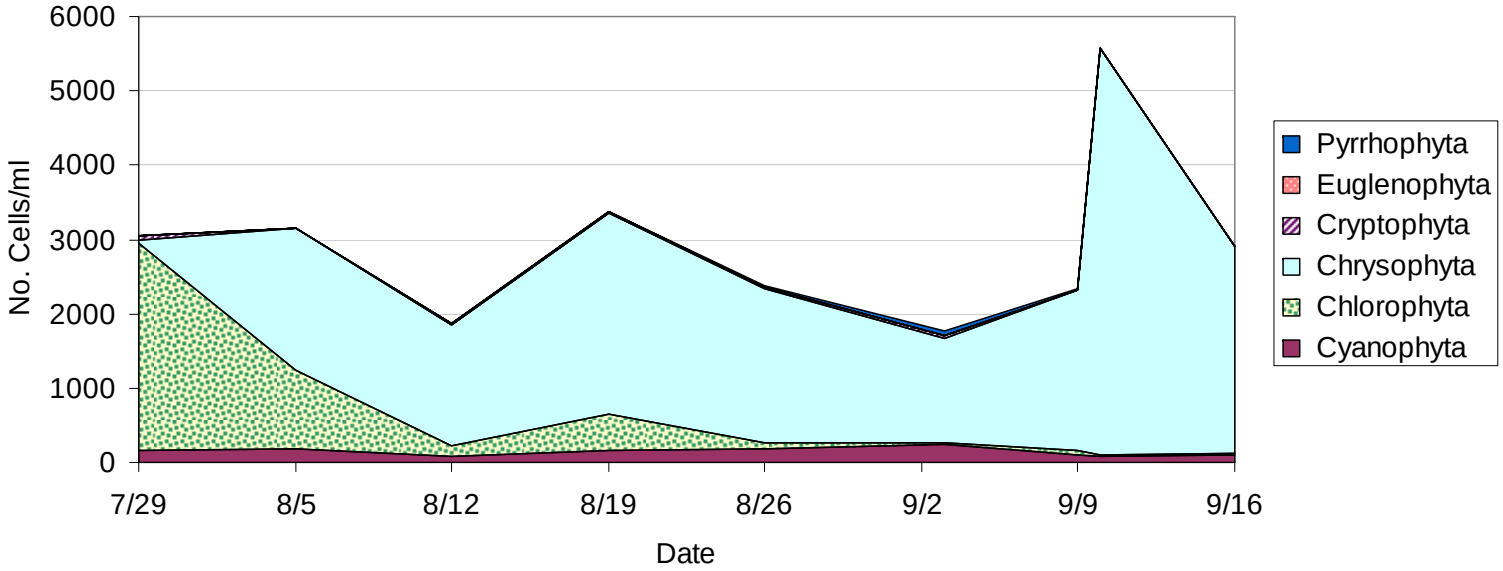


Figure 3.16 Phytoplankton community composition of filtered (25 µm) water collected from Paradise Lake for fathead minnow toxicity testing (2007)

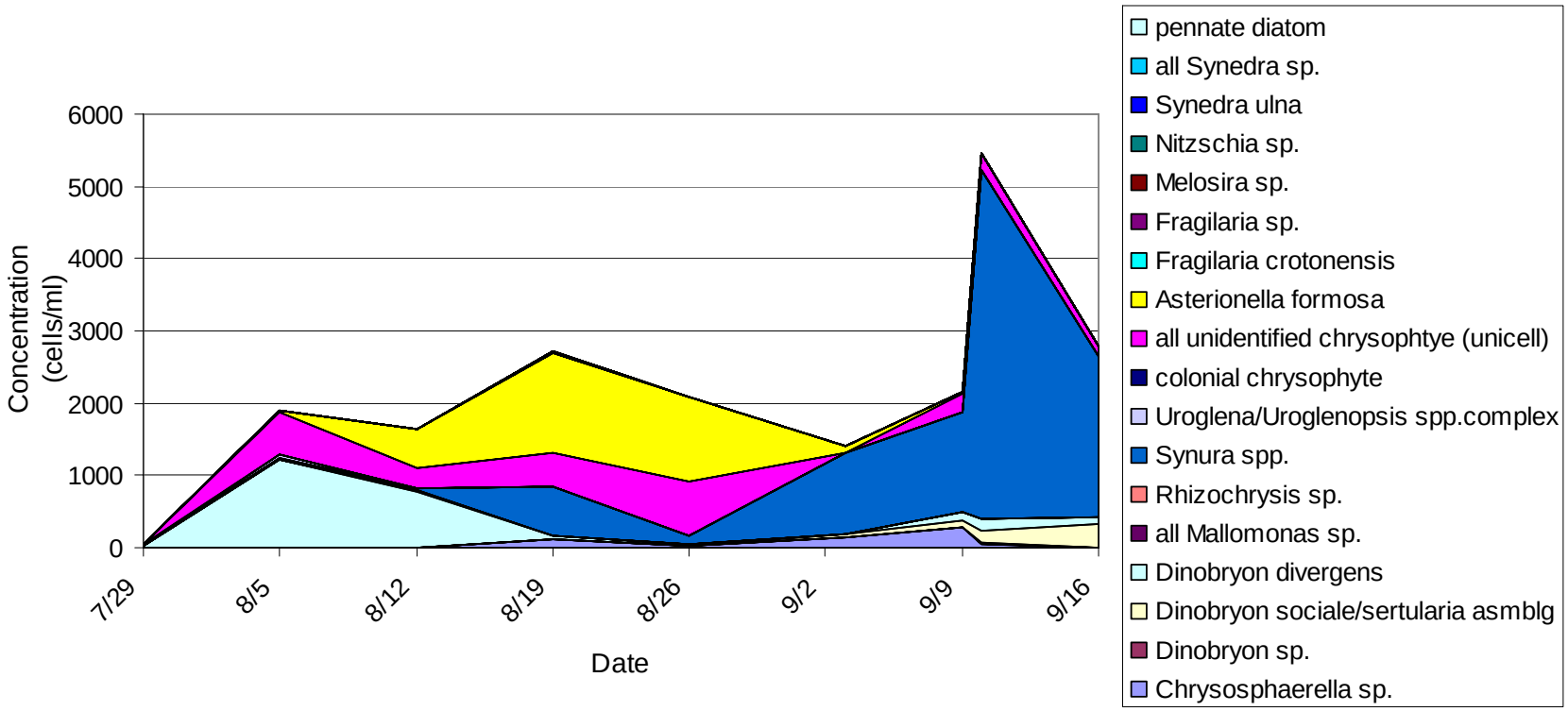


Figure 3.17 Chrysophyte composition of filtered (25 µm) water collected from Paradise Lake for fathead minnow toxicity test.

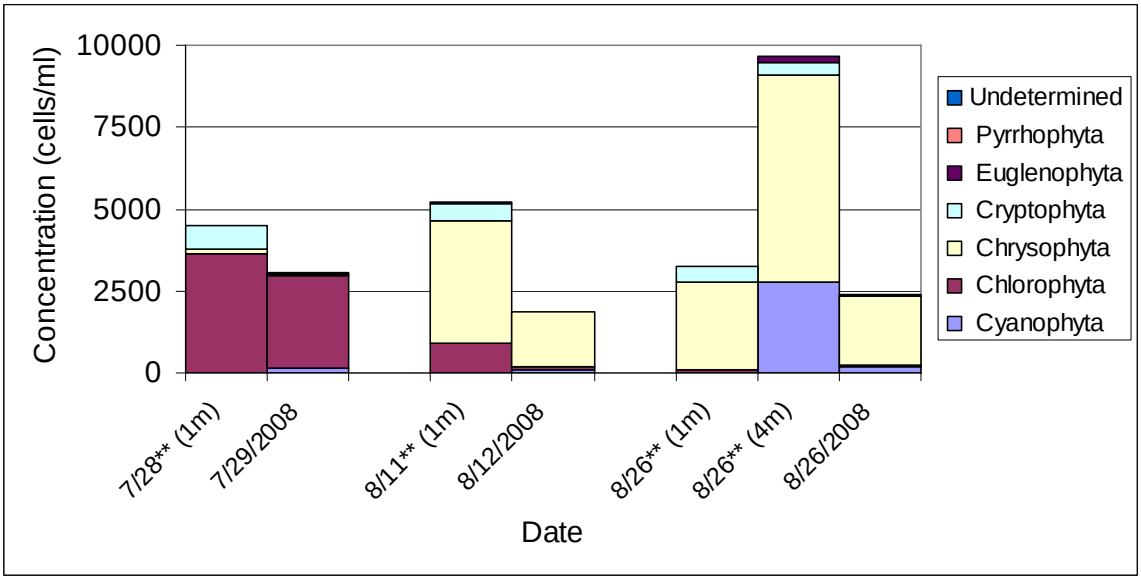
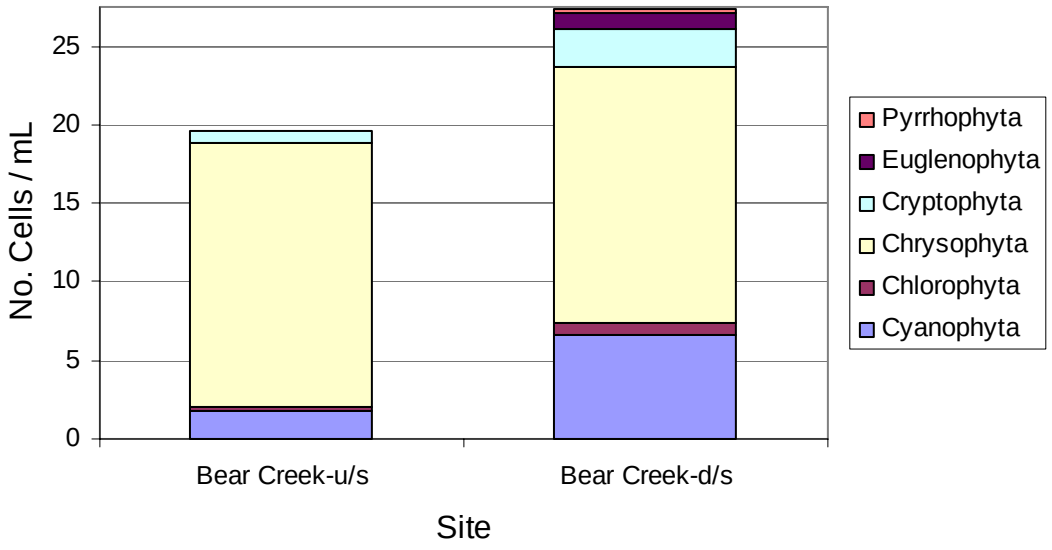
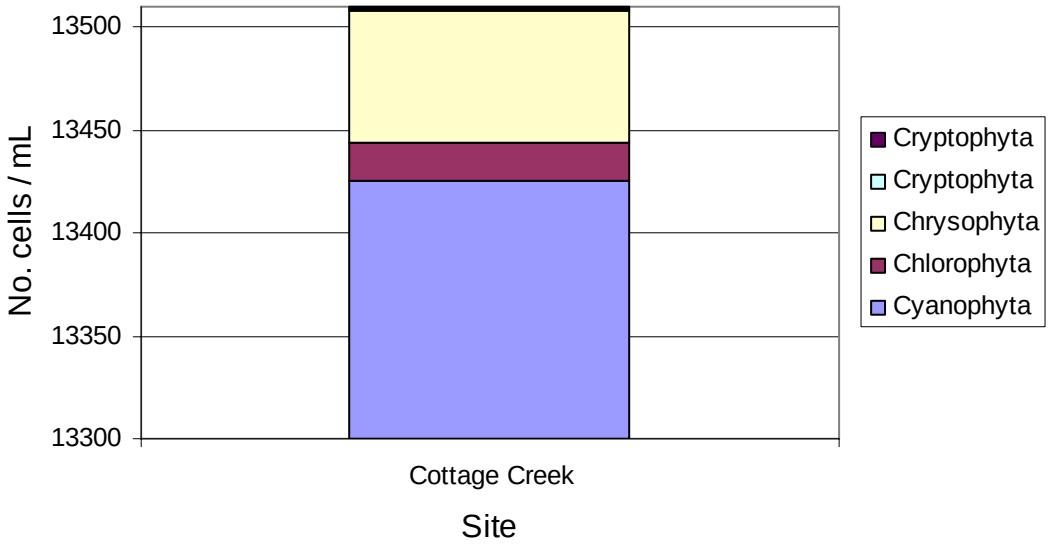


Figure 3.18 Plankton composition comparison between filtered and raw sample water collection methods, asterisks indicate raw sample collection method. Parenthesis values indicate depth of water collection for raw water samples. Sieved samples were collected at 1.0, 1.5, 2.0, and 5.0-m depth and composited.



A



B.

Figure 3.19 Phytoplankton community composition of water collected from Bear Creek (A) and Cottage Lake Creek (B) relocation sites 9/12/2007 for screening level fathead minnow toxicity testing.

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Chapter V: Conclusions

Results from this study indicate that heightened *M. falcata* mortality is occurring downstream, but not upstream, of Paradise Lake and that early fall is a critical time for this mortality. Two lines of evidence support these statements. The caged mussel relocation experiment supported observations made during the survey that suggested that non-predation related mortality occurred downstream, but not upstream of Paradise Lake. Mortality observed in both the caged mussel relocation and the screening level fathead minnow toxicity screening suggest that a toxic substance or stressor is occurring in Paradise Lake and the Bear Creek – downstream mussel relocation site starting in late August and early September.

The freshwater mussel survey revealed that most *M. falcata* beds in Bear Creek, downstream of Paradise Lake have experienced severe decline since they were last surveyed in 2002. Declines were the most drastic in the upper watershed, where live mussel densities were highest in 2002. The only sites where a decline was not observed were in the lower section of the creek where mussel densities were low in 2002, sites 8 and 10. Given the low numbers of mussels located lower in the watershed, conspicuous numbers of empty shells would not signal that conditions had suddenly become unfavorable for mussels. The failure to detect a change in the downstream reach indicates that conditions may differ between lower Bear Creek and upper Bear Creek – downstream of Paradise Lake. More explicit investigation that documents mortality in these downstream areas is needed to explore this possibility.

The size distribution of empty shells suggests that all size classes of mussels experienced mortality and the sudden abundance of empty shells did not reflect natural senescence among similarly aged individuals. The 2007 survey only examined a limited area upstream of Paradise Lake, but this area was more heterogeneous and contained 1 bed dominated by shells, 1 bed composed of mixed shells and live mussels, and 1 bed dominated by live mussels. At the shell-dominated

location, there was physical evidence that predation was driving mortality in this location. These signs of predation were not apparent at the resurveyed sites, suggesting different causes of mortality at the resurveyed sites and at the newly established sites.

The hypothesis that different mechanisms are driving *M. falcata* mortality downstream and upstream of Paradise Lake is supported by results from the caged mussel relocation. Differences in survivorship were highly significant between the BC-DS site when compared to either the BC-US site or the CL-C site. Additionally, mortality at the BC-DS site provided a strong signal of a significant stressor at the BC-DS site in early fall. Mussels that had been relocated to the site in late November/early December 2006 (Phase 1) and in early May 2007 (Phase 2) experienced approximately 40% mortality by early December 2007, with the onset of mortality in early fall, 2007.

Screening level fathead minnow bioassays suggested the presence of a toxin in Paradise Lake. The cause of the fathead minnow mortality was not determined and was not demonstrated by this investigation to be linked to the mussel mortality observed downstream of the lake. However, the temporal proximity of fathead minnow mortality to the onset of *M. falcata* mortality, combined with relocated *M. falcata* mortality downstream of the lake and not upstream of the lake, suggests that these events might be connected and merit further investigation.

Lower glycogen levels were observed in *M. falcata* individuals with visibly weakened adductor muscles, and differences in the digestive gland were noticed in dead mussels collected from the BC-DS location, but neither monitoring glycogen nor tissue morphology provided an early-warning that the BC-DS relocated mussels were stressed. The apparently good condition of the tissue morphology of some mussels collected after death, especially among the digestive gland epithelial cells, suggested that mortality was relatively rapid in mussels collected in September and October and

there was not a prolonged pathological sequence of events leading to mortality. Additional investigation of the surviving mussels may provide additional information about the unknown stressor and its mode of mortality.

This study addressed the following questions:

1. *How much M. falcata mortality has occurred and what is the spatial extent of die-offs along Bear Creek?*

In 9 of the 10 *M. falcata* baseline survey beds established in 2002, by 2007 at least 93% of the bed contained empty shells. The only location not dominated by empty shells was the furthest downstream bed, where mussels were sparse in both 2002 and 2007. In 2002, site 8 was dominated by empty shells, but in 2007 there was a statistically insignificant increase in the number of live animals encountered. The largest change in live mussel density between 2002 and 2007 was experienced in the 3 most upstream beds, where mussel densities were the highest in 2002. Due to the large number of empty shells observed at the resurveyed sites, changes in the number of live animals encountered are hypothesized to be driven by mortality. Out of the 3 beds surveyed upstream of Paradise Lake, 1 bed was dominated by empty shells. Field observations indicated that mortality at this location appeared to be driven by predation. In the beds downstream of Paradise Lake, mussels appeared to have died *in situ* and predation did not appear to be the primary cause of mortality.

2. *Do healthy M. falcata become diseased when relocated to Bear Creek?*

M. falcata were moved to 2 sites along Bear Creek: Downstream of Paradise Lake and upstream of Paradise Lake. Only mussels moved to the downstream site became diseased (as indicated by mortality).

3. *If healthy freshwater mussels become diseased when relocated to Bear Creek, when is the onset of symptoms and is there a pathological sequence of events that lead to mortality?*

The first indication that mussels had departed from their normal condition (were diseased) was the onset of mortality among mussels at the BC-DS site. Among both groups of mussels that had been at the relocation site for 9 (Phase 1) and 4 months (Phase 2), the onset of mortality occurred in early September, and mortality rates were highest in September and October. While mussels collected post-mortem were generally characterized by a highly vacuolated digestive gland, some increase in necrotic cells, and lower rates of intact gill, these tissue changes did not appear in BC-DS mussels collected before death.

4. *Could toxic algae be responsible for mussel mortality?*

While no algal species commonly known to be toxic were observed in Paradise Lake, the possibility of a toxic algae was not ruled out by this study. Significant toxicity observed in the screening level fathead minnow bioassays suggests the presence of a toxin or stressor in Paradise Lake. Toxicity was detected in the fathead minnow tests for Paradise Lake water at generally the same time *M. falcata* experienced the onset of mortality at the downstream site. However, a direct link between toxicity in Paradise Lake and downstream *M. falcata* mortality was not established by this investigation.

Suggestions for Further Research

Results from this study have established that conditions in Bear Creek are causing *M. falcata* mortality when relocated to a site downstream, but not upstream of Paradise Lake and that the cause of this mortality may originate in Paradise Lake or in proximity to Paradise Lake. It has not been determined what is causing mortality, or

if the cause of this mortality is part of a natural process (e.g., a freshwater algal toxin) or anthropogenic (e.g., point source pollution). The understanding of this problem would be increased by additional research that would continue to pinpoint the areas experiencing toxic effects and the timing of toxicity and a better understanding of the potential stressors. The relocated mussels and fathead minnows were sensitive indicators of a change in conditions, and broadened use of the bio-indicators are recommended for further investigation:

- Continue to monitor established caged mussels to see if the remaining mussels experience mortality and to identify time periods with the highest mortality rates.
- Explore other freshwater bivalve species that can be used as bio-indicators for mortality in the lentic environment that characterizes Paradise Lake. *Anodonta* mussels would be a good candidate, because they are native to the area, inhabit lakes, and have been observed in both Paradise Lake and at the BC-US site.
- Explore using a bivalve species that can be lab-cultured in for bioassay monitoring, including working to culture *M. falcata*. Lab-culture would allow a larger number of organisms to be used, enabling more replication over space and over time to investigate the toxic event.
- Replicate the caged-mussel study over time by deploying naïve caged mussels over consecutive years to determine if early fall is consistently the time when the onset of mortality occurs, and to investigate a relationship between environmental conditions such as water quality (contaminants), stream temperature and rainfall on an interannual time-scale.
- Replicate the caged-mussel over a broader spatial distribution, including sites in the inlet and outlet of Paradise Lake, and sites lower down in the watershed corresponding to each survey site.
- Conduct more extensive fathead minnow toxicity assays and conduct fathead minnow testing and caged-mussel relocations at the same site to help verify

that the fathead minnows and mussels are responding to the same toxic event. Increase the frequency of fathead minnow testing, range of dates when testing occurs (ideally year round) and extend the spatial area examined by adding multiple sites both upstream and downstream of Paradise Lake. Candidate sites include the inlet and outlet of Paradise Lake and sites corresponding to each survey site….this should be coupled with more water quality or algal toxin identification

- Continue to document the phytoplankton community in Paradise Lake, and document the phytoplankton community at the BC-US and BC-DS sites, and any other sites were caged mussel relocations are conducted.
- Utilize results from both bivalve and fathead minnow tests to direct additional water quality sampling, and archive water from fathead minnow tests for further analysis. Water filtered through C-18 solid-phase extraction cartridges and stored at -80ºC can be used to test for both potential algal toxins and organic contaminants.
- Complete a more comprehensive survey and more frequent mussel surveys in the Bear Creek drainage– include tributaries to Bear Creek, mainstem Bear Creek upstream of Paradise Lake, and Cottage Lake Creek. The Bear Creek subbasin is an urbanizing area, and closely monitoring mussel populations may provide an indication of whether these developments are harming aquatic resources. This study indicates that in order to detect the onset of decline in the mussel population monitoring needs to be conducted more frequently than every 5 years.

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Appendix A: Histology Examples for Reproductive Stage Characterizations

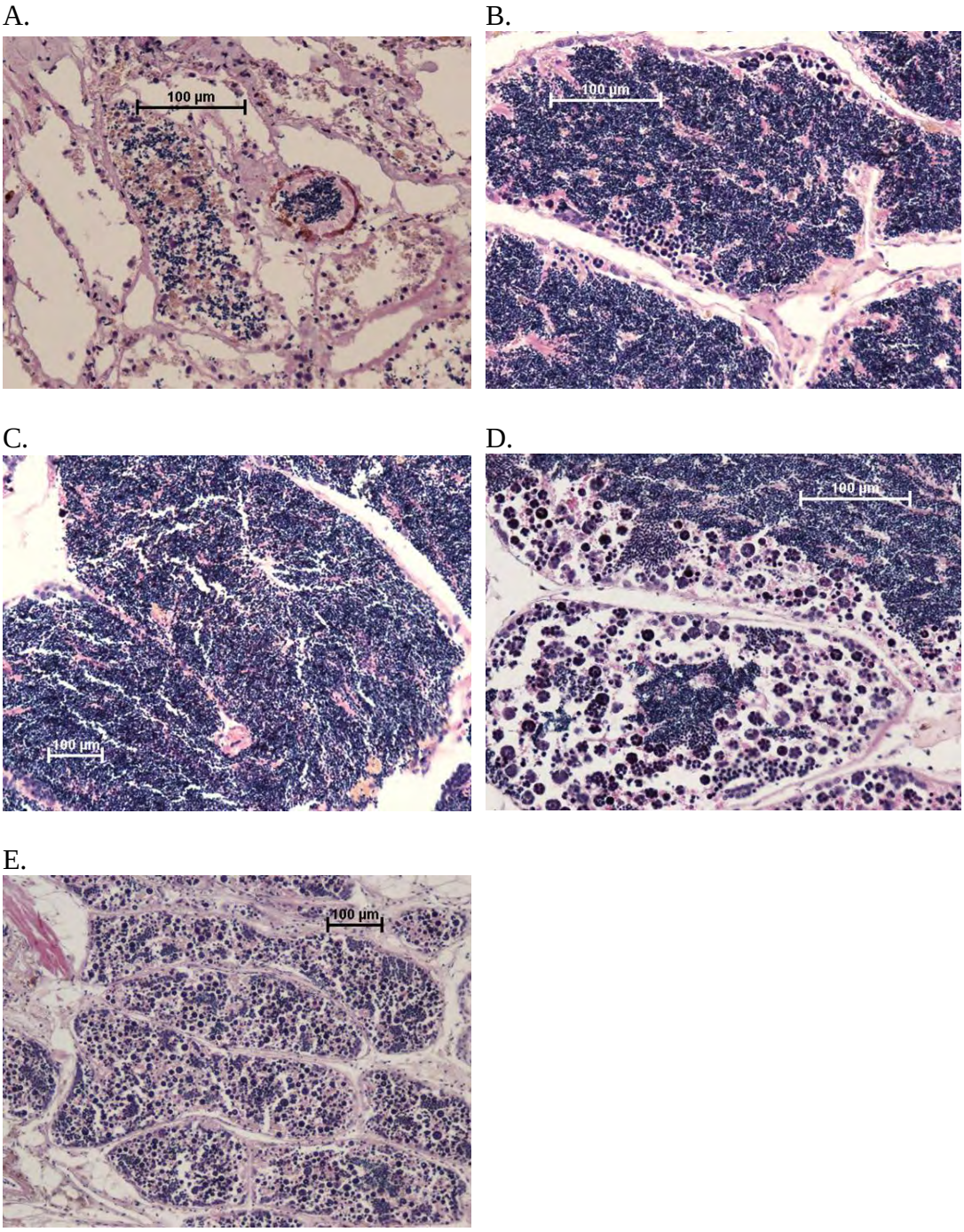


Figure A.1 Male reproductive stages observed among *M. falcata* collected during study period: A. stage 1 (20x), B. stage 2 (20x), C. stage 3 (20x), D. stage 4 (20x), E. stage 5 (10x).

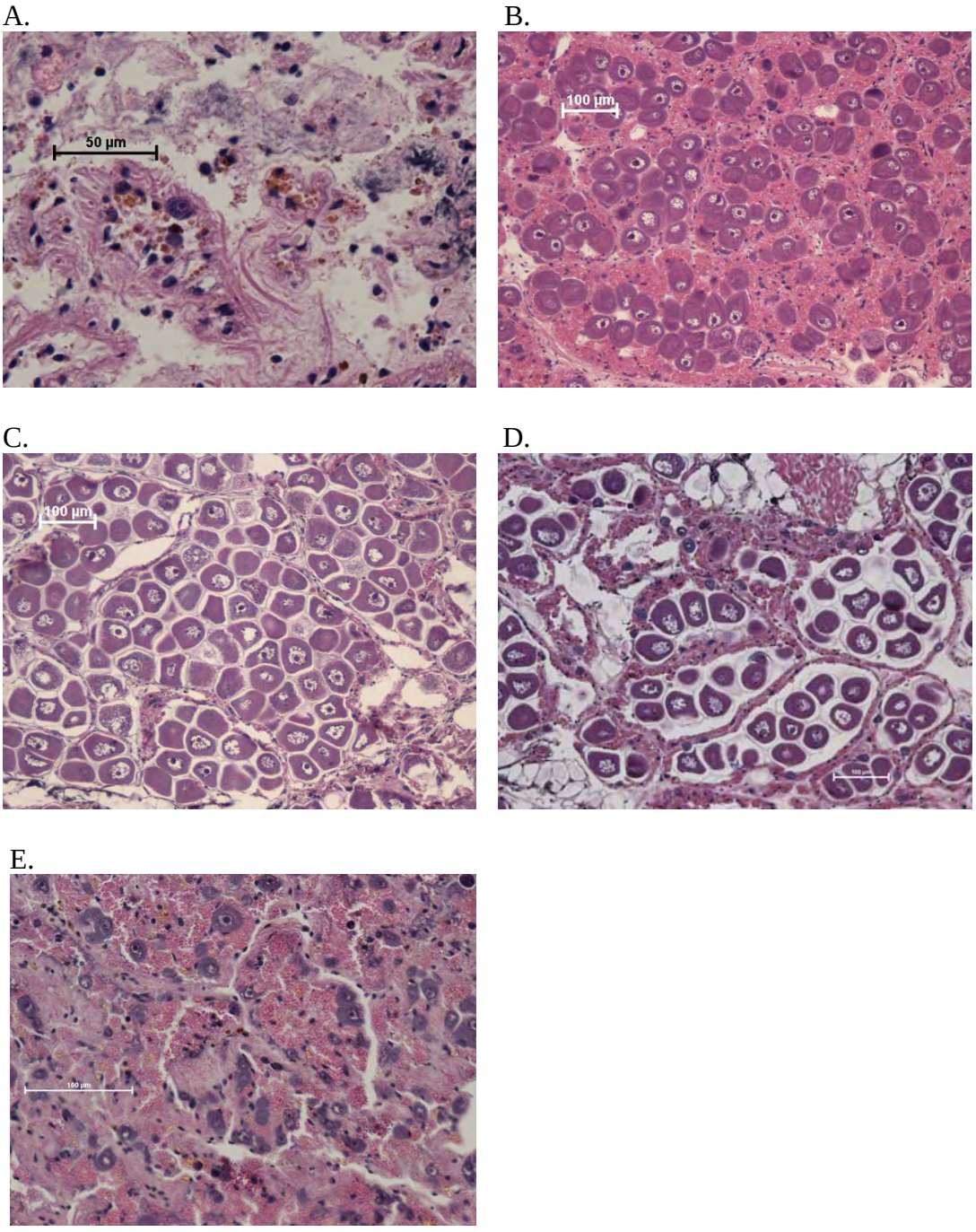


Figure A.2 Female reproductive stages observed among *M. falcata* collected during study period: A. stage 1 (40x), B. stage 2 (10x), C. stage 3 (10x), D. stage 4 (10x), E. stage 5 (20x).

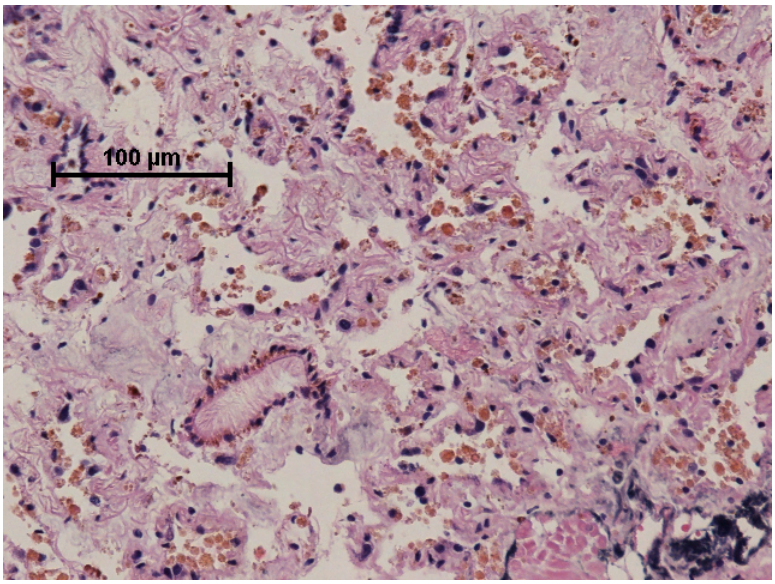


Figure A.3 Stage 0 reproductive stage among *M. falcata* collected during study period (20x)

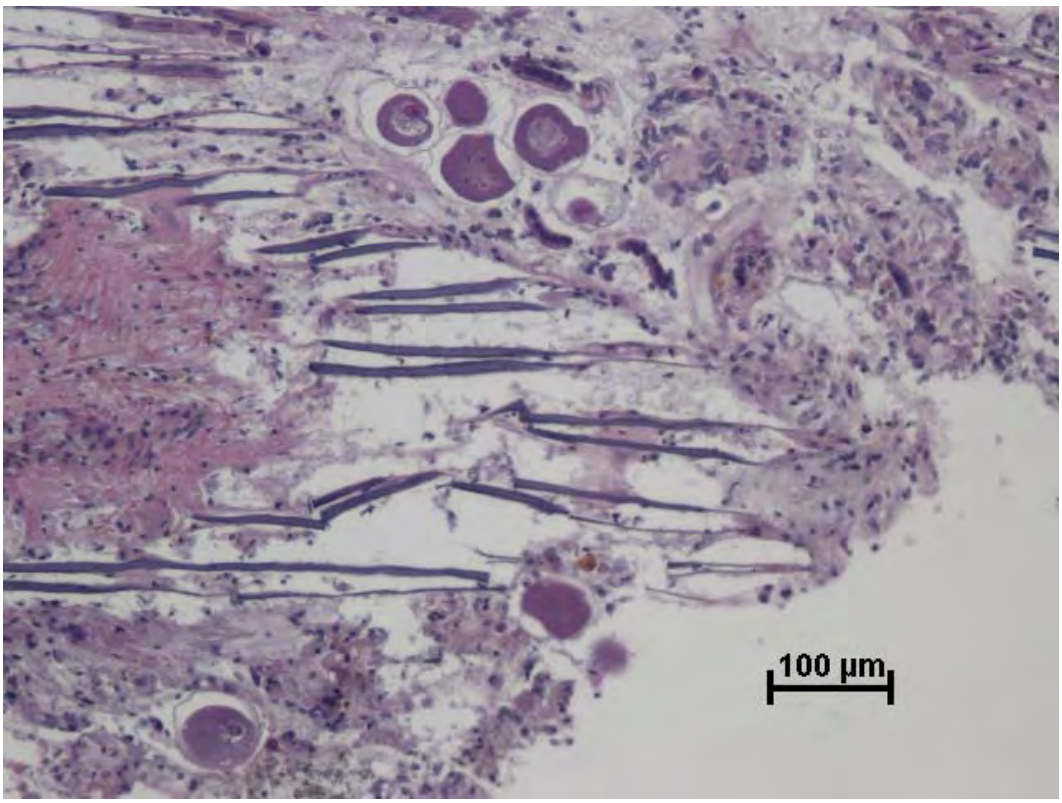
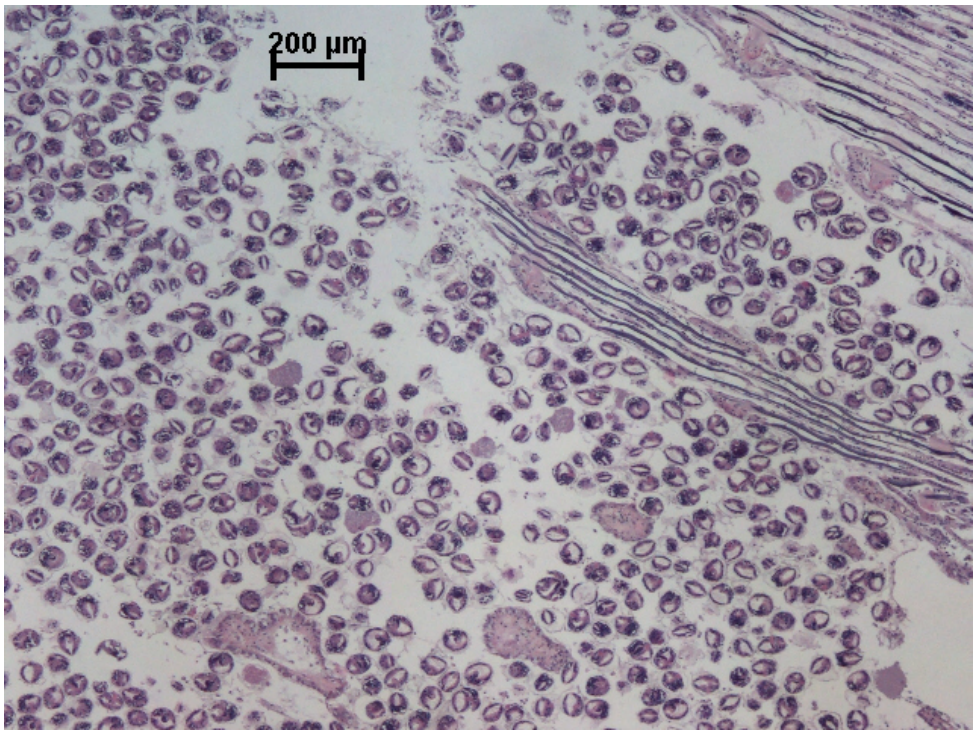


Figure A.4 Unfertilized eggs in marsupial gill (10X).

A.



B.

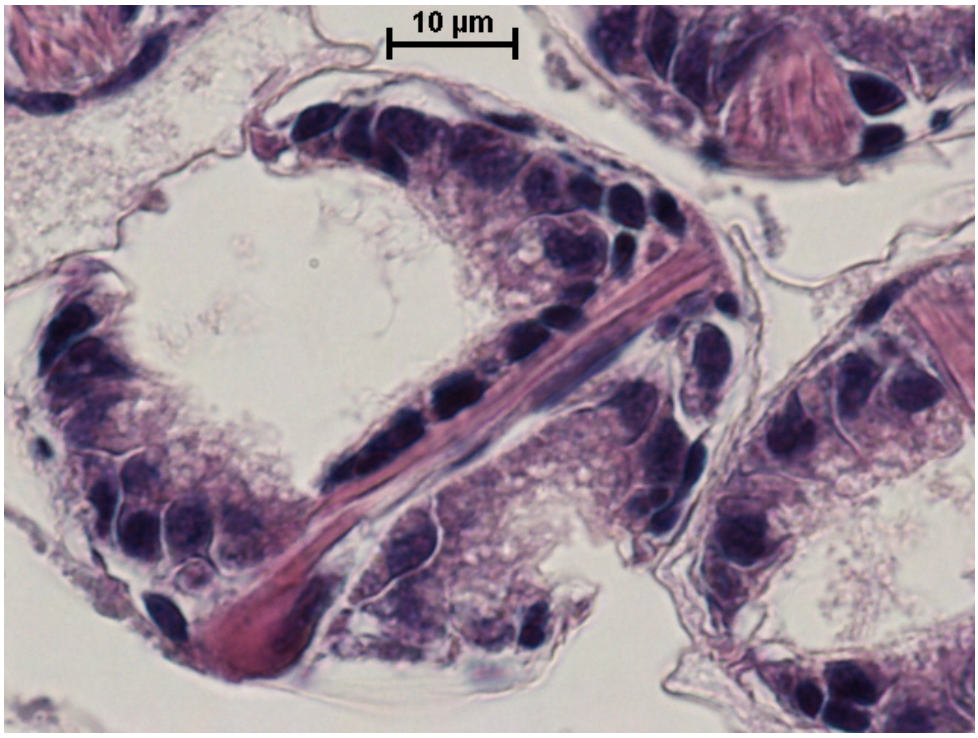


Figure A.5 Larvae observed in the visceral mass, 10X (A) and 100X (B).

Appendix B: Histology Examples for Digestive Gland and Gill Characterization

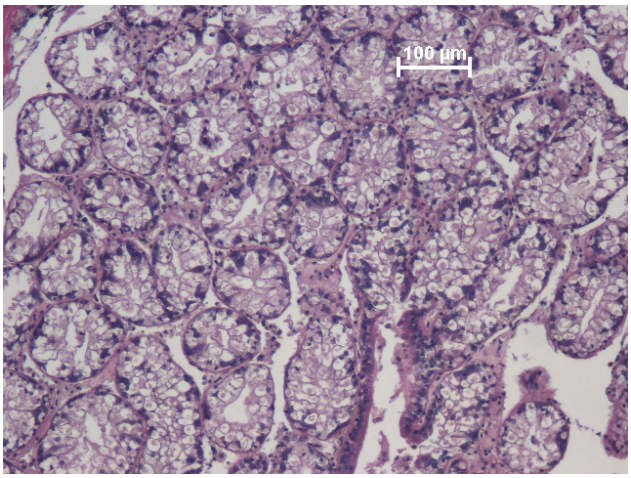


Figure B.1 High digestive gland vacuolization (10X).

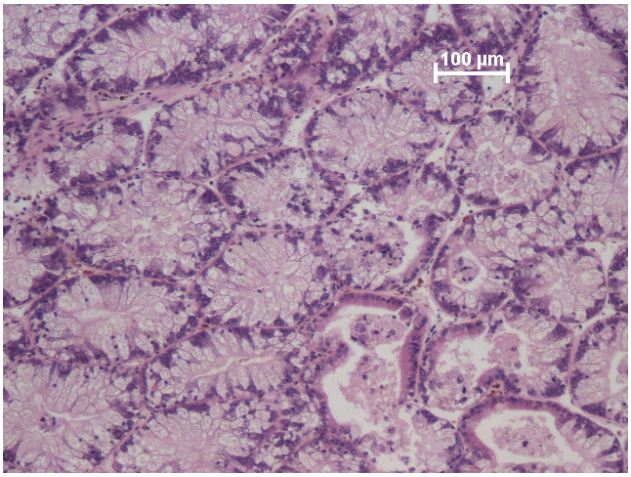


Figure B.2 Medium digestive gland vacuolization (10X).

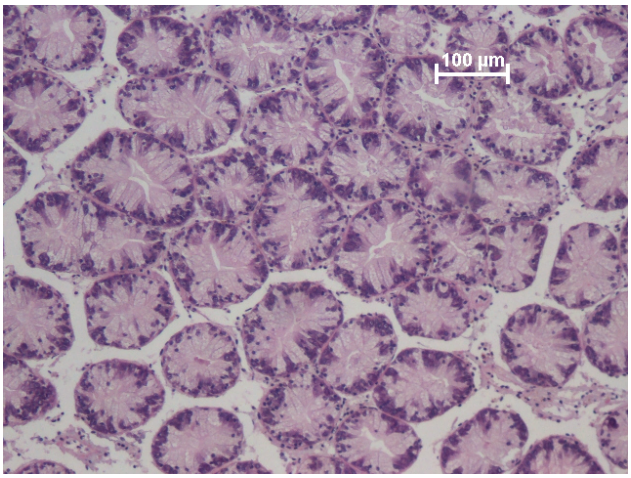


Figure B.3 Low digestive gland vacuolization (10X).

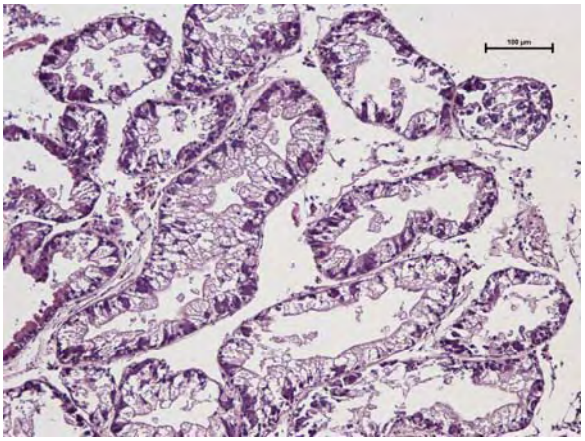


Figure B.4 High digestive gland dilation (10X)

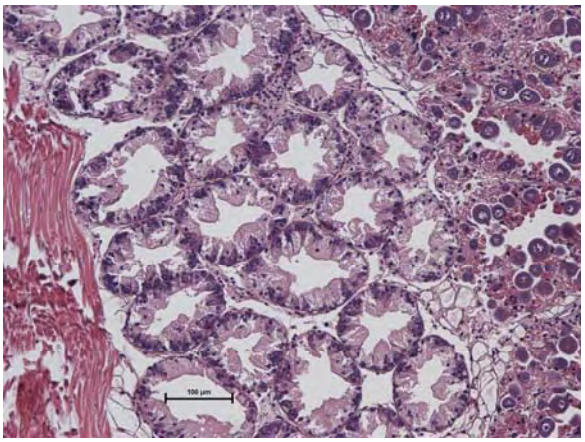


Figure B.5 Medium digestive gland dilation (10x)

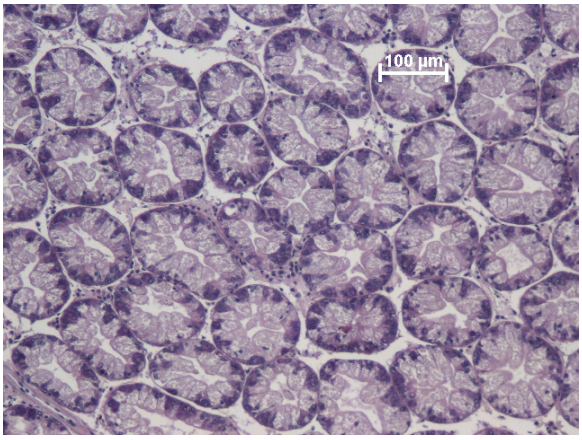


Figure B.6 Low digestive gland dilation (10X).

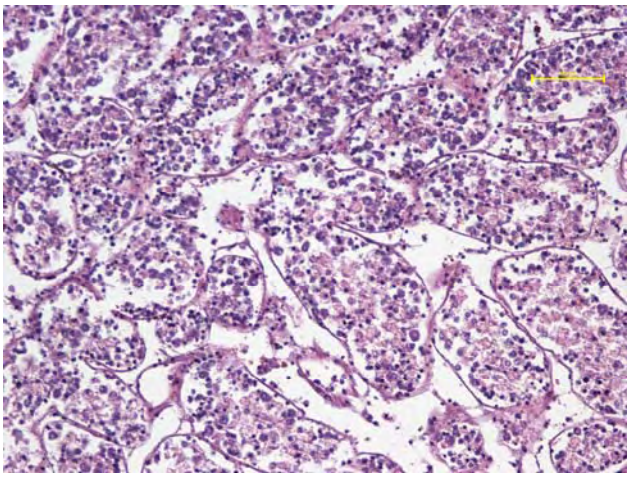


Figure B.7 High cell necrosis and sloughing

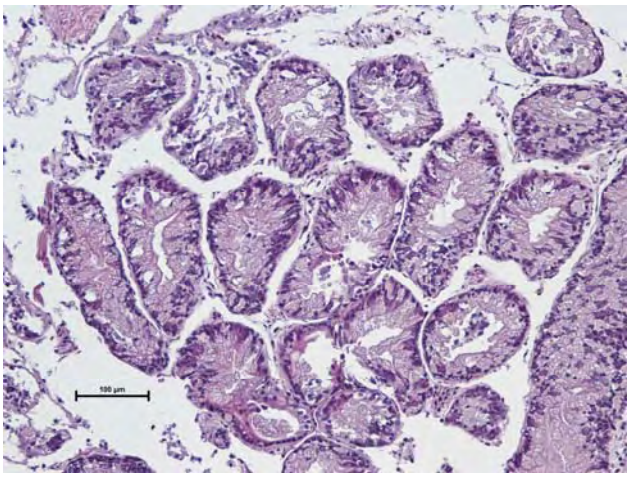


Figure B.8 Medium cell necrosis and sloughing

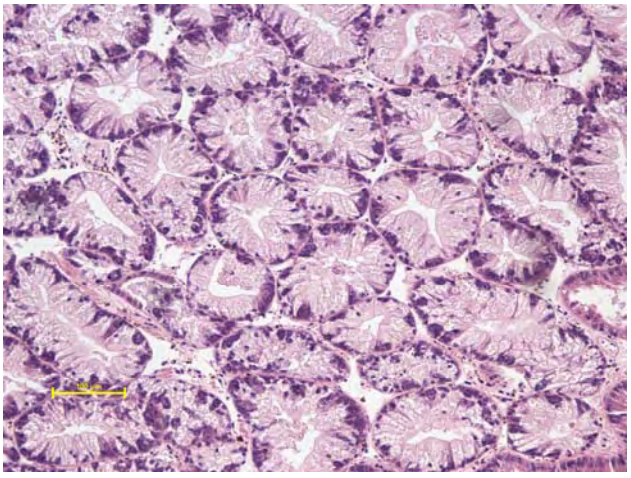


Figure B.9 Low cell necrosis and sloughing

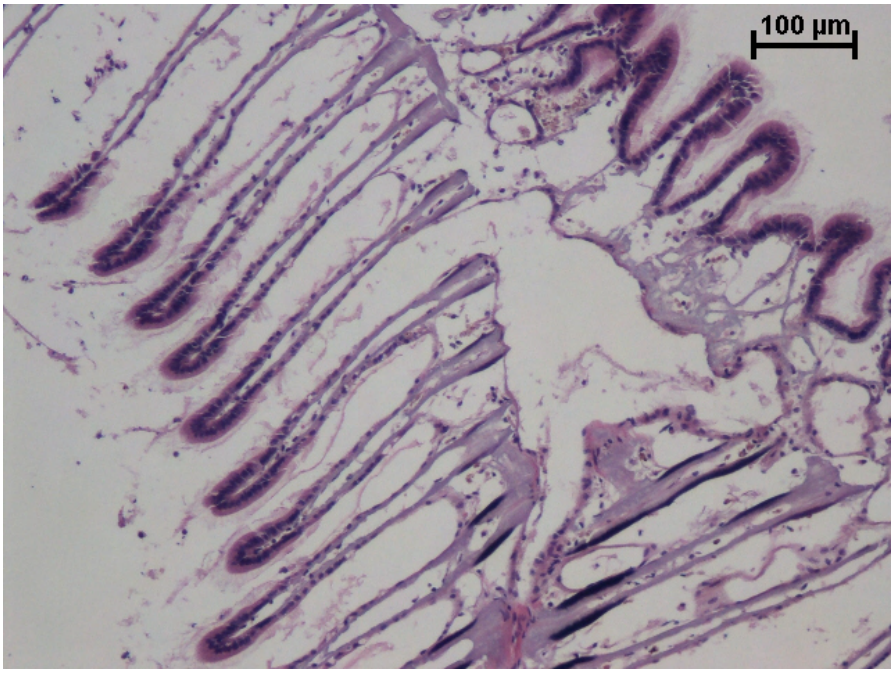


Figure B.10 Intact gill epithelium

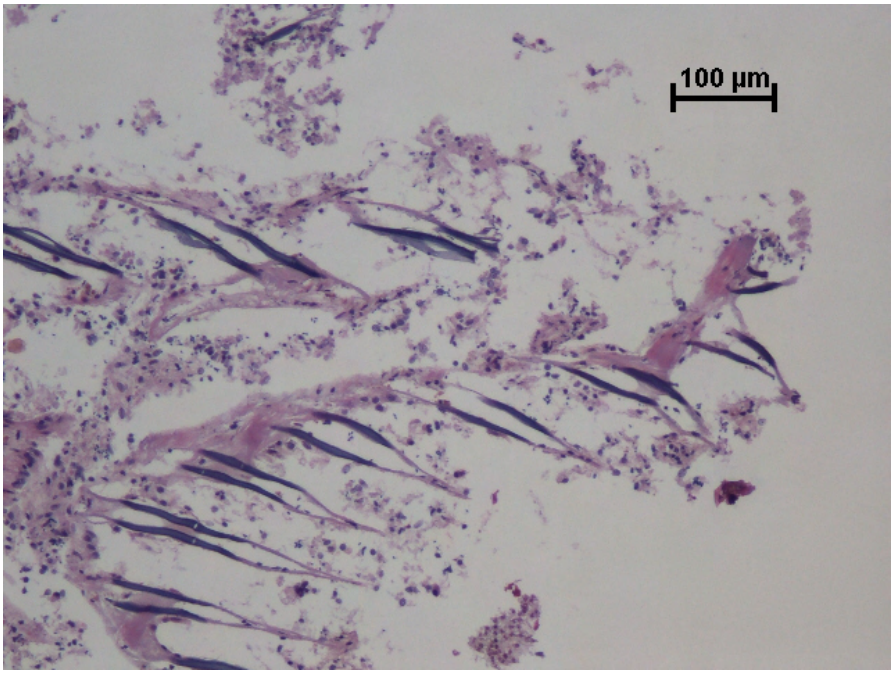


Figure B.11 Gill epithelium not intact