AN ABSTRACT OF THE THESIS OF

Richard W. Stocking for the degree of Master of Science in Microbiology presented on February 23, 2006.
Title: Distribution of *Ceratomyxa shasta* (Myxozoa) and Habitat Preference of the Polychaete Host, *Manayunkia speciosa* in the Klamath River

Abstract approved: _____

Jerri L. Bartholomew

Ceratomyxa shasta is a myxozoan parasite of salmonids and requires the freshwater polychaete, *Manayunkia speciosa* to complete its life cycle. The parasite's distribution is currently limited to the Pacific Northwest region of North America and has been reported to cause substantial losses of both wild and hatchery salmonids. The spatial and seasonal distribution of *C. shasta* can vary considerably both within and between river systems. This variation was thought to be a result of specific habitat requirements limiting polychaete distribution and abundance. Field studies were conducted in the Klamath River basin where *C. shasta* is suspected to have caused high losses in migrating juvenile salmonids. The purpose of this study was to document the host-parasite distribution of *C. shasta* in the river, assess its ability to cause disease, and study aspects of the polychaetes habitat ecology. This is the first study to report the broad-scale distribution of *M. speciosa* in a river and the various characteristics of those populations.

The seasonal distribution of *C. shasta* in the Klamath River was investigated by exposing separate groups of *C. shasta*-susceptible rainbow trout (*Oncorhynchus mykiss*)

at monthly intervals during the study. The spatial distribution was assessed by one basin-wide exposure. The ability of *C. shasta* to cause disease in native Klamath River salmonids was investigated by exposing fall Chinook salmon (*Oncorhynchus tshawytscha;* Iron Gate Hatchery strain), along with the known susceptible strains of rainbow trout. The distribution and habitat preferences of *M. speciosa* were investigated by collecting benthic habitat samples from a variety of habitat types. Where populations were identified, sampling was conducted at a finer scale to study aspects of those populations such as size, density, and age-structure. The distribution and prevalence of *C. shasta* infection in populations of *M. speciosa* were determined by using a pooled prevalence strategy on several polychaete populations throughout the Klamath River.

Ceratomyxa shasta, with few exceptions, was only detected in exposure groups from the main-stem Klamath. The parasite could be detected from April until November when water temperatures reached 6˚C. It is likely the parasite could be detected beyond the temporal limits of this study. Prevalence of infection was high with little mortality in rainbow trout exposures groups above Iron Gate dam (Upper Klamath River). Mortality was 100% for rainbow trout exposure groups below Iron Gate Dam (Lower Klamath River). Fall Chinook salmon demonstrated a high level of resistance to the parasite above Iron Gate Dam compared to the rainbow trout, but suffered nearly 50% mortality below the dam. This suggests that resistance of native stocks to the parasite can be overwhelmed in the Lower Klamath River and provides further evidence that infectious dose is high relative to the Upper Klamath River.

The polychaete host, *M. speciosa*, was found to occur throughout the Klamath River and was often located in slow flowing depositional habitats such as pools and reservoirs. River populations were highly aggregated into small areas whereas reservoir populations were large, widespread and centered at the inflow area. Sand-organic matter substrates and mat-forming epilithic algae were primary microhabitats. Flow velocity, habitat stability and life traits such as dispersal ability appear to be primary factors limiting distribution and abundance of the polychaete. Populations of *M. speciosa* tested for the prevalence of *C. shasta* infection demonstrate a low mean prevalence of 0.27% with areas of elevated infection (4.8 and 8.3%) located just downstream of Iron Gate Dam. This suggests that this area may be the primary source of infectious actinospores contributing to the high juvenile salmonid mortality observed in the Lower Klamath River.

Distribution of *Ceratomyxa shasta* (Myxozoa) and Habitat Preference of the Polychaete Host, *Manayunkia speciosa* in the Klamath River

by
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APPROVED:

Major Professor, representing Microbiology

Chair of the Department of Microbiology

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Richard W. Stocking, Author

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CONTRIBUTION OF AUTHORS

Dr. Jerri L. Bartholomew was involved in the entire study process, including the sample design, interpretation of data and development of manuscripts. Dr. Rich Holt was involved in field and lab aspects of the fish exposure studies and development of manuscript. Scott Foott provided assistance in the field and manuscript comments. Harriet Lorz was involved in the Puget Sound fish exposure study, manuscript development and assisted with maintenance of experimental fish.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDIX FIGURES

LIST OF APPENDIX FIGURES (Continued)

Distribution of *Ceratomyxa shasta* (Myxozoa) and Habitat Preference of the Polychaete Host, *Manayunkia speciosa* in the Klamath River

CHAPTER 1: INTRODUCTION

Ceratomyxa shasta

Ceratomyxa shasta Noble, 1950 is a myxozoan parasite with two distinct spore stages alternating between two obligate hosts (Bartholomew et al. 1997). The myxospore stage, which develops in the salmonid host, is infective to the polychaete host *Manayunkia speciosa* Liedy 1858. The actinospore stage, infective for salmonids, develops in the polychaete host. In the fish, *C. shasta* migrates to the intestinal tissues causing necrosis and hemorrhaging. Additional clinical signs include anemia, exophthalmia (protrusion of the eye) and swelling of the gut due to excessive ascites, a condition referred to as ceratomyxosis. Resistance to *C. shasta* has been documented in salmonid species that historically occur where the parasite is enzootic. The mechanisms of this resistance are not entirely known but are genetically inherited as crosses between susceptible and resistant strains produce offspring with intermediate resistance (Bartholomew 1998; Bartholomew et al. 2001). Studies have demonstrated that resistant strains can succumb to infection with an increase in exposure duration indicating that resistance can be overwhelmed when the exposure dose is high (Ratliff 1981; Ibarra et al. 1994). The rate of disease progression in susceptible species is positively correlated with

water temperature during the latent period (period between infection and development of clinical signs of disease) (Udey et al. 1975).

DIAGNOSIS OF *Ceratomyxa shasta*

Detection of *C. shasta* in fish has been refined to the point where even early presporogonic stages can be identified. Several techniques can be used to detect presence of *C. shasta* in fish such as the indirect fluorescent antibody technique (IFAT) and histology (Bartholomew et al. 2004). However, this review will only cover techniques used during this study. Identification of the characteristic kidney bean-shaped myxospore from intestinal material by light microscopy constitutes visual verification of infection. If mature myxospores are not detected, infection can be determined by assay of a portion of the gut tissue by polymerase chain reaction (PCR) following procedures developed Palenzuela and Bartholomew (2002). The PCR assay is a highly sensitive and accurate means of detecting the presence *C. shasta*. The primers used are highly specific for the 18sRNA gene of *C. shasta* and do not amplify DNA from other myxozoan species (Palenzuela et al. 1999).

In the environment, presence of the actinospore can be detected by exposing known susceptible strains of rainbow trout (*Oncorhynchus mykiss*) in waters of interest and then monitoring the exposure groups for clinical disease and infection, as described above. By using two or more species whose known susceptibilities vary and by adjusting the exposure time, it is possible to qualitatively assess relative actinospore concentrations. For areas where the actinospore dose is suspected to be very low,

increasing the exposure duration increases the chance of detecting the parasite. Parameters such as prevalence of infection (percentage of fish infected), mortality (percentage of fish that died due to infection) and mean time to death (average number of days mortality occurred) are used to estimate parasite distribution and relative abundance the environment.

DISTRIBUTION AND IMPACTS OF *Ceratomyxa shasta*

The salmonid parasite, *C. shasta*, is enzootic to many of the large river systems in the Pacific Northwest including the Columbia River basin as well as the Fraser, Klamath, Rogue and Sacramento rivers (Hoffmaster et al 1988; Bartholomew 1989b). However, its distribution within these systems varies considerably both spatially and temporally. For example, the parasite could be detected in the main-stem of the Columbia River upstream to the Snake River confluence and in the Snake River upstream to Oxbow Reservoir. Similar studies have demonstrated *C. shasta* in the Rogue River up to Rkm 196.3 and in the Willamette River (a tributary of the Columbia River) upstream to Rkm 260.7 (Bartholomew 1989). These data suggest that the polychaete host is restricted by some unidentified factors limiting its distribution. In addition, the geographic isolation of *C. shasta* to these regions indicates that the polychaete host may also be confined to certain Pacific Northwest watersheds.

Exposure studies conducted over time demonstrate that occurrence of the actinospore stage is a function of season (Ching and Munday 1983; Hendrickson et al. 1989). Onset of the infection cycle varies from system to system but generally begins in

April or May when water temperatures reach about 10˚C and continues into November or December when water temperatures drop to 4 - 6˚C. As water temperature increases the infection prevalence increases and mean time to death decreases (Ching and Munday 1983). Likewise, as water temperature decreases, the mean time to death increases, however, infection prevalence can remain high indicating that infectious dose changes in response to water temperature.

The effect of *C. shasta* infection on native salmonids varies from season to season but has been connected to juvenile and pre-spawning adult mortality in the Columbia River system (Bartholomew 1998; Bartholomew et al. 1992; Sanders et al. 1970). Studies have documented as many as 29% of migrating smolts infected with *C. shasta* suggesting that substantial mortality may occur prior to entering the estuary (Jacobson et al. 2002). Treatments for ceratomyxosis are limited because infection generally occurs in the natural environment. Hatcheries using waters containing the actinospore may use ozone treatment to reduce incidence of disease, however, it has been suggested that stocking salmonids naturally resistant to *C. shasta* is the best management practice (Bartholomew 1998). The impacts of *C. shasta* on stocks of salmonids in other river systems have not been documented as well as on Columbia River stocks. For example, Hendrickson et al. (1989) documented the seasonal occurrence of *C. shasta* in rivers of northern California but did not report estimates of prevalence or mortality. The main-stem Klamath River was found to be infectious for most of the year. The impacts of *C. shasta* on Klamath River salmonids remained unclear until 2001 when the culmination of drought conditions and high losses of juvenile Chinook salmon demonstrating signs of ceratomyxosis (Foott

et al 2002) prompted a series of studies designed to understand the life cycle and estimate the contribution of *C. shasta* to salmon mortality in this system.

KLAMATH RIVER: BACKGROUND

In the field of fisheries, few species receive so much attention as the wild and native stocks of salmonids in the Pacific Northwest, which are increasingly under pressure from loss of habitat, over fishing and hatchery practices. This is especially evident for the heavily depleted stocks of wild salmon in the Klamath River basin which once boasted the third largest salmon fishery along the Pacific coast of North America (Snyder 1931, Hamilton et al. 2005). In the Klamath region, the struggle for water between the agriculture industry, hydroelectric industry, fishermen and tribal fisheries, would escalate to near violence during a severe drought in 2001 when the Bureau of Reclamation was prompted to move Klamath River water from agriculture back to the river to aid a struggling run of returning adult salmonids (Nation Research Council 2004). In 2002, the situation worsened when greater than 33,000 pre-spawning adult fall Chinook salmon (*Oncorhynchus tshawytscha*) died as a result of elevated water temperatures and massive infections from a variety of pathogens (Nation Research Council 2004). Reports of out-migrating juvenile Chinook salmon succumbing to ceratomyxosis captured media attention prompting concern about the future of the fishery (John Driscoll, Eureka Times-Standard, June 20, 2004). Monitoring studies conducted by the United States Fish and Wildlife Service (USFWS) found that between 28 and 50% of smolts caught in screw-traps in the Lower Klamath River were infected with *C. shasta*

(Foott et al. 2002). Most of the infected fish that had been examined by histology exhibited severe tissue damage suggesting high mortality was likely to occur during the migration period as a result of ceratomyxosis. Biologists implicated *C. shasta* as a priority pathogen and a potentially limiting factor to the survival of Klamath River salmonids (Foott et al. 2004).

STUDY OBJECTIVES

The mortality of *C. shasta* on Klamath River salmonids are suspected to have increased within the last decade. It is not clear if these "elevated" impacts are a result of drought conditions, changes to river ecology brought about by the hydroelectric projects, or simply heightened awareness stemming from improved monitoring activities. The goal of this research was to document the distribution and relative abundance of *C. shasta* in the Klamath River and elucidate an important factor of the life cycle that has not been studied: the distribution and habitat requirements of the polychaete host, *Manayunkia speciosa*. It is thought that a better understanding of factors limiting the polychaete's distribution and abundance may provide stakeholders with a means of reducing the incidence of ceratomyxosis. These goals were addressed by the following objectives:

Objective 1) Document the spatial and seasonal distribution of the *C. shasta* actinospore in the Klamath River basin and assess relative susceptibility of native salmonids to infection. *Approach*: Conduct 3-4 d exposures of known susceptible rainbow trout in various tributary and main-stem Klamath River locations once a month over a period of

6-7 months and monitor the exposure groups for signs of infection. When available, expose specific pathogen free species of native Klamath River salmonids simultaneously for comparison.

Objective 2) Document the spatial distribution of polychaete host populations in the Klamath River and determine the habitat preference(s) of this species. *Approach*: Conduct large-scale (kilometers) habitat sampling to determine basin-wide distribution and evaluate the frequency of occurrence and relative densities of *M. speciosa* by habitat type in conjunction with measures of various abiotic parameters such as flow velocity, depth and substrate type and small-scale habitat sampling to determine population characteristics by habitat type.

Objective 3) Estimate average infection prevalence of *C. shasta* in populations of *M. speciosa* and evaluate if patterns in population-levels prevalence exist. *Approach*: Using polychaetes from populations collected during the habitat survey, a variable pool size strategy can be used with the PCR assay to estimate the population prevalence of *C. shasta*. The estimate obtained from this method is a percentage of the population that is infected with the parasite with associated confidence levels.

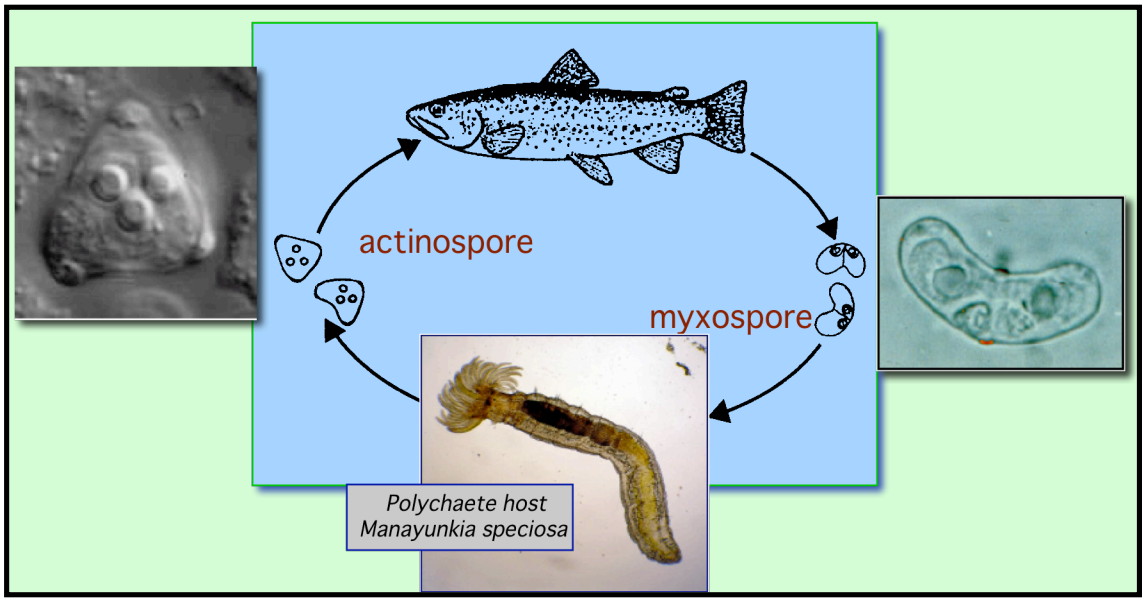

Figure 1.1. Life cycle of the myxozoan parasite, *Ceratomyxa shasta*, with insets of the two spore stages and the polychaete host, *Manayunkia speciosa*. For additional information concerning the life cycle, see Bartholomew et al. (1997).

CHAPTER 2: SPATIAL AND TEMPORAL OCCURRENCE OF THE SALMONID PARASITE *Ceratomyxa shasta* (MYXOZOA) IN THE OREGON-CALIFORNIA KLAMATH RIVER BASIN

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ABSTRACT

Ceratomyxa shasta has been implicated as a significant source of salmonid mortality in the Lower Klamath River (below Iron Gate dam). A study on the prevalence of *C. shasta* and its geographic and spatial distribution throughout the Klamath River basin was conducted to determine when and where juvenile salmon encounter lethal parasite doses. *Ceratomyxa shasta*-susceptible rainbow trout (*Oncorhynchus mykiss*) were exposed to the parasite in the Klamath River and held for 3-4 d at seven locations between Beaver Creek and Keno Reservoir in April, June, July, September, and November 2003. A Klamath River strain of fall Chinook salmon (*O. tshawytscha*) was held in three locations in the Upper Klamath River in April, June and July. In June 2004, rainbow trout exposures were conducted for 4 d at 18 locations from Klamath Lake to the mouth of the Klamath River, including several major spawning tributaries, with one fall Chinook exposure occurring in the Lower Klamath River. Rainbow trout mortality due to infection for groups exposed in the Upper Klamath River was reduced (<8.0 %) and delayed (mean day to death 40-110 d) compared to mortality in groups exposed in the Lower Klamath River (>98%, mean day to death 33-36 d). Experimental fall Chinook salmon did not become infected in the Upper Klamath River exposure groups, but infection was detected in Chinook salmon groups exposed in the Lower Klamath River with nearly 50% succumbing to infection. These dramatic differences in mortality

between the Upper and Lower Klamath River could not be explained by differences in water temperatures during exposure and are likely a result of differences in infectious dose. Lack of infection in groups exposed in tributaries support the hypothesis that the parasite life cycle, and the invertebrate host, is largely confined to the main-stem Klamath River.

INTRODUCTION

Ceratomyxa shasta Noble, 1950 (Myxozoa) causes the disease ceratomyxosis in trout and salmon of the Pacific Northwest, USA and has been reported to be a significant contributor to mortality of adult and juvenile fish (Hoffmaster et al. 1988; Bartholomew 1998). In 1997, the life cycle of the parasite was elucidated with the discovery of the invertebrate host (Bartholomew et al. 1997). *Ceratomyxa shasta* requires the annelid host *Manayunkia speciosa* Leidy (Polychaeta: Sabellidae), and a salmonid host to complete its life cycle, with a different spore stage developing in each host. The continued isolation of *C. shasta* to a limited geographic range in the Pacific Northwest in an era when other fish pathogens have been widely dispersed may indicate that the polychaete host has very specific habitat requirements, limited dispersal abilities, or both.

Currently, the most sensitive means of demonstrating the presence of *C. shasta* is by exposing known susceptible strains of salmon or trout (sentinel fish) in lakes or rivers for a period of time and then monitoring the exposed groups for infection. Results are limited to qualitative interpretations because exposures are conducted during a relatively brief period, in a specific location within a body of water, and are not suited as a quantitative measure of spore densities. Sentinel fish exposures used to document the geographic and seasonal occurrence of *C. shasta* have shown that the parasite exhibits a highly variable distribution and prevalence throughout Northern California, Oregon, Washington, British Columbia and Idaho (Bartholomew et al. 1989). In watersheds where *C. shasta* is enzootic, resistance in native trout and salmon has developed due to selective pressures (Johnson 1975; Zinn et al. 1977; Buchanan et al. 1983; Bartholomew 1998). Exposures

studies have demonstrated that ability to resist infection and mortality due to infection is a function of infectious dose (Ratliff 1981; Ching et al. 1984) exposure duration (Ibarra et al. 1992) and temperature (Udey et al. 1975). Although development of the parasite can occur in all age-classes, the effects appear to be greatest on populations of juvenile salmon emigrating during periods of high parasite densities (Bartholomew et al. 1992).

In the Klamath River, Hendrickson et al. (1989) demonstrated that *C. shasta* could be detected at the estuary beginning early April when water temperatures reached 15˚C and from then until mid-December when temperatures dropped to 7˚C. That study also demonstrated that the infective stage, though present in the main-stem Klamath from Copco Reservoir to the estuary, could not be detected in any of the tributaries to the Lower Klamath River. Beginning in the early 1990's, Klamath River fisheries biologists noted high numbers of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) manifesting signs of ceratomyxosis. Although prior quantitative data of ceratomyxosis in the field was lacking for comparison, these observations renewed interest to find sites of elevated *C. shasta* infectivity and assess the potential impacts of the parasite on Klamath River salmonids. The purpose of this study was to describe the spatial and temporal distribution of *C. shasta* within the Klamath River.

METHODS

Sentinel exposures spanned two sample seasons: April to November 2003 (temporal distribution study) and June 2004 (spatial distribution study).

Study Area (Figure 1; Table 1)

The Klamath River begins at Klamath Lake in South Central Oregon, extends southwest through Northern California and turns west-southwest to the northern California coast. The river is approximately 423 km in length and for the purposes of this study, has been divided into upper and lower reaches. The Upper Klamath River (UKR) extends from Klamath Lake to Iron Gate Dam and is characterized by its system of five dams: Link, Keno, J.C. Boyle, Copco, and Iron Gate respectively.

The Lower Klamath River (LKR) extends from Iron Gate Dam to the mouth of the river. The 2003 sentinel exposures occurred in the main-stem Klamath River beginning at Keno Dam and extending downstream to Iron Gate with only one exposure occurring below Iron Gate Dam just above the Beaver Creek confluence. The 2004 sentinel exposures encompassed portions of the main-stem lower and upper-river reaches and included several major tributaries. Site selection was influenced by accessibility and ability to protect fish holding cages from vandalism. Table 1 and Figure 1 show the locations and abbreviated reference terms for all sites.

Experimental Procedures

Ceratomyxa shasta-susceptible rainbow trout were obtained from Oregon Department of Fish and Wildlife Oak Springs and Roaring River hatcheries. These strains have similar susceptibility to the parasite and are standard controls in *C. shasta* distribution and susceptibility studies. Klamath River fall Chinook salmon was obtained from California Department of Fish and Game, Iron Gate Hatchery. Fish obtained from the

hatcheries weighed, depending on rearing phase, between 2.5 and 26.0 grams. The fish were transferred to specific pathogen-free (SPF) well water at the John L. Fryer, Oregon State University Salmon Disease Laboratory (OSU-SDL). Fish were fed a diet containing 2 - 4% oxytetracycline (approximately 3.5 g oxytetracycline/45 kg fish/day) in the form of TM100 (Pfizer, Atlanta, GA) for 10 d prior to exposure. Fish were then transported to the exposure sites, placed in 0.3 x 1 m cylindrical holding cages and held for 3 – 4 d in the river without feeding. After exposure, the cages were retrieved from the river, and the groups placed in individual coolers with bubbled oxygen. The fish were then transported to the OSU-SDL where each exposure group was held in a 100 L tank receiving 13 - 16˚C SPF water until termination (approximately 70 – 90 d PE) when all fish were euthanized.

Preventative treatments for bacterial infections were administered within 1 d post-exposure (PE), and included resuming a diet consisting of TM100 medicated feed and one mg/L Furanase (Aquarium Products, Glenburnie, MD) bath treatment 1 hr daily for 3 d. After two weeks PE, fish received a 1 hr formalin bath at 125 – 170 mg/L for three consecutive days to remove external parasites. Dead or moribund fish were collected daily and examined for signs of infection. Negative control fish (non-exposed) were handled the same as the experimental groups.

At each exposure location, latitude and longitude coordinates were collected using a Garmin 72GPS unit. Dissolved oxygen (mg/L) and temperature (˚C) were measured using YSI model 55 (Yellow Springs Instruments, Yellow Springs, OH). Water temperatures during fish exposures were recorded using Optic StowAway (Onset

Computer Corporation, Pocasset, MA) submersible temperature loggers placed in the fish holding cages. Data was downloaded to an Excel spreadsheet where mean temperatures and corresponding standard deviations (SD) were calculated.

Seasonal Distribution Study (Figure 1)

Ceratomyxa shasta-susceptible rainbow trout from the Oak Springs Hatchery (stock # 53.02) were used in the April, June, and July exposures and Roaring River Hatchery (stock #72.02) were used for September and November exposures. Exposures were conducted for 3 – 4 d in April through November 2003 with 50 - 70 rainbow trout per cage exposed in seven locations in the upper river and with an additional location during September and November in the LKR above the Beaver Creek confluence (KBC). Only one exposure occurred in the Copco Reservoir (CPR) in September. Klamath River fall Chinook were exposed in the Keno Reach (KRC), Boyle bypass Reach (BBR), and the Boyle peaking Reach (BPR) in April, June and July of 2003.

For April through September exposures, sub-samples of 15 fish were collected at 15 d post exposure and tested for infection by PCR analysis. Sub-samples of exposure groups were not collected prior to termination for the November exposure groups. At termination (70 – 90 d PE), a minimum of 25 fish (or all remaining if less than 25) from each group was sampled for *C. shasta*.

Geographic Distribution Study (Figure 2.1, Table 2.1)

In June 2004, a single 4 d exposure was conducted using Roaring River Hatchery rainbow trout for all locations and fall Chinook salmon were exposed in the LKR at KBC. In addition to the 2003 exposure locations, 11 sites were added to encompass a broader range of the main-stem Klamath River including several key tributaries. Seventy fish per cage were exposed and fish were treated the same as for the seasonal study except that exposure groups were not sampled prior to termination.

Determination of Infection

All groups, including the negative control group, were terminated with a lethal dose of MS222 (tricaine methanesulfonate). A sample of 10 fish per exposure group was visually examined for spores by microscopy. If any fish were identified as positive, an additional 15 fish were examined by microscopy. Dead or moribund fish as well as fish sampled for infection were first examined by wet-mount. The wet-mount was prepared by inserting a sterilized inoculating loop of the appropriate diameter into the anogenital pore to a depth of approximately 1.0 - 1.5 cm. The sample collected was smeared onto a glass microscope slide and observed at 100 X or 250 X magnifications for 3 min. Fish were considered positive if the characteristic kidney bean-shaped myxospore was observed. Fish not demonstrating clear spore stages were not considered visually positive due to the difficulty of visually differentiating early presporogonic stages from host cells or other myxozoans.

If spores were not observed then intestinal tissue was excised, digested, and assayed by a single round polymerase chain reaction (PCR) amplifying a 638bp product using

methods described by Palenzuela and Bartholomew (2002). The following modifications were made to the protocol: an additional 2-3 mm segment of the alimentary canal, just posterior to the pyloric ceca attachment, was excised and included with the 5mm segment of the posterior intestine.

Percent prevalence of infection was calculated as the number of exposed fish that tested positive for infection (by microscopy and/or PCR analysis), including sampled fish and mortalities, divided by the total number of fish examined for infection (X 100). Percent mortality was calculated as the number of fish that died PE that were visually positive for *C. shasta* by microscopy, divided by the total number of fish that had survived the treatment period (X 100). These fish generally displayed clinical signs of the disease; fish that died prior to the time when *C. shasta* infections became patent were excluded from the analysis. Mortalities due to *C. shasta* are included in the prevalence estimates. The first 10 fish sampled at the termination period are to detect presence/absence of the parasite while additional samples increase precision of prevalence estimates. The mean day to death in days PE, for each exposure group was calculated as the geometric mean of all days with *C. shasta* positive mortalities.

RESULTS

2003 *Seasonal Distribution in the Upper Klamath River* (Table 2.2)

April — Death due to infection occurred only at BPR (see Table 2.1 for site identification) with a single fish dying at 68 d post exposure. Infection prevalence ranged from 40 to 73% for all groups of rainbow trout exposed below Keno Dam with the lowest

detectable infection occurring in fish held at BDM. Infection prevalence was highest in the group held at BPR (73.3%). Infection prevalence was considerably lower in April relative to all other seasonal exposures. Fall Chinook salmon exposed at KRE, BBR, and BPR tested negative for infection. Water temperatures ranged from 8.8 – 10.0˚C. Unexposed rainbow trout and fall Chinook salmon tested negative for the parasite.

June — Death due to infection was observed in rainbow trout held at BBR (7.5%) and CPV (4.8%), but not at BPR, where mortality had occurred in April. Infection prevalence of 81 – 100% was observed during the June exposures. Temperatures during the June exposures also increased to an average of 20˚C with the lowest temperatures (14.8˚C) occurring at BBR, a site that is influenced by cold-water springs. Despite the cooler temperatures, fish held at this location exhibited greater loss due to infection. Exposures at BDM had the lowest detectable infection prevalence (81.3%) while fish exposed at KRE demonstrated high infection prevalence (100%) without any resulting mortality. All fish exposed at KDM died during exposure and as such were not included in analysis. *Flavobacterium columnare* was isolated from samples of these fish suggesting that death in this group was likely a result of high temperatures (>23.0˚C) and columnaris infection. Fall Chinook salmon exposed at sites KRE, BBR, and BPR tested negative for infection. Unexposed rainbow trout and fall Chinook salmon tested negative for the parasite.

July — Water temperatures at KDM, KRE, and BDM exceeded the lethal threshold of 25˚C, which prevented fish exposures at these locations. Because water temperatures

downstream of the John Boyle reservoir are influenced by cold-water springs, we were able to conduct exposures from BBR to CPV. Infection prevalence among rainbow trout exposed at sites between CPV and BBR was >97% and mortality due to infection was much higher than that of previous exposures (9.1 – 52.4%). It is interesting to note that the site with the coolest water temperatures (BBR) also sustained the highest mortality levels (52.4%) during the July exposures. Fall Chinook salmon exposed at KRE, BBR, and BPR tested negative for infection. Unexposed rainbow trout and fall Chinook salmon tested negative for the parasite.

September — Percent mortality with clinical ceratomyxosis ranged from 0 to 23% for rainbow trout exposed at sites above Iron Gate Dam. Infection prevalence was >90% at all sites except KDM (0%) and CPR (22%). All mortalities occurred in groups held in the free-flowing stretches of the Klamath River. Mortality due to infection (3.0%) was observed in the KRE exposure group for the first time. Water temperatures at KRE during September were significantly less than temperatures during the June exposure (17.0˚C vs. 23.3˚C) when mortality was not observed. Exposure groups held at BDM and CPV had high infection prevalence although mortalities in these groups were not observed. Rainbow trout exposed below Iron Gate dam at KBC, a site where exposures had not previously been conducted, sustained 100% mortality. Unexposed fish tested negative for the parasite.

November — Prevalence of infection was >96% for all groups of rainbow trout exposed in the main-stem Klamath River except KDM (0%). Mortality was not observed in any of the groups exposed above Iron Gate Dam. When compared to the September exposures, mortality decreased by 17% in rainbow trout held below Iron Gate Dam at KBC (83%). Temperatures decreased by approximately 7˚C for all sites with CPV and KBC averaging the highest temperatures (11˚C). Unexposed fish tested negative for the parasite.

2004 *Geographic Distribution* (Table 2.3, Figure 2.1 & 2.2)

June 2004 infection patterns for rainbow trout exposed at sites above Iron Gate Dam are similar to results in the 2003 exposures. The infection prevalence for groups exposed at main-stem sites above Iron Gate Dam ranged from 85 - 100%, except KDM where a single fish was positive. Mortality in rainbow trout groups exposed above Iron Gate Dam ranged from 0.0 – 4.3% compared to below Iron Gate Dam, where mortality ranged from 98 – 100%. Additionally, the mean day to death for exposure groups above Iron Gate Dam was significantly higher (40-110 d) than for groups exposed below Iron Gate Dam (34-36 d). Mortality with clinical ceratomyxosis in the fall Chinook group exposed at KBC was less than that of the rainbow trout group (49% vs. 100% respectively). Infection was not detected in any of the fish exposed in tributaries except Hunter Creek, where a single fish tested positive. Unexposed rainbow trout and fall Chinook salmon tested negative for the parasite.

DISCUSSION

Although the reasons underlying the increased observance of ceratomyxosis in Klamath River fall Chinook salmon are not clear, this study illustrates dramatic differences in infection patterns between the Upper Klamath River (UKR) and Lower Klamath River (LKR). In the LKR, complete mortality of all sentinel rainbow trout and near 50% loss of juvenile Chinook salmon as a result of ceratomyxosis suggests a shift in host-parasite balance when compared to UKR exposure results. Infection patterns in the UKR were more variable (mortality ranged from 0 – 52.4%) with areas of elevated infectivity in the free-flowing river stretches and areas of the least infectivity within the reservoirs. An exception is the Williamson River, a river system that flows into Klamath Lake, where exposure groups have also succumbed to both high infection prevalence and high mortality relative to other UKR exposures (A. Hemmingson, Oregon Department of Fish and Wildlife, personal communication; author's unpublished observations). From Klamath Lake to the estuary, with the exception of Hunter Creek, sentinel fish only became infected in the main-stem channel implying that the parasite life cycle is almost exclusively confined to this area. The proximity of Hunter Creek to the mouth of the Klamath River makes it subject to tidal influence where Klamath River water may enter the creek with infectious spores.

Differences in infection severity between sentinel groups exposed above and below Iron Gate Dam were quite apparent. Mortality due to *C. shasta* infection was both greatly reduced and delayed in rainbow trout groups exposed in the UKR when compared to groups exposed in the LKR. Although stocks of salmonids native to rivers where the

parasite is enzootic acquire resistance to the parasite (Johnson 1975; Zinn et al. 1977; Buchanan et al. 1983; Bartholomew 1998), several studies report an increase of mortality in resistant strains with an increase in exposure frequency and duration (Ratliff 1981, Ibarra et al. 1992). The native Klamath River fall Chinook were more resistant than the susceptible rainbow trout used in these exposures, however, their resistance were clearly overwhelmed in the LKR with nearly 50% of the exposure group succumbing to ceratomyxosis. *Ceratomyxa shasta* infection patterns follow seasonal trends (Hendrickson et al. 1989, Ching and Munday 1984, Ratliff 1983) and these were evident in this study. As Klamath River water temperature increased in the spring, infection prevalence and mortality also increased. Interestingly, we found that as water temperatures decreased in the fall, mortality decreased but infection prevalence remained high. Such an extended period of infection without resulting mortality may be due to decreased parasite abundance and/or improved water conditions benefiting the salmonid host (e.g. lower water temperatures).

The temperature ranges over which *C. shasta* can develop within its polychaete host and remain infective for its salmonid hosts are not known. Studies conducted on *Myxobolus cerebralis* (the causative agent of whirling disease in salmonids) and its invertebrate host *Tubifex tubifex,* have demonstrated positive correlates between temperature, infection rates of *T. tubifex*, and triactinomyxon (TAM) production and release (Blazer et al. 2003). *Myxobolus cerebralis* TAM release occurs between 9 and 17˚C and optimally at 13 - 15˚C (El-Matbouli et al. 1999; Blazer et al. 2003), indicating that the parasite responds to temperature changes. The minimum and maximum

temperatures at which *C. shasta* has been detected by sentinel exposures range from 4 - 23˚C (Ching and Munday 1984; Hendrickson et al. 1989, current study). An optimal temperature has been difficult to determine due to problems with maintaining the polychaete host in culture but data from seasonal exposures in this study support earlier findings that 10˚C is a critical threshold for the production and release of the infectious stage.

Water temperatures during exposure help to explain some of the site-specific trends that we observed, but fail to explain the dramatic differences between sites. Given the short exposure duration, the high mortality and reduced mean day to death in groups held in the LKR are not likely a result of water temperature, but more likely a result of high actinospore density relative to the UKR. This is also supported by results of exposures at the spring-fed Boyle bypass Reach, which during the study had the coolest water temperatures in the Klamath River and yet, fish held at this location often demonstrated the highest infection prevalence and *C. shasta*-related mortality of all UKR exposure groups. This suggests that parasite densities are higher at the Boyle bypass relative to other UKR sites.

The presence of four reservoirs in the upper basin likely has a significant influence on the abundance and distribution of the *C. shasta* actinospore. Ratliff (1983) demonstrated that the infectious stage (actinospore) is viable for less than 10 d under laboratory conditions. Because of their higher capacity and longer retention time relative to the free-flowing stretches, the reservoirs may serve to dilute incoming spore densities and impede passage of the fragile actinospore by means of spore sedimentation. For example, Keno

Reservoir, where only one fish became infected, receives inflow from Link River, which contains the *C. shasta* actinospore. Keno Reservoir has a retention time of 6 d (PacifiCorp, 2004) at an average annual flow of 1600cfs (cubic feet per second). By comparison, Boyle Reservoir has a retention time of 1.1 d at the same average flow (PacifiCorp, 2004) and exposure groups consistently tested positive for infection, although infection prevalence was always less than that of the Keno Reach, which drains into the reservoir. In general, mortality was reduced and delayed in the reservoir groups when compared to groups exposed in the free-flowing stretches of the river.

If high spore densities resulted in the high mortality documented in exposure groups held in the LKR, then it seems likely that continuity of water flow (absence of obstructions) is an important factor in explaining the differences between the UKR and the LKR. If we assume a steady input of viable actinospores (i.e. polychaete populations are equally distributed and equally infected) with distance downstream, a constant flow rate and volume, and a spore viability time of less than 10 d, then the lack of any obstructions to flow would permit a net increase in spore densities with distance downstream. This should hold true for the LKR since water leaving Iron Gate Dam takes 4 - 6 d to reach the estuary (i.e. viable actinospores can traverse the entire LKR). These assumptions would not hold true for the UKR due to the retention time of reservoirs. If we were to test this using the mean day to death of LKR exposure groups as an inverse measure of relative spore densities, our data suggests that densities do not increase with distance downstream (i.e., mean day to death does not decrease). By contrast, the mean day to death does not increase either, indicating that actinospores contributions are not

constant with distance downstream of Iron Gate Dam or that any further actinospore contributions are diluted by flow additions from tributaries. Thus, the spatial distribution and parasite burden of polychaete populations would have a significant effect in the LKR, which comprises nearly 73% in length of the entire main-stem Klamath River and is not blocked by impoundments.

Decreased water levels during peak actinospore release may also have a compounding effect on *C. shasta* infections. Droughts and partitioning of water has forced reductions in summertime flows below Iron Gate Dam. These reductions may have a number of consequences including increasing water temperatures or decreasing water temperature fluctuations, which would presumably affect parasite development rates in both the salmonid and polychaete host. Flow reductions may also concentrate parasite spores and increase salmonid migration time, subsequently increasing the exposure dose and exposure duration.

Studies are currently underway to document the spatial distribution and abundance of the polychaete host in the Klamath River and determine infection prevalence in selected host populations. Such information will be paramount to understanding the differences of infection severity observed between the UKR and LKR. The USFWS and Tribal fisheries biologists are also working to assess the effects of *C. shasta* on wild stocks of salmonids and quantify juvenile mortality. These data, combined with the information gathered from this and future studies, will provide fisheries managers with information on how to reduce impacts on Klamath River salmonids.

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TABLES AND FIGURES

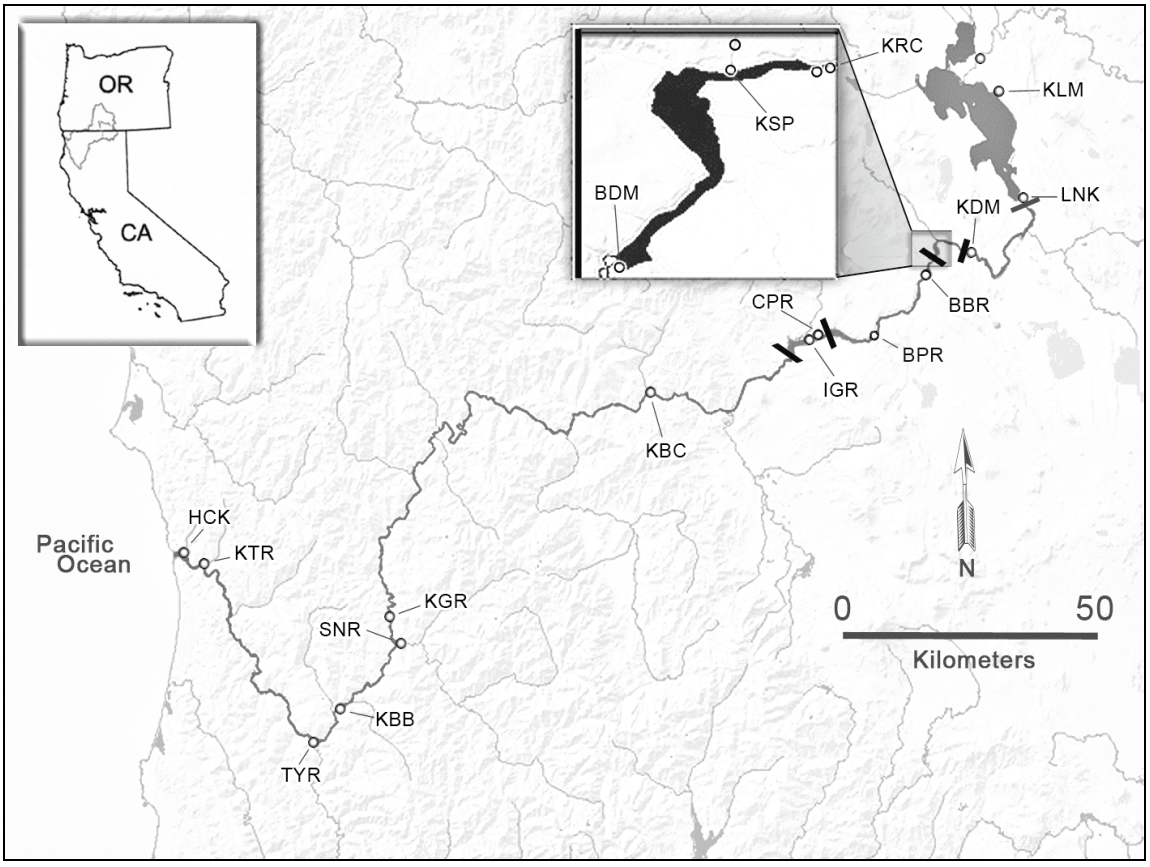

Figure 2.1. — Map of Klamath River 2003 and 2004 sentinel exposure sites. Site names, site identification, and the river kilometer for each site are given in Table 1.1. Bars denote a dam.

TABLE 2.1. — Sites where fish exposures occurred in the Klamath River basin to detect *Ceratomyxa shasta*. Sites listed with river kilometer (Rkm) and site identification. Pos: actinospore detected; Neg: negative

Exposure Site	(Rkm)	Site ID	Results
Upper Klamath River Main-Stem			
Klamath Lake at Modok point	415.1	KLM	Pos
Link River	409.0	LNK	Pos
Keno Dam	374.9	KDM	Pos
Keno Reach	368.6	KRC	Pos
Keno Eddy	368.5	KRE	Pos
Klamath at Spencer Creek	366.0	KSP	Pos
Boyle Dam	361.5	BDM	Pos
Boyle bypass Reach	354.5	BBR	Pos
Boyle peaking Reach	332.3	BPR	Pos
Copco Dam	328.6	CPR	Pos
Copco Village	327.2	CPV	Pos
Iron Gate Dam	305.8	IGR	Pos
Lower Klamath River Main-Stem			
Klamath at Beaver Creek	259.1	KBC	Pos
Klamath at Green Riffle	113.1	KGR	Pos
Klamath at Big Bar	81.1	KBB	Pos
Klamath at Terwer	8.0	KTR	Pos
Tributaries to the Klamath River			
Spencer Creek	0.8	SPC	Neg
Salmon River	0.1	SNR	Neg
Trinity River	0.1	TYR	Neg
Hunter Creek	0.1	HCK	Pos

TABLE 2.2 Results of 3 to 4 d exposures of known susceptible rainbow trout conducted in April through November 2003 to detect Ceratomyxa shasta in the Klamath River.

	April 25th - 29th			June 5th - 9th			July 21st - 24th			Sept 22nd - 26th			Oct 30th - Nov 4th		
Site ID	%Prev[a]	%Mort[b]	˚C	%Prev	%Mort	˚C	%Prev	%Mort	˚C	%Prev	%Mort	˚C	%Prev	%Mort	˚C
KDM	0	0	8.9	ND	ND	23	-	-	-	0	0	16.7	0	0	8.7
KRE	50	0	8.8	100	0	22.3	-	-	-	100	3	17	100	0	8.3
BDM	40.7	0	8.9	81.3	0	ND	-	-	-	96	0	17.3	96	0	8.2
BBR	50	0	10	100	7.5	14.8	100	52.4	16	93	11	12.7	100	0	9.4
BPR	73.3	2.5	9.5	95	0	20.9	97.4	25.6	22.6	94	23	15.9	100	0	8.4
CPV	43.3	0	10	100	4.8	20	100	9.1	21.8	97	0	17	100	0	11
CPR	-	-	-	-	-	-	-	-	-	22	0	ND	-	-	-
KBC	-	-	-	-	-	-	-	-	-	100	100	17.2	100	83	11.1

[a] %Prev = Percent prevalence is the number of fish in a group diagnosed with C. shasta divided by the number of fish examined in that group (x100). [b] %Mort = percent mortality is the number of fish in a group that died with clinical ceratomyxosis divided by the total number exposed in that group (x100). Mean temperatures = ˚C for the 3 to 4d exposures. A minimum of 50 rainbow trout was exposed per site. Fish that died within 2 d post-exposure were not examined. Sites CPR and KBC were added during the Fall exposures. ND = No Data. Sites are listed from upriver to downriver. A bar indicates no exposure occurred.

TABLE 2.3. Exposures of juvenile rainbow trout (Rbt) and fall Chinook salmon (Chf) conducted June, 2004 to detect *Ceratomyxa shasta* in the Klamath River. Sites labelled top-down with up-river sites going down river.

Site ID	% Prev	(n)	% Mort	(N)	μDOD	˚C
Upper Klamath River Main-Stem						
KLM	90.0	(30)	4.3	(70)	71	18.60
LNK	92.3	(26)	1.4	(70)	69	19.30
KDM	3.8	(26)	0.0	(70)		20.70
KRC	100	(26)	1.4	(70)	40	19.80
KRE	89.7	(29)	1.4	(70)		19.90
KSP	96.3	(27)	1.4	(70)	110	17.90
BDM	89.3	(28)	0.0	(70)		21.60
BBR	93.1	(29)	2.9	(70)	91	14.20
CPV	92.6	(54)	0.0	(70)		18.20
IGR	100	(26)	1.4	(70)	88	19.00
Lower Klamath River Main-Stem						
KBC(Rbt)	100	(12)	100	(12)	35	20.60
KBC(Chf)	68.6	(35)	48.6	(35)	25	20.60
KGR	98.2	(57)	98.2	(57)	33	20.60
KBB	98.2	(56)	98.2	(56)	35	20.10
KTR	100	(70)	100	(70)	35	ND
Tributaries to the Klamath River						
SPC	0.0	(26)	0.0	(70)		16.50
SNR	0.0	(27)	0.0	(58)		17.30
TYR	0.0	(25)	0.0	(70)		ND
HCK	3.7	(27)	1.4	(70)		12.20

Seventy rainbow trout (RbT) exposed at each location with 70 fall Chinook (ChF) exposed at one location. Mean days to death (mDOD) is the calculated geometric mean of the days post exposure where death due to ceratomyxosis had occurred ± SD (standard deviation). The number of exposed fish that were sampled (n) at the 90d termination period were taken from the number of fish that survived the exposure period (N). %Prev = perecent prevlance: the number of fish that were infected with *C. shasta* divided by the number of fish examined (x100). %Mort = percent mortality: the numbe of fish that died due to infection divided by the number of fish examined (x100). Site ID can be found on Table 2.1 and locations on Figure 2.1.

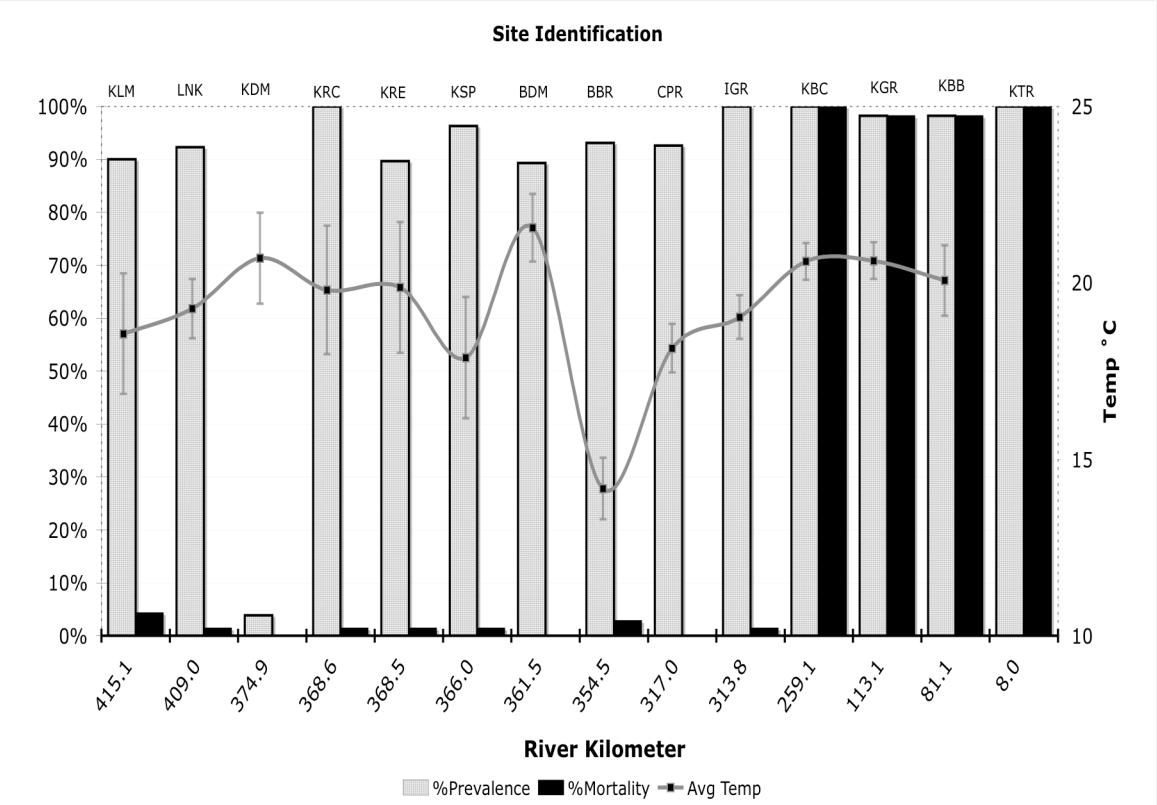

Figure 2.2. Percent prevalence and percent mortality of rainbow trout (*Oncorhynchus mykiss*) exposed in Klamath River (June 2004) locations reading from the uppermost site (left) to the lowest (right). On top axis, the Site ID and name can be found in Table 1. Iron Gate Dam separates sites IGR and KBC.

CHAPTER 3: DISTRIBUTION AND MACROHABITAT PREFERENCE OF *Manayunkia speciosa* (POLYCHAETA, SABELLIDAE) AND INFECTION PREVALENCE WITH THE PARASITE *Ceratomyxa shasta* (MYXOZOA) IN THE KLAMATH RIVER

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DISTRIBUTION AND MACROHABITAT PREFERENCE OF *Manayunkia speciosa* (POLYCHAETA, SABELLIDAE) AND INFECTION PREVALENCE WITH THE PARASITE *Ceratomyxa shasta* (MYXOZOA) IN THE KLAMATH RIVER

ABSTRACT

A survey for *Manayunkia speciosa,* the freshwater polychaete host for the myxozoan parasite *Ceratomyxa shasta,* was conducted from 2003 to 2005 as part of an integrated study of the epidemiology of ceratomyxosis in Klamath River (Oregon-California, USA) salmonids. Substrate samples (N = 257) were collected from Klamath Lake to the mouth of the Klamath River to document occurrence and relative abundance of the polychaete and estimate the prevalence of *C. shasta* within selected polychaete populations. Large populations were consistently present and spatially structured at the inflow to the main-stem reservoirs indicating preference for this habitat. Small but dense aggregations of *M. speciosa* were found within pools (51.6%), eddy-pools (47.0%) and runs (40.0%). Habitat stability and flow velocity appear to strongly influence polychaete distribution. Twelve of 71 populations identified were tested for *C. shasta* revealing a mean infection prevalence of 0.27%. Two heavily infected populations (4.9% and 8.3%) were identified below Iron Gate Dam which explain the high infectious spore densities demonstrated in concurrent studies and observations of *C. shasta*–induced mortality in naturally resistant Klamath River fall Chinook salmon (*Oncorhynchus tshawytscha*).

INTRODUCTION

Manayunkia speciosa Leidy, 1858 is a freshwater polychaete described from east and west coast river systems of North America and the Great Lakes region (Spencer, 1976). The polychaete was reported in the Willamette River, Oregon when Bartholomew et al. (1997) identified *M. speciosa* as the obligate invertebrate host for a myxozoan parasite of salmonids, *Ceratomyxa shasta* Noble, 1950. The distribution and impacts of *C. shasta* on populations of wild and hatchery salmonids in the Pacific Northwest have been documented (Ratliff 1981; Ching and Munday 1984; Hoffmaster et al. 1988; Bartholomew et al. 1989; Hendrickson et al. 1989; Stocking et al. 2006). However, little information on the ecology of the polychaete host has been published and its confirmed distribution in the Pacific Northwest has only been reported from a few locations in California (Hazel 1966), Oregon (Hazel 1966; Bartholomew et al. 1997) and Alaska (Holmquist 1973).

Reports of *M. speciosa* have occasionally appeared in the literature over the last 150 years, mostly as notes and short communications (Spencer 1976). This lack of information may be a result of interest in larger or more economically important invertebrate species (Mackie and Qadri 1971) or collection methods that may have been inadequate to retain *M. speciosa* (Hazel 1966). In live samples, the polychaete is easily overlooked given its slow movements, tendency to attract and coat its sticky body with fine material, its small size (0.2 to 3.0 mm length) and semi-transparent body (Holmquist 1973). The inconspicuous nature and discontinuous occurrence of the polychaete

indicates that *M. speciosa* might have a greater geographic distribution than has been reported (Pettibone 1953; Spencer 1976).

Myxozoan parasites utilize a two-host life cycle, involving a fish and an aquatic invertebrate, with a spore stage developing in one host that is infectious for the other. The myxospore stage, which develops in the fish, generally requires a specific invertebrate host (Ozer et al. 2002; Kent et al. 2001). Because of this specificity, the distribution of *M. speciosa* can be indirectly inferred from sentinel fish exposure studies documenting the *C. shasta* actinospore (stage infectious for fish) (Stocking et al. 2006). These exposure studies (Bartholomew et al. 1989; Hoffmaster et al 1988) demonstrate that the *C. shasta* life cycle is mostly confined to large river systems with a connection to the coast. Results from exposure studies in the Klamath River suggest that the polychaete host is largely confined to the main-stem river (Hendrickson et al. 1989; Stocking et al. 2006).

The current study was conducted in the Klamath River where *C. shasta* has been implicated as a potentially limiting factor to survival of Klamath River fall Chinook salmon (*Oncorhynchus tshawytscha*) (Foott et al. 2004). Concurrent research in our laboratory indicates that the high *C. shasta* induced mortality observed in Klamath River fall Chinook are attributable to high spore densities detected in water samples from the Lower Klamath River (Stocking et al. 2006; Hallett and Bartholomew 2006). These high parasite densities could be explained by increased polychaete host population densities and/or by increased proportions of infected polychaetes within these populations. To determine what contributed to these severe infections, our study was designed to

document the distribution and relative abundance of *M. speciosa* throughout the main-stem Klamath River, gather information about macrohabitat preference and identify polychaete populations that may be of epidemiological importance.

METHODS

Study Area

The Klamath River begins at Klamath Lake in south central Oregon and runs south-southwest through northern California to the Pacific Ocean. Notable features in the Upper Klamath River (UKR) include Klamath Lake, a large and shallow hypereutrophic water body, and four reservoirs downstream of Klamath Lake: Keno, Boyle, Copco and Iron Gate. The Lower Klamath River (LKR), making up approximately 73% of the total length of the Klamath River, flows un-obstructed through the Cascade Range and receives substantial flow contributions from the Shasta, Scott, Salmon and Trinity Rivers. Substrate within this region is mainly bedrock, boulder and cobble. For the purposes of this study, the Klamath River has been divided into the UKR extending from Klamath Lake downstream to Iron Gate Dam and the LKR extending downstream from Iron Gate Dam to the coast (Figure 3.1). The LKR was further subdivided by reaches between tributaries: from Iron Gate Dam (IGD) to Interstate-5 (I-5), Scott River confluence, Indian Creek confluence, Salmon River confluence, and the Trinity River confluence and to the estuary (Figure 3.1, Table 3.1).

In June – July 2003, sampling was conducted mainly in the UKR (Table 3.1), especially in the Boyle and Keno reservoirs, with additional collections in the LKR

approximately 14 – 21 river kilometers below Iron Gate Dam. Additional samples were collected during October 2003 from Boyle Reservoir and the Keno eddy (Rkm 368.5). In June – August 2004, samples were collected in both the UKR and LKR with efforts focusing on the reservoirs. For seasonal monitoring of known polychaete populations, samples were collected from the Williamson River inflow into Klamath Lake, Keno eddy and Tree of Heaven (Rkm 278.3) during March 2005. The last sampling season occurred in the LKR from July – August 2005 (Table 3.1).

Sampling Technique

Polychaetes were collected from hard substrates by scraping with a 60 μm mesh zooplankton tow net fitted with a 3.2 m telescoping rod and a stainless steel lip attached to the metal net frame. For soft sediments in wadeable areas, the net was used to disturb the fines into suspension and this material was collected with the net. Collected material was placed into a 1gal resealable bag by inverting the mesh net and washing material into the bag with 95% ethanol. Sampling time was standardized to 30 seconds of effort for both methods. For approximation of field densities, the area sampled during the 30 s period was estimated to be 0.318 ± 0.056 m^2 for flat surfaces. For collection of soft substrates from non-wadeable lentic areas, a weighted ponar grab with a 0.18 m^2 opening was used. The material was then placed into a large specimen pan, allowed to settle, drained of excess water and then washed into a 1 gal, resealable bag with 95% ethanol. After 14 d of storage, all samples were drained and refilled with 95% ethanol. When

possible, live specimens were collected and transferred to a cooler and acclimated to 6˚C for observation.

Microhabitat sample sites were selected by looking for large substrates such as cobble and boulder or fine substrates such as sand and silt. Other substrates like pebble, gravel, mud and vascular macrophyte beds were only sampled on occasion because initial sampling revealed these to be inadequate habitats for *M. speciosa*. Reservoir samples were collected in a line-transect manner beginning at the inflow and running to the end of the reservoir. Macrohabitat type (MHT), embeddedness, dominant and co-dominant substrate were recorded at each site (Arend 1999). Reservoirs were divided longitudinally as riverine (inflow) with cobble-pebble substrate, transition with gravel and sand substrates and lucustrine with mud substrate. Lotic habitats were categorized as pools, runs, riffles, and eddies. Klamath Lake samples were characterized simply as littoral or profundal. Depth of sample was measured with a sounding pole or weighted 50 m-fabric measuring tape depending on depth and water velocity. When possible, water flow was measured 12 cm above the substratum using a Marsh-McBirney Flowmate 2000 (Frederick, MD) portable current velocity meter connected to a rod in wadeable areas, or to a 20-lb bullet anchor in non-wadeable areas. Date, time and GPS locations were recorded for each sample. Maps of all sample sites were generated through ArcMap® (ArcGIS 9.1). Temperature (˚C) and dissolved oxygen (mg/L) were recorded for each sample site using YSI meter Model 55 (Yellow Springs Instruments, Yellow Springs, OH).

Sample Processing

Contents of the sample bag were emptied into a sub-sample tray (20 x 28 cm) and mixed. The contents of three randomly selected grids (5.0 x 5.6 cm) were then transferred to individual Petri dishes where polychaetes were counted to estimate sample density. Polychaetes were sorted under a dissection microscope at approximately 20X. Sample densities were calculated by averaging the sub-samples and multiplying the average by 20 (the total number of grids in the sub-sample tray). Sample densities for both the pole and ponar methods were divided by the sample area that each method collects to estimate field densities. For samples containing high amounts of fine suspended sediment, the contents of a grid were transferred to a 150 ml-measuring cylinder, stirred, and allowed to settle for 5 min. The suspended material was extracted with a 10 ml pipette, filtered through 40 μm mesh and observed for polychaetes. The process was repeated until the suspension was clear enough to view contents of the Petri dish under a dissecting microscope. The number of juveniles (<1.1 mm), adults out of tubes, and adults in tubes were counted along with the number of eggs per tube if present. For each site, polychaetes were sorted into a 1.5 ml centrifuge tube labeled with site number and filled with 95% ethanol. Identification of *M. speciosa* was based on the characteristic tentacular crown, pygidium, and abdominal setigerous segments (Foster 1972, Pettibone 1953).

Prevalence of Infection

Polychaetes from six populations in the UKR and six in the LKR were chosen to estimate prevalence of infection with the parasite *C. shasta*. Populations were selected

based on spatial distance and whether or not a sufficient number of polychaetes (≥300) had been collected. Three sites in the UKR (Rkm 368.5, 368.2 and 368.0) and three in the LKR (Rkm 189.5, 187.9 and 187.4) were chosen to examine prevalence differences at smaller spatial scales. To estimate prevalence, the 1.5 ml tube containing polychaetes from a site was emptied into a small Petri dish. Polychaetes were randomly selected with fine forceps from the dish and sorted into a 1.5 ml tube representing a pool. Pool sizes (number of polychaetes per tube) varied based on the number of polychaetes present, as did the number of pools (tubes) used (Zendt and Bergersen 2000; Williams and Moffitt 2001). Pool size and the number of each size tested generally consisted of 2 – 4 pools with 50 worms each, 4 – 5 pools with 20 or 25 worms each, 10 pools of 10 worms each and 8 - 10 pools of 5 worms each (Table 3.3). Tubes were marked with the site identification and numbered according to pool size. For PCR controls, polychaetes and fish tissue microscopically identified as positive for infection were used as positive controls. Uninfected fish tissues were used as negative controls. The controls were handled and prepared the same as samples.

The tubes were uncapped in an incubator set at 37˚C for 24 hrs, allowing the alcohol to evaporate completely. After evaporation, samples were digested with 500 µl of DNA extraction buffer with 10µl Proteinase-K. The tubes were capped and incubated at 37˚C with gentle rocking for 6 hr. After digestion, samples were spiked with 5 µl of RNaseA incubated for 30 min at 37˚C, and then boiled at 100˚C for 5 min. A 1:100 dilution of each sample was made using HPLC (molecular grade) water, then assayed for *C. shasta* by adding 1.0 µl of diluted sample to 19.0 µl of PCR master mix using methods

described by Palenzuela and Bartholomew (2002). Results of the assay were visualized on 1.0% agarose gel stained with ethidium bromide and included a 1-kb+ ladder. Each pool (gel lane) was determined as positive or negative for presence of *C. shasta* by presence or absence of a 628 bp product. To estimate percent prevalence, pool sizes, number of pools tested, and the number of pools positive were entered into the Ausvet-Pooled Prevalence Calculator (Sergeant 2004) following the link to *variable pools sizes and perfect test*. Prevalence here is expressed as the percentage of the population that is positive for *C. shasta* infection.

Statistical Analysis

Simple tables and graphs were generated using Excel for Windows, whereas, more in-depth analyses were performed using Stata 9.0 for Mac OS-X (StataCorp 2005). Density estimates (ln-transformed) were weighted by frequency of occurrence in a one-way ANOVA with Bonferroni correction. Lotic and lentic habitats were analyzed separately and pooled by category. For logistic regression, samples with zero values were omitted and ln-transformed to determine if correlations exist between ln-density and parameters such as flow velocity, depth, distance, and infection prevalence.

RESULTS

Macrohabitat Features

Manayunkia speciosa was identified in samples collected from Klamath Lake to the mouth of the Klamath River (Figure 3.1) and distribution was not confined to any

particular region of the river. The polychaete could be found throughout the main-stem channel inhabiting slow flowing macrohabitats but not stagnant habitats. *Manayunkia speciosa* was identified in 51.6% of pools, 47% of eddies and 40% of runs sampled (Figure 3.2A), however, eddies were not as common a habitat feature as pools and runs (Table 3.2). The polychaete was only present in 20% of the riffles sampled and here only a few polychaetes were collected along the edge-water. Frequency of occurrence increased from the fastest flowing habitats to the slowest. Estimates of polychaete field densities were highly variable for both the plankton net and the ponar grab. Mean densities were less than 300 m^{-2} for lotic habitats. However, the highest maximum field densities of all habitats (40,607 m^{-2}) occurred in a single pool at the Tree of Heaven (Figure 3.2A). Results from ANOVA show that differences between lotic groups were not significant (Table 3.2). From Iron Gate Dam to the Trinity River confluence, populations were often discretely confined and densely aggregated within a very small area (5 – 10 m^2) relative to size of the habitat. Below the Trinity River confluence, populations were more widespread within a habitat but far less dense (mean = 163.5 ± 68.1 m^{-2}; max = 308.2 m^{-2}). Due to the inaccessibility of the area, fewer samples were collected per Rkm than above the Trinity River.

The distribution of *M. speciosa* in the lentic component was markedly different from that of the lotic component. The polychaete was consistently found to occur broadly throughout the Williamson River inflow to Klamath Lake and each of the reservoir inflow areas (the Keno Reservoir inflow was not sampled due to logistic constraint). Estimated mean field densities at the reservoir inflow (riverine) areas (804 m^{-}

^{2}) averaged higher than lotic samples (Table 3.2A,B), but maximum polychaete densities were lower in the riverine area (16,054 m^{-2}) than in pools (40,607 m^{-2}) and similar to those from runs (15,119 m^{-2}). Mean densities, maximum densities and frequency of occurrence decreased from riverine to lucustrine areas. The most extensively sampled reservoir was J. C. Boyle where *M. speciosa* was widespread throughout the inflow (Figure 3.5). In this reservoir, the polychaete was also found at the bottom of a deep (6.3 m) channel-constriction, separating two lucustrine areas, on boulder-bedrock substrate associated with freshwater sponge (Figure 3.5). In two samples within the lucustrine area of the J.C. Boyle Reservoir, *M. speciosa* was found, not on the benthos, but on concrete bridge supports with freshwater sponge (Figure 3.2B). In Iron Gate Reservoir, *M. speciosa* was also found at a channel constriction on boulder-gravel substrate where flow increased from <0.01 m s^{-1} to 0.03 m s^{-1}. Results from ANOVA confirmed a significant difference between riverine, transition, and lucustrine habitats (Table 3.2). Regression analysis demonstrates a correlation between ln-density and distance from the inflow (Figure 3.4). Correlations were found between polychaete densities, flow velocity and depth ($P \leq 0.05$), but could not be separated from the effects of inflow distance.

Microhabitat Features

Plotting flow velocity against ln-density for all samples containing the polychaete showed that *M. speciosa* is found most often at velocities between 0.02 and 0.05 m/s where the polychaete was most commonly associated with two microhabitats (Figure 3.3). One microhabitat was sand embedded with fine benthic organic matter (FBOM).

This habitat was generally clustered at the slower velocities. Another important microhabitat was a mat-forming epilithic alga identified to the genus *Cladophora*. This algae adhered strongly to any hard substrate (e.g. mussel shells), was slightly compressible, and about 1.0 cm thick. Examination of the algae found it to contain large quantities of FBOM, diatoms, sand and silt. Polychaete densities within this material varied widely but regression analysis did show an inverse relationship between 1n-density and velocity ($P<0.01$, Figure 3.3). *Manayunkia speciosa* was found in a wider range of flow velocities where *Cladophora* occurs than within the sand-FBOM habitat. Polychaetes were sometimes associated with sponge but rarely with beds of vascular macrophytes or areas containing heavy deposits of coarse organic matter. *Manayunkia speciosa* was rarely found in medium and large grained sands that lacked FBOM, but was often found on such substrates if noticeable amounts of FBOM were present. Moderately dense populations (3,896/m^2) of *M. speciosa* were found at the mouth of the Williamson River with large deposits of sand-FBOM. The depth of samples containing the polychaete ranged from 0.2 m in edge-water riffles to 6.5 m in the reservoirs. Examination of live specimens revealed a diet consisting of very fine (2 – 3 μm) detritus and small diatoms.

Apparent Prevalence of Infection

Prevalence estimates of the parasite *C. shasta* in populations of *M. speciosa* were low (~0.27%) for most sites tested (Figure 3.6). In the UKR, parasite prevalence at the mouth of the Williamson River (Rkm 441), Klamath Lake (Rkm 425) and above the Boyle Reservoir inflow (Rkm 369) was estimated at 0.45%, 1.02% and 0.69%

respectively (Table 3.3). Two samples collected within the Boyle Reservoir inflow were not positive for infection nor were samples collected below the Boyle Reservoir (Rkm 352) or from the Iron Gate inflow (Rkm 327). Two samples collected in the LKR: one site at I-5 (Rkm 290) and the other at the Tree of Heaven (Rkm 278) exhibited an infection prevalence of 4.9% and 8.3% respectively. One site at Rkm 212 showed a prevalence of 0.79% and infection was not detected in populations tested downstream of this site at Rkm 189.5, 187.9 and 187.5 (Figure 3.6). Regression analysis did not detect a significant correlation between prevalence of infection and density, flow velocity or percent adults. Control tissues from infected polychaetes and infected fish amplified as expected. Negative control tissues were not positive for *C. shasta* DNA.

DISCUSSION

Distribution and habitat preference

The geographic range of *C. shasta* is shaped by both its hosts, and its confinement to the Pacific Northwest of the U.S. and Canada can be explained by its specificity for Pacific salmon as the vertebrate host. However, the range of the parasite is known to be discontinuous within the larger range of Pacific salmon and this is likely explained by factors that define the distribution of the invertebrate host, *M. speciosa*. Prior to this study, little was known about this organism or its habitat requirements that would provide insight into observed patterns of parasite distribution. This study, conducted to explain the epidemiology of *C. shasta* in the Klamath River, demonstrated *M. speciosa* throughout the main-stem Klamath River from Klamath Lake to the mouth of the river. In

the Lower Klamath River, the polychaete was aggregated into small patchy populations that were most concentrated between I-5 (Rkm 293) and the Trinity River confluence (Rkm 70). In this area, discreet, and sometimes, very dense populations were located within slow-flowing habitats with *Cladophora* sp., sand-silt and fine benthic organic matter (FBOM) or large substrates that were heavily embedded with this material. Below the Trinity River, polychaete populations were sparse with considerably lower densities. The absence of *M. speciosa* from many habitats with slow flows and FBOM suggests that its distribution may be limited by its ability to disperse and colonize these habitats.

The patchy and inconsistent distribution of *M. speciosa* in the lotic environment in both the UKR and LKR makes predictive assessments of habitat preference difficult. Intensive sampling efforts in several lotic habitats above the Trinity River revealed that populations tended to be highly aggregated into a small area (5 – 10 m^2). Such populations are easy to miss and thus some suitable habitats may not have been represented in our sampling scheme. In addition, conventional aquatic invertebrate sampling techniques (e.g. kick-nets) were not effective tools for sampling *M. speciosa* because of its small size and the range of substrates and depths at which the polychaete could be found. Another confounding factor to determining preference was that *M. speciosa* was not confined to any particular category of macrohabitat, but was found in a variety of locations such as the edge-water of riffles, bridge supports in lucustrine areas, on mussel shells and hard substrates covered with *Cladophora* sp., or just hard substrates embedded with fine materials. The macrohabitat preference of *M. speciosa* needs to be considered in light of its dispersal abilities because an organism that is unable to actively

move toward favorable habitats may settle in unfavorable or marginal habitats (Fonseca and Hart 2001). Thus, the tools used here to determine preference are based on the frequency of occurrence and relative densities of *M speciosa* by macrohabitat as well as the biological and ecological characteristics exhibited by this polychaete. The population size and structure of *M. speciosa* at the riverine areas of reservoirs and river mouths provides contrast to the lotic populations.

Manayunkia speciosa was consistently present at the riverine transition (lotic-lentic interface) into man-made reservoirs of the Klamath River at moderate (804/m^2) to high (16,054/m^2) densities. In JC Boyle reservoir, where a large number of samples were collected to study spatial patterns, population densities rapidly increased with distance from the inflow into the reservoir and then steadily decreased. These results parallel other studies, which also found the polychaete at the mouths of major rivers (Hiltunen 1965; Hazel 1966; Mackie and Qadri 1971; Poe and Stefan 1974). The spatial structuring of lotic-lentic interface populations suggests that optimal habitats are present at the densest areas, which indicates that the lotic-lentic interface is the preferred macrohabitat. It is suggested that polychaete distribution in the lotic environment can be explained in terms of patch dynamics. For example, it has been noted that habitat similarities exist between the lotic-lentic interface and the riffle-pool interface (Willis and Magnuson 2000) and that the quantity and quality of this transitional "ecotone" will not be distributed equally over space and time in the river environment (Pringle et al. 1988). Thus, the spatiotemporal heterogeneity of lotic systems places a burden on aquatic organisms to be

effectively resilient to disturbances (high flow events) and/or possess mechanisms (e.g. good dispersal) to take advantage of those changes (Townsend 1989).

Micro and macrohabitat stability or resilience is likely a key factor influencing distribution of the polychaete. In March 2005, we recorded a population in sand-FBOM at the Tree of Heaven with the highest maximum densities of all pools sampled (40,607/m^2). For monitoring purposes, this site was sampled again in July of 2005 after a high flow event (May – June 2005) and the population was absent. A reference polychaete population in *Cladophora* located approximately 11.5 Rkm upstream of the Tree of Heaven, was also sampled at the same time and had not been impacted by the high flow event. Patches of *Cladophora*, where *M. speciosa* was consistently identified, were observed to not grow away from the substrate but spread around the exposed surface and was sufficiently porous enough to collect fine materials and provide space for *M speciosa* and other meiofauna such as nematodes. The ability of *M. speciosa* to persist in certain microhabitat types during disturbances and not in others indicates that lotic populations are influenced by microhabitat stability.

Stability at the macrohabitat scale also influences polychaete distribution. The lotic-lentic interface could be considered the most stable macrohabitat sampled during this study. Here, polychaete populations receive a constant supply of flowing water where extreme events are buffered by the reservoir. Albeit, daily fluctuations of flow because of hydropower needs may influence small-scale spatiotemporal distribution within this habitat, however, the unique life traits of *M. speciosa* can apparently circumvent

disadvantages and may have allowed this polychaete, to survive and specialize for such habitats.

Manayunkia speciosa is a member of the Sabellidae, which are unique in having species that occur exclusively in freshwater systems (Rouse and Pliejel 2001). The subclass Fabriciinae has at least two freshwater representatives: *M. speciosa*, identified within nearctic freshwater systems of both the Pacific and Atlantic coasts of North America, and *M. baikalensis,* found in the Lena and Amur basins of Siberia (Klishko 1996). Another closely related polychaete, *M. aestuarina*, an inhabitant of brackish water estuaries and mud flats, occurs roughly along the same longitudinal gradient as *M. speciosa* in North America and Western Europe (Light 1969; Bishop 1984). Fabriciid polychaetes are sedentary, or discreetly motile, tube-dwellers with no desiccation resistant or free-swimming stages (Pettibone 1953; Holmquist 1973; Fauchald and Jumars 1979; Rouse and Pleijel 2001).

Most Fabriciinae are both suspension feeders and facultative surface deposit feeders, utilizing a diet of fine organic detritus and micro-algae that are only seasonally abundant (Rouse and Pleijel 2001; Fauchald and Jumars 1979). Polychaetes that utilize this strategy, referred to as “interface” feeders, are known to switch feeding modes during flux of seston quantity and quality (Taghon and Green 1992). Because they require large quantities of organic matter, interface feeders are considered food limited (Lopez and Levinton 1987). These species are able to select particulates of the appropriate size for ingestion while larger sizes are either rejected or used as material for tube construction (Lewis 1968). Suspension feeding with delicate feeding appendages is likely the reason

M. speciosa is found within slower flowing habitats (sand-FBOM). In the case of *Cladophora* sp., the habitat appears to collect material the polychaete can use as food and provide a buffer from high flow events.

Information on *M. speciosa* reproductive ecology is sparse, however, all known Fabriciinae are gonochoristic, continuous or semi-continuous breeders (Rouse and Pliejel 2001). Female *M. aestuarina* brood small clutches of large non-feeding larvae (Bell 1982) and several clutches may be produced by the same female during the reproductive phase (Bick 1996). During this study, tubes containing 2-3 *M. speciosa* larvae were found during March, July, August and November, with the highest numbers of tubes containing eggs and juveniles occurring during March and November. Large investments of resources into a small number of offspring are traits characteristic of K-selected strategists, which are poor dispersers but good competitors, in contrast to R-selected strategists, which are good dispersers (Townsend 1989). The combination of iteroparity, a simple diet of organic matter, and tolerance to water temperatures near freezing point (Holmquist 1973) would permit populations of *M. speciosa* to continue growing when most other aquatic macro-invertebrate populations have declined.

Because the type locality of *M. speciosa* occurs along the Atlantic coast, Hazel (1966) suggested a possible introduction of *M. speciosa* to freshwaters of the Pacific coast via fish transplants. However, this was considered not likely to occur in the isolated arctic lakes of Alaska where *M. speciosa* has been identified (Croskery 1978; Rolan 1974; Holmquist 1973; Light 1969). One explanation for the geographic distribution of the polychaete is that freshwater tolerant members of Fabriciinae were translocated

during the last marine incursion and adapted to a solely freshwater existence when the oceans receded giving rise to *M. speciosa* and *M. baikalensis* (Holmquist 1973; Croskery 1978; Klishko 1996). Additional evidence supporting a long residence time of *M. speciosa* along the Pacific coast comes indirectly from epidemiological studies. Many salmonid species native to Pacific Northwest watersheds, where *C. shasta* is endemic, have developed genetic resistance to the parasite (Bartholomew 1998; Bartholomew et al. 2001) indicating that the life cycle, and therefore the polychaete, was present in these systems before the introduction of non-native species. This helps to explain the coastal freshwater distribution of *M. speciosa*, but also gives insight as to why, if the polychaete has been present in these systems for hundreds if not thousands of years, it has not managed to spread to more mainland freshwater systems. The polychaete is not well suited to dispersal and evidence from this study and others suggest that *M. speciosa* is habitat specific.

Host-parasite distribution

In a previous study, we developed a hypothesis of polychaete-parasite distribution that might explain the high *C. shasta* induced mortality observed in naturally resistant Klamath River Chinook salmon (Stocking et al. 2006). We concluded that the mortality observed in the LKR was likely a result of elevated actinospore densities relative to the UKR where exposed fish rarely died due to infection. Results from an environmental *C. shasta* QPCR assay, developed to quantify spore density in the water column, demonstrate that parasite densities at sites in the LKR were significantly higher than in

the UKR (Hallett and Bartholomew 2006). This could be explained by a greater proportion of infected polychaetes in the LKR relative to the UKR. To test this assumption, we assayed polychaetes from several populations in the UKR and LKR for presence of *C. shasta*. Generally, myxozoan infection prevalence in naturally exposed invertebrate populations tends to be very low (0.1 – 2.0%), thus prohibiting tests on individuals (Ozer et al. 2002; Zendt and Bergersen 2000; Rognlie and Knapp 1998; Markiw 1986). Therefore, we targeted populations of *M. speciosa* where sufficient numbers had been acquired during the sampling effort to estimate infection prevalence.

Methods for pooling samples have been widely applied in large animal and insect vector epidemiology since the 1950's (Brookmeyer 1999; Cowling et al. 1999; Colon et al 2001; Wicki et al. 2000; Raizman et al. 2004) and have recently become important to fish health professionals (Allen and Bergersen 2002; Williams and Moffitt 2001). The advent of molecular technology and statistical software has allowed these methods to be easily applied to populations of fish and aquatic invertebrates (Williams and Moffitt 2005). We acknowledge that samples used to estimate prevalence were collected at different times and may not display an accurate representation of actual host-parasite distribution and prevalence at any particular point in time. However, the results present us with two general factors to consider. One is simply that prevalence of infection in most polychaete populations sampled was quite low, averaging only about 0.27%. Another factor revealed by the data shows a zone of high infection prevalence (4.9% and 8.3%) directly downriver from Iron Gate Dam. In a concurrent study, Hallett and Bartholomew (2006) reported concentrations of >20 spores L^{-1} in this same river reach, in contrast to

<1.0 spores L^{-1} above Iron Gate Dam. This area, where high mortality of exposure fish occurs, is also a spawning area where adult Klamath River fall Chinook salmon are concentrated.

A likely scenario suggests that adult salmon, carrying *C. shasta* spores infectious to the polychaete (myxospores), spawn, die and release these spores into the environment. Populations of *M. speciosa* within or near the spawning grounds receive the highest doses. This provides one explanation for the high *C. shasta* induced mortality observed in fish exposed within this area and supports the hypothesis that elevated spore densities from heavily infected populations are the cause of salmonid ceratomyxosis in the LKR. The fact that all fish groups exposed throughout the LKR succumbed to infection (Stocking et al. 2006) suggests that actinospores from these highly infected populations can remain viable during the 5d journey from Iron Gate dam to the estuary. Interestingly, an area of high infection also occurs in the Williamson River, a tributary to Klamath Lake, where concentrations of 10 – 20 spores L^{-1} were reported (Hallett and Bartholomew 2006). Anadromous salmon are blocked from this region, so the only salmonid hosts present are native redband rainbow trout (*O. mykiss*). Polychaetes sampled at the mouth of the Williamson River showed a relatively low infection prevalence of 0.45%. However, the prevalence of *C. shasta* infection in polychaete populations above these sites was not determined and may be considerably higher than at the river mouth.

This study addresses factors that are paramount to understanding *C. shasta* in the Klamath River by relating the epidemiology of the parasite with the ecology of its

invertebrate host. Additional information on the life history characteristics of *M. speciosa* and *C. shasta* infection dynamics will be necessary to develop a conceptual model better suited to explain how a "hot-zone" developed below Iron Gate Dam and what, if anything, can be done to minimize the impacts of *C. shasta* on native Klamath River salmonids. For example, would rapid high flow events (spates) decrease polychaete and/or parasite densities or would longer, more moderate flows decrease juvenile salmon exposure time? A recent high flow event, which occurred during the drafting of this manuscript, may provide a natural experiment in which this very question can be evaluated.

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TABLES AND FIGURES

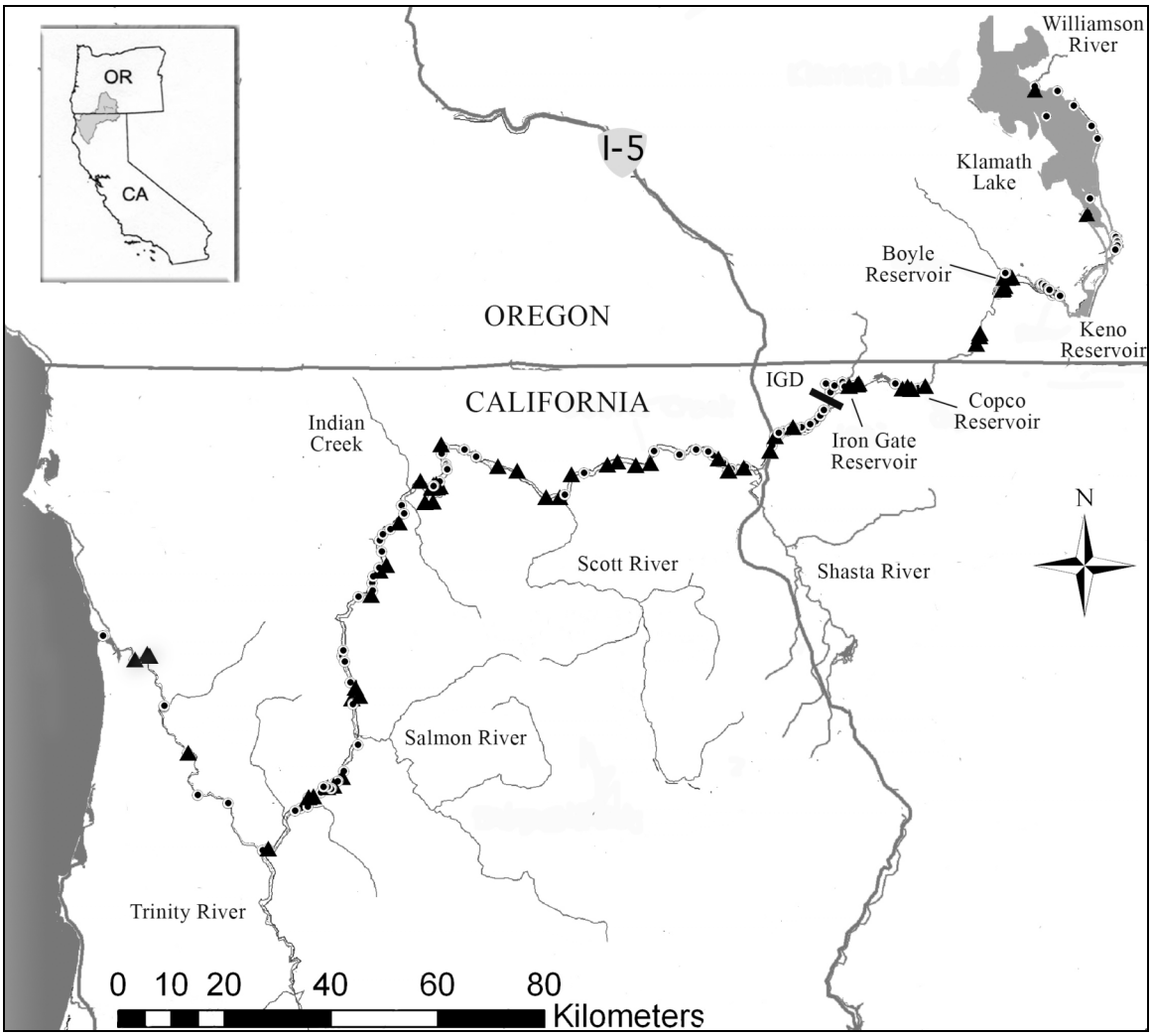

Figure 3.1. Map showing 2003-2005 sample sites in the Klamath River CA-OR. Circles are sample sites where *Manayunkia speciosa* was not identified and filled triangles are sites where the polychaete was identified. For this study, Iron Gate Dam (IGD) divides the Upper Klamath River from the Lower Klamath River. The Lower Klamath River was further divided and sampled from IGD to I–5, to Scott River, to Indian Creek, to Salmon River, then from Trinity River to the estuary (Table 1).

Table 3.1. Date and location of *Manayunkia speciosa* sampling conducted in the Klamath River from 2003 to 2005.

Sample Period	Region	Location	River kilometer	Number of sites
July - August 2003				
	UKR	Keno Reservoir	374.9	19
	UKR	Boyle Reservoir	361.6	13
	UKR	Boyle Peaking Reach	328.3 - 354.7	4
	LKR	I-5 to Scott River	292.9 - 230.1	15
June - August 2004				
	UKR	Klamath Lake	407.3 - 422.3	8
	UKR	Boyle Reservoir	361.6	33
	UKR	Keno Reservoir	382	5
	UKR	Below Boyle Reservoir	342.8	3
	UKR	Copco Reservoir	319.6 - 328.3	10
	UKR	Iron Gate Reservoir	305.7 - 316.7	17
	LKR	IGD to I - 5	305.7 - 292.9	24
	LKR	Salmon River to Trinity River	106.2 - 69.8	24
	LKR	Trinity River to Estuary	69.8 - 0	11
July - August 2005				
	LKR	I - 5 to Scott River	292.9 - 230.1	16
	LKR	Scott River to Indian Creek	230.1 - 171.8	25
	LKR	Indian Creek to Salmon River	171.8 - 106.2	19
	LKR	Salmon River to Trinity River	106.2 - 69.8	11

Note: Region UKR = Upper Klamath River and LKR = Lower Klamath River. Iron Gate Dam = IGD and Interstate-5 = I - 5. River Kilometer values are the approximate area where samples were collected.

Table 3.2. Summary of one-way ANOVA results for *Manayunkia speciosa* occurrence frequency and ln-density estimates from four lotic macrohabitat type (MHT) and three lentic MHT collected from the Klamath River during 2003-2005.

MHT	† Number of MHT	¥ Freq. of Occur. MHT	Total (**N**)	§ Freq. of Occur. (**N**)	ç Mean ln-Density (**N**)	Std. Dev.
Lotic (F = 1.21, P = 0.31) $df_{3,193}$						
Riffle	15	0.20	17	0.18	-0.52352	1.94
Run	60	0.40	82	0.29	0.46915	2.89
Eddy	17	0.47	24	0.33	0.43606	2.78
Pool	64	0.52	84	0.39	0.91916	3.21
Lentic (F = 13.15, P <0.01) $df_{2,94}$						
Riverine	3	1.00	25	0.80	2.91341	4.01
Transition	4	0.75	43	0.33	0.83427	3.17
Lucustrine	4	0.25	29	0.07	-0.23476	0.81

Note: † The total number of MHT that were sampled. ¥ The ratio of MHT's where *M. speciosa* was identified. § The ratio of all samples (**N**) of a MHT in which *M. specoisa* was identifed. ç All density values, including zero values, were natural log-transformed (ln-density + 0.50) with standard devations.

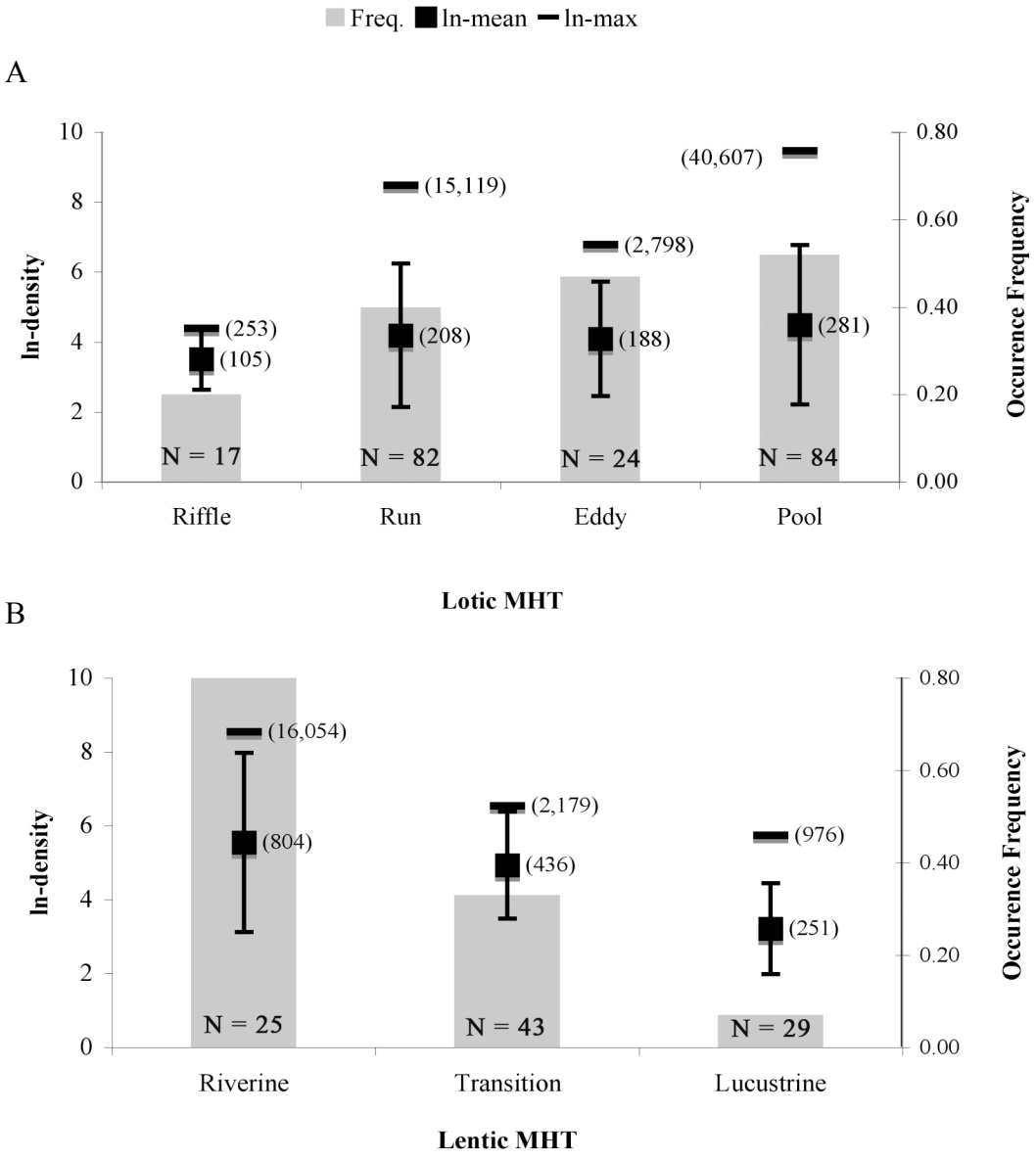

FIGURE 3.2. Mean density (natural log-transformed), max density and frequency of occurrence of *Manayunkia speciosa* by (A) lotic macrohabitat and by (B) lentic macrohabitat. Density values are based on only those samples that contained *M. speciosa*. Values in parenthesis are estimated mean and maximum field densities / m^2.

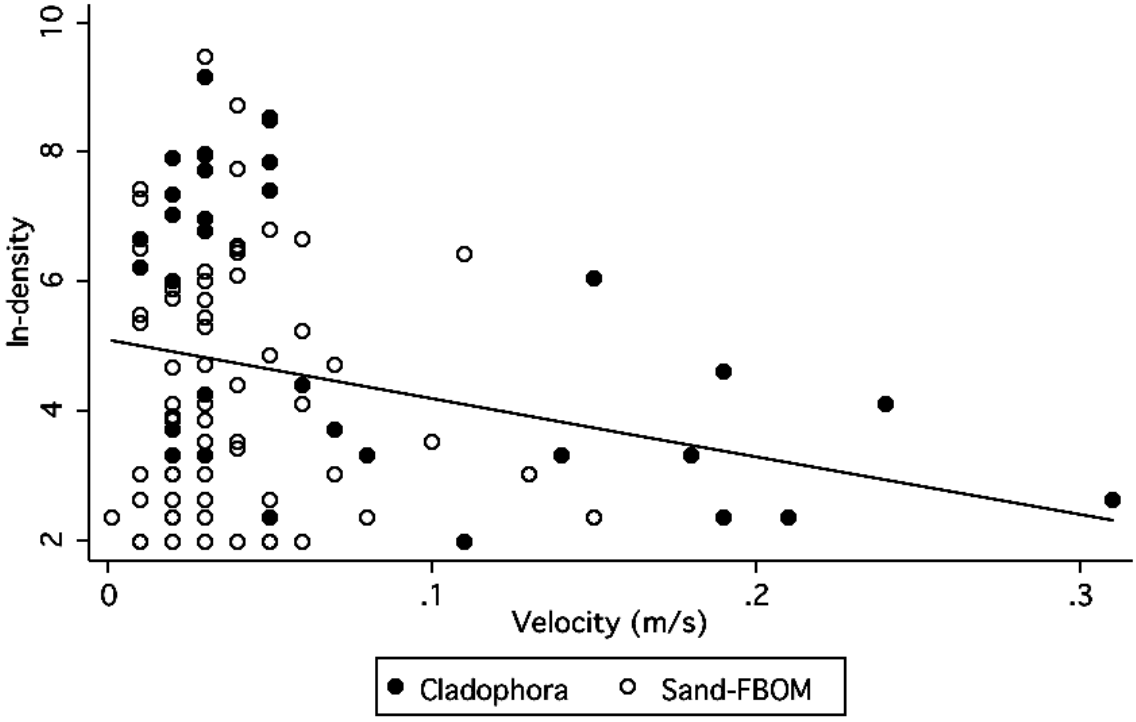

FIGURE 3.3. Relationship between *Manayunkia speciosa* density (natural log-transformed) and flow velocity within two microhabitats: Cladophora (a mat-forming, epilithic algae) and sand with fine benthic organic matter (FBOM) collected from the Klamath River. Simple linear regression: within cladophora (r^2 = 0.30, P <0.01, n = 32) and sand (r^2 = .02, P = 0.26, n = 69).

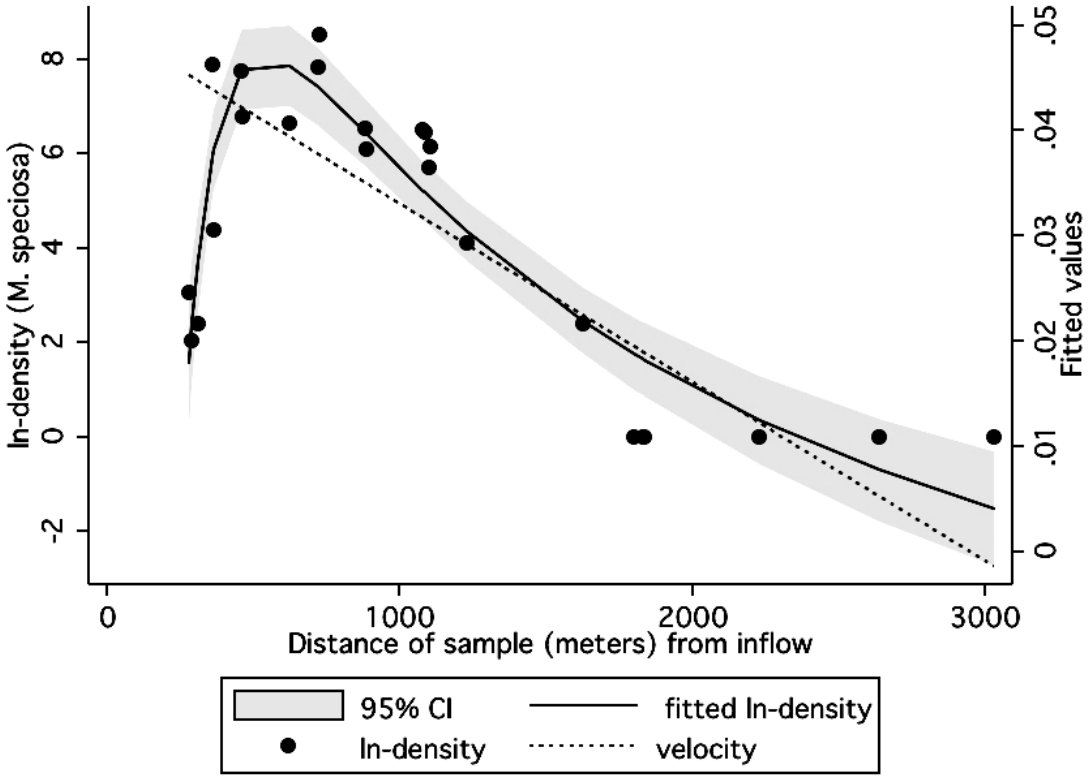

FIGURE 3.4. Density (log-transformed) distribution of *Manayunkia speciosa* sampled from the J.C. Boyle reservoir starting at the inflow (see Fig. 4). The *ln*-density distribution was fitted using fractional polynomial regression ($r^2 = 0.54$, $P = <0.001$, $n = 24$) with 95% confidence intervals (CI). Velocity was fitted (dashed line) by simple linear regression.

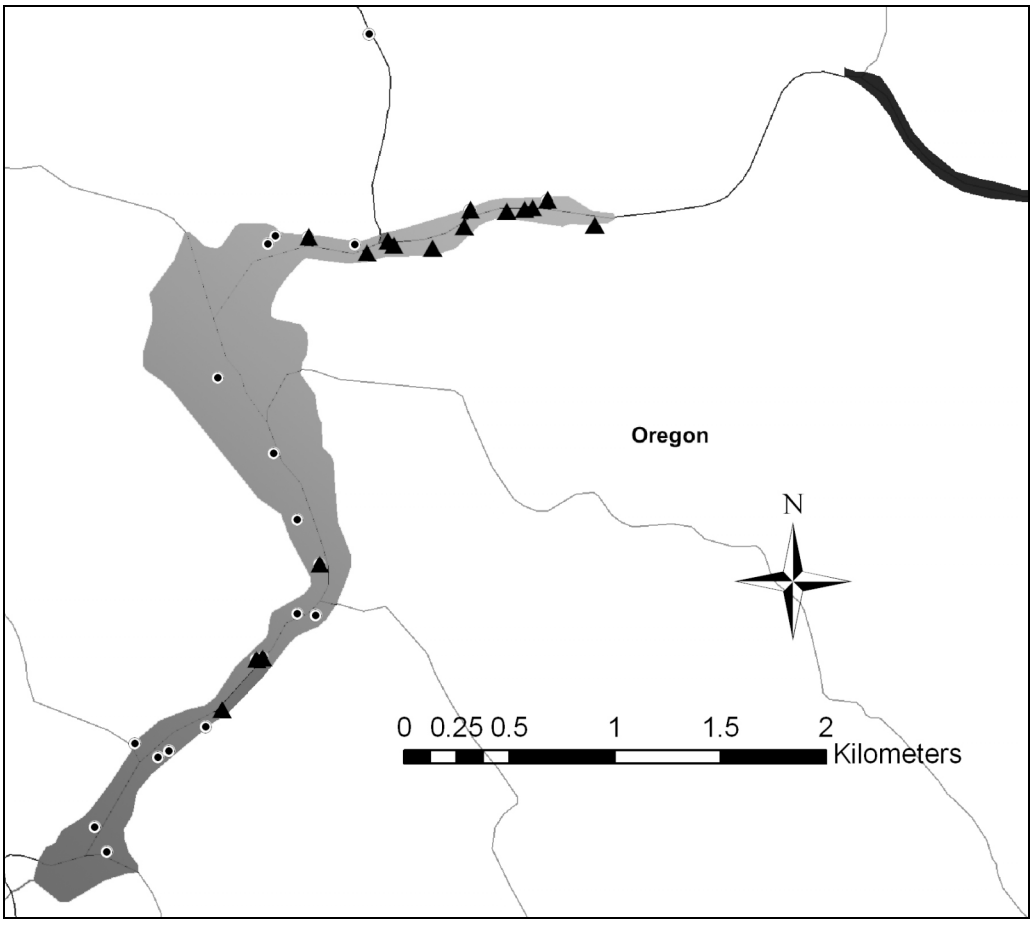

FIGURE 3.5. Samples collected June 2003 from the J.C. Boyle reservoir located near Klamath Lake (see Fig.1). Circles are sample sites where *Manayunkia speciosa* was not identified and triangles are sites where *M. speciosa* was identified. The inflow to the reservoir is located in the northeast corner and flows to the southwest.

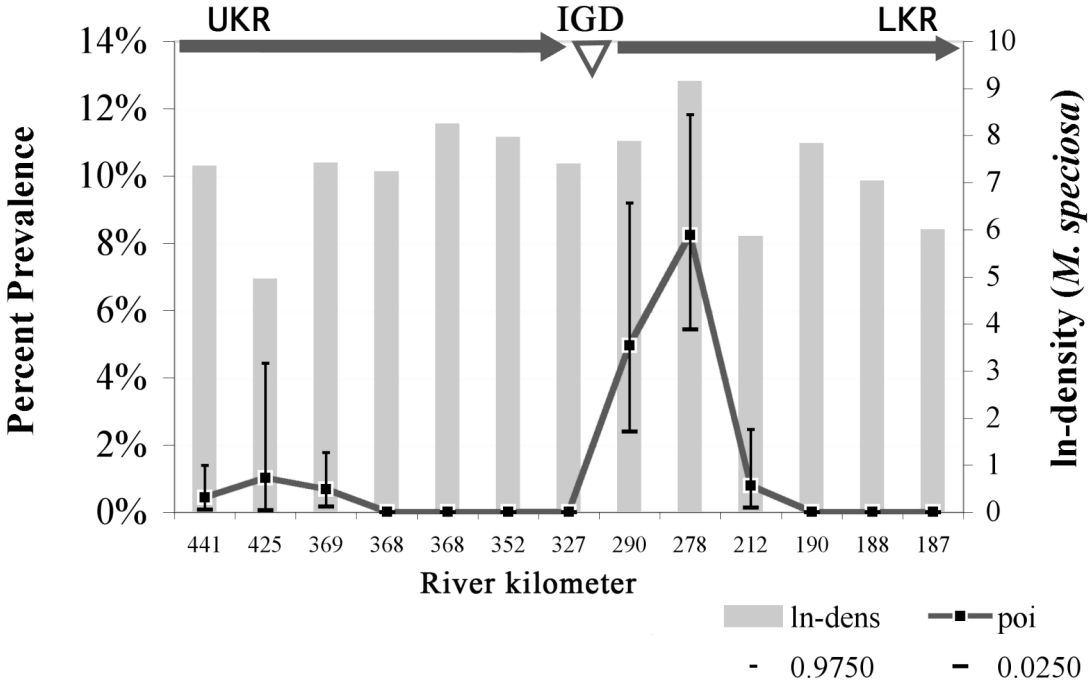

FIGURE 3.6. Estimates of *Ceratomyxa shasta* infection prevalence (poi) and associated confidence levels within selected populations of *Manayunkia speciosa* collected from the Klamath River. Sites sorted on the x-axis from Klamath Lake (Rkm 441) going downriver towards the mouth (see Table 3.3). Abbreviations UKR = Upper Klamath River, IGD = Iron Gate Dam, and LKR = Lower Klamath River.

Table 3.3. Summary of samples used to estimate the apparent prevalence of *Ceratomyxa shasta* within selected populations of *Manayunkia speciosa* collected from the Klamath River, OR - CA.

Rkm	Date Coll	Percent Adults	Pool size and number of pools tested (n)	Pools Positive	Total†	% Prev¥
441.000	Jul-04	84.8	50(4), 25(4), 10(10), 5(10)	0, 0, 1, 1	450	0.45
425.000	Jul-04	84.2	10(5), 5(10)	0,1	100	1.02
368.500	Oct-03	63.7	50(4), 25(4), 10(10), 5(10)	0, 0, 1, 2	450	0.69
368.220	Jul-04	63.4	50(4), 25(4), 10(10), 5(10)	0, 0, 0, 0	450	0
368.215	Jul-04	62.5	50(4), 25(4), 10(10), 5(10)	0, 0, 0, 0	450	0
352.200	Jul-04	48.2	50(4), 20(5), 10(10) 5(10)	0, 0, 0, 0	510	0
327.150	Jul-04	55.8	25(4), 20(5), 10(10), 20*(10)	0, 0, 0, 0	500	0
289.805	Jul-05	43.0	50(4), 20(5), 10(8), 40*(1)	4, 2, 4, 0	420	4.96
278.250	Mar-05	76.4	10(15), 5(30), 1(45)	7, 7, 3	345	8.24
211.918	Jul-05	87.9	50(2), 20(5), 10(8), 5(5)	2, 0, 0, 0	305	0.79
189.500	Aug-04	90.4	50(4), 25(4), 10(10), 5(10)	0, 0, 0, 0	450	0
187.971	Aug-04	96.1	50(4), 25(4), 10(10), 5(10)	0, 0, 0, 0	450	0
187.489	Jul-05	85.7	50(2), 20(5), 10(8), 5(5)	0, 0, 0, 0	305	0

Note: Values with asterisk are juveniles only where all other values are both juveniles and adults. Pool size refers to the number of polychaetes in that pool. Pools positive refers to number of each pool size that tested positive for *C. shasta*. † Total number of polychaetes tested. ¥ The percentage of the Total tested that was estimated to be positive for *C. shasta*.

CHAPTER 4: SURVEILLANCE FOR *Ceratomyxa shasta* IN THE PUGET SOUND WATERSHED WA, USA

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SURVEILLANCE FOR *Ceratomyxa shasta* IN THE PUGET SOUND WATERSHED WA, USA

ABSTRACT

Discovery of fish exhibiting clinical signs of ceratomyxosis in Washington State prompted concern over the potential impact of the myxozoan parasite *Ceratomyxa shasta* on native stocks of steelhead (*Oncorhynchus mykiss*). To investigate these concerns, a survey of 16 freshwater systems within the Puget Sound watershed, including Lake Washington, was conducted by sentinel exposure of susceptible fish. Fish were exposed for 7d during September 2003 and May 2004, and then returned to a holding facility to monitor for signs of disease. Mortality caused by the parasite occurred only in the exposure group held at the University of Washington Hatchery, which receives its water from Portage Bay of Lake Washington. Fish from all other sites were negative for *C. shasta*, both visually and by PCR assay, with the exception of a single fish held at the Deschutes River -Tumwater Falls Hatchery in September 2003. A single deformed spore was detected but infection could not be confirmed by PCR and the parasite was not detected from any other fish held at that site during either the September or the May exposures.

INTRODUCTION

Ceratomyxosis is a disease of salmonids caused by the myxozoan parasite *Ceratomyxa shasta*. Clinical signs of the disease include abdominal distension as a result of excessive ascites production, lethargy, loss of appetite, and swollen vent often accompanied with hemorrhaging (Conrad & Decew 1966; Schafer 1968; Bartholomew et al. 1989). These signs develop as the parasite migrates to the lower intestinal tract causing inflammation and extensive tissue damage. In cases of severe infection, *C. shasta* can colonize other soft tissues including the spleen, liver and kidneys. Where the parasite is enzootic, effects on hatchery and wild populations of salmonids can range from minimal to severe depending on the susceptibility of the species (Bartholomew 1998), water temperature during exposure (Udey et al. 1975), and duration or frequency of exposure (Ratliff 1981; Ibarra et al. 1994).

As with other known myxozoan life cycles, the *C. shasta* life cycle is complex: the freshwater polychaete *Manayunkia speciosa* hosts the actinospore (infectious) stage (Bartholomew et al. 1997) while the myxospore stage can develop in most species of salmonids. Presence of the invertebrate host determines whether the parasite becomes established in a watershed, thus, the distribution of *C. shasta* is limited by the distribution of the polychaete host. In North America, *C. shasta* is enzootic within several large river systems of the Pacific Northwest of the U.S. and Canada. Occurrence is best documented in the Columbia River basin where *C. shasta* has been implicated as a contributor of mortality to out-migrating smolts (Ratliff 1983; Bartholomew et al. 1992). In Washington, streams where the parasite is known to be present include the Columbia

River, Cowlitz River and La Camas Creek, the latter two being Columbia River tributaries.

The first indication that *C. shasta* was established in waters around Puget Sound, WA was the diagnosis of ceratomyxosis in June 2002 from a steelhead smolt (*Oncorhynchus mykiss*) in a downstream migrant trap on the Cedar River. Subsequently, *C. shasta* was diagnosed from a juvenile cutthroat trout (*O. clarki*) collected from a trap on Bear Creek, and from juvenile Chinook salmon (*O. tshawytscha*) being held at the University of Washington hatchery as part of an unrelated study (J. Thomas, Washington Fish and Game, personal communication). This investigation was designed to examine the distribution of *C. shasta* in the Lake Washington-Puget Sound area and assess the potential impacts of the parasite on local stocks of steelhead.

METHODS

Fish exposure and handling: *Ceratomyxa shasta*-susceptible Twin Lakes cutthroat trout (1.0 g, Lakewood Hatchery, WA) were used in exposures conducted in 2003 and Spokane rainbow trout (2.5 g, Eells Springs Hatchery, WA) were used in the 2004 exposures. Groups of 60 fish were held in live cages for 7d in 16 separate freshwater locations around Puget Sound including Lake Washington. Exposure sites in rivers feeding into Puget Sound were located upstream of the estuaries to avoid saltwater intrusion. Thirteen sites were tested by exposure in 2003 and 8 sites were tested in 2004 (Table 4.1, Figure 4.1).

Groups of 60 fish from each stock were also exposed for 7d in the Willamette River, Oregon (RM 124), where *C. shasta* is known to occur, to confirm the relative susceptibility of fish stocks used in this study. Non-exposed, negative control groups (60 of each stock) were maintained at the Salmon Disease Laboratory, Oregon State University (OSU-SDL) on pathogen free water during and after the exposure period.

Following the exposure period, experimental fish were returned to holding facilities at the OSU-SDL and maintained on 16˚C pathogen free water. Fish were prophylactically treated for bacterial pathogens by bath treatment with Furanase® (1.0 mg/L, 1 h) and feeding a diet supplemented with oxytetracycline in the form of TM100® for 2 wk post-exposure. A dilute formalin bath was administered when external parasites were detected. After treatment, fish were maintained on a standard diet for 97d or until mortality occurred.

Diagnosis of C. shasta infection: Fish that had died during the holding period were necropsied. A wet mount of intestinal contents of the lower gut was prepared by inserting a sterilized inoculation loop of appropriate diameter into the anogenital pore and lower gut then smearing the contents onto a glass slide. The material was then examined under 200x magnification for 3min. If *C. shasta* spores were identified, the fish was considered positive for infection. If spores were not observed, a sample of the posterior intestine was collected for PCR assay (Palenzuela et al. 1998).

At termination of the experiment (approx. 97d), surviving fish were euthanized using buffered tricaine methanesulfonate (MS222). A subset of 20 fish from each group in 2003 and 10 fish from each group in 2004 were necropsied and examined for infection

by microscopy. If infection was diagnosed in an exposure group, then samples of all remaining fish from that exposure were collected for PCR assay. PCR assay was conducted according to Palenzuela et al. (1998). Percent prevalence is calculated as the number of infected fish identified both visually and by PCR assay, divided by the number of fish examined within that group (e.g. mortalities and fish that survived to the termination period) x 100. Percent mortality is calculated as the number of fish deaths associated with ceratomyxosis divided by the number of fish examined x 100.

RESULTS

2003—Post exposure mortality was high in the Deschutes River Tumwater Falls group with 53 of 60 fish dying prior to 90d post exposure (PE); however, the cause of death was not determined. *Ceratomyxa shasta* was detected in only a single fish, and the infection appeared to be at a very low level as only one spore was identified, few presporogonic stages were recognized and all fish were negative by PCR. The parasite was not detected from fish exposed at any other location and infection was not detected in experimental fish at termination of the study. Mortality and incidence of *C. shasta* infection in positive control groups exposed in the Willamette River was low, with an infection prevalence of 6.0%. The parasite was not detected in the non-exposed control groups.

2004— *Ceratomyxa shasta* was detected at a low prevalence (9.1%) among fish held at the University of Washington Hatchery. Infection with *C. shasta* was not detected from groups held at the Tumwater Falls Hatchery on Deschutes River water. Infection

prevalence in the Spokane rainbow trout positive control group held in the Willamette River was 65.8%. The negative control groups tested negative for infection.

DISCUSSION

Sentinel exposure studies currently provide the most reliable information about *C. shasta* distribution when coupled with molecular technology such as the PCR assay. However, because exposures are conducted during a discreet and relatively brief period, and in a specific location within a body of water there are limitations to these studies. For a parasite like *C. shasta* that exhibits a patchy geographic, and often temporal distribution, it can be difficult to determine whether failure to detect the parasite is a result of low abundance, timing or complete absence from the waters being tested. Thus, detection of the parasite in exposed fish confirms that the *C. shasta* life cycle is established, but inability to detect the parasite does not necessarily confirm its absence. In this survey, exposure locations were selected close to the river mouths to detect parasites released from upriver. The inability to detect the parasite from most of the waters tested suggests that *C. shasta* is either not present, or present in low abundance.

In 2003, *C. shasta* was detected in only a single fish from all exposure locations, and from only 6.0% of those held at the control location. This low infection level suggests either that, parasite numbers were extremely low at the exposure site, or the strain of fish used had some degree of resistance to *C. shasta*. Results of concurrent *C. shasta* studies in Oregon's Klamath and Willamette Rivers in 2003 also indicated that parasite levels had been lower than normal during the spring and summer, suggesting that

regional environmental factors in the Pacific Northwest may have influenced exposure results in this study. Additional studies were conducted in 2004 to verify the 2003 results. The inability to re-isolate *C. shasta* from 2004 exposure groups at any of the Deschutes River sites could be explained by occurrence of very low parasite levels, exposure timing, or by human error in the initial isolation.

Detection of *C. shasta* from fish exposed at the University of Washington Hatchery demonstrates that the life cycle is established in Lake Washington. However, additional sentinel exposures at various locations in the lake during periods from late spring to late fall would provide more information on the extent of parasite establishment. Because the parasite could not be detected in the Cedar River and Bear Creek tributaries of Lake Washington, sites where infected smolts had previously been identified, it is likely that the smolts were exposed in Lake Washington and migrated upstream. However, this study does not rule out the tributaries as possible sources of infection and this should be further investigated.

Results of this survey indicate that the life cycle of *C. shasta* is not widely established in the Puget Sound watershed. If the parasite is present in rivers entering the Puget Sound, then it is not as abundant as in other enzootic areas, such as the Columbia and Klamath rivers. As such, the parasite is not likely to have contributed significantly to declines in steelhead populations throughout Puget Sound. However, additional studies to define parasite distribution and seasonal abundance and to determine the susceptibilities of resident stocks would provide information that would allow a better assessment of potential parasite effects.

ACKNOWLEDGMENTS

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TABLES AND FIGURES

FIGURE 4.1. Washington map showing locations of exposure sites. The location where fish were infected with *Ceratomyxa shasta* (UW Hatchery) is identified as a triangle. An unconfirmed detection (Deschutes River) is identified as a cross.

Table 4.1—Exposure results of the *Ceratomyxa shasta* survey of Lake Washington and Puget Sound, WA tributary streams for 2003 - 2004. Twin Lakes cutthroat trout were used for exposures in September 2003. Spokane rainbow trout were exposed in May 2004. Control site for exposed controls in Oregon

Region	Site	Expos. Date[a]	No. Recovered	No. Mortalities	Mortalities Cs+ (visual)	Mortalities Cs+ (PCR)	No. Survivors	Survivors Cs+ by PCR	% Prev.[b]
N. Puget Sound	Nooksack	3-Sep	59	0	0	-	59	0 (20)	0
	Samish	3-Sep	58	3	0	0 (1)	55	0 (20)	0
	Skagit	3-Sep	57	16	0	0 (6)	41	0 (20)	0
	Stillaguamish	3-Sep	40	10	0	ND	30	0 (20)	0
Lake Washington	Cedar	3-Sep	60	2	0	0	58	0 (20)	0
	Cedar	4-May	59	5	0	0 (4)	54	0 (10)	0
	Bear	3-Sep	58	4	0	0 (3)	54	0 (20)	0
	Bear	4-May	67	0	0	0	67	0 (10)	0
	UW Hatchery	4-May	55	7	2	0 (4)	48	3 (48)	9.1
S. Puget Sound	Green	3-Sep	52	5	0	0 (4)	47	0 (20)	0
	Duwamish	4-May	47	5	0	0 (4)	42	0 (10)	0
	Puyallup	3-Sep	59	2	0	ND	57	0 (20)	0
	Chambers	4-May	62	4	0	0 (4)	58	0 (10)	0
	Deschutes	3-Sep	60	53	1 (spore)	0 (53)	7	0 (7)	1.6
	Deschutes	4-May	12	3	0	0	9	0 (9)	0
	Black	4-May	57	4	0	0 (3)	53	0 (10)	0
	Kennedy	3-Sep	65	1	0	0	64	0 (20)	0
	Nisqually	3-Sep	57	1	0	0	56	0 (20)	0
Hood Canal	Skokomish	3-Sep	50	8	0	0 (6)	42	0 (20)	0
Control site	Willamette	3-Sep	50	4	3	3 (4)	46	0 (20)	6
	Willamette	4-May	38	16	12	3 (4)	22	10 (22)	65.8

ND = No Data. Cs+ = *Ceratomyxa shasta* positive.[a] Exposure Date: in both 2003 and 2004, fish were held for 7d in live cages at each site. [b] Percent prevalence = all Cs+ fish (survivors + morts) / number examined (x100)

CHAPTER 5: SUMMARY

Fish exposure studies conducted in the Klamath River revealed dramatic differences in *Ceratomyxa shasta* induced mortality between exposure groups. Known susceptible strains of rainbow trout (*Oncorhynchus mykiss)* exposed above Iron Gate Dam became infected as a result of exposure to the parasite but sustained low mortality (<10%). By contrast, groups of the same strain exposed below Iron Gate Dam all died due to infection. During these studies, fall Chinook salmon (*O. tshawytscha*) were also exposed above Iron Gate Dam and did not become infected. However, 68% of the fall Chinook exposed below Iron Gate Dam became infected, with nearly 50% of the group succumbing to infection. Native salmonids, such as the Klamath River Chinook, that co-occur where the parasite is endemic are expected to exhibit a level of resistance to *C. shasta* greater than that of non-native species (Bartholomew 1998) as was apparent from our results. However, the high mortality of the Chinook salmon exposure group below Iron Gate Dam indicates that their resistance to the parasite was compromised.

Studies have identified water temperature and infectious dose as two factors that can potentially overwhelm resistance in native species and strains (Ratliff 1981; Ibarra et al. 1992; Udey et al. 1975). Water temperature during exposure above and below Iron Gate had been monitored during the study and differences were not considered substantial enough to explain the mortality differences, thus it was hypothesized that infectious dose was the primary factor involved. This hypothesis was later validated by

Hallet and Bartholomew (2006) who developed a test for quantifying *C. shasta* spores from water samples. The factors that lead to the high spore densities were unknown and it was hypothesized that greater numbers of infected polychaetes were present in the Lower Klamath River relative to the Upper Klamath River. Concurrent studies set out to document the polychaetes habitat requirements, distribution and infection prevalence with the parasite.

The polychaete host, *Manayunkia speciosa*, was found throughout the Klamath River main-stem where its distribution by habitat type was discontinuous in the lotic (river) component but consistent in the lentic component (lake-reservoir). The polychaete occurred frequently in pools and runs (52% and 40% respectively) and densities in some of these habitats could be high: as many as 12,000 individuals were collected in a single 30-second sample from a pool below Iron Gate Dam. These populations tended to be very compact or aggregated into a small area within the depositional areas of the habitat. The polychaete's distribution in the reservoirs was consistent with 100% of the reservoir inflows sample containing specimens. These populations were also widespread at the inflow and structured so that the density distribution of *M. speciosa* increased rapidly within the first 500m within the reservoir and then steadily decreased. This pattern is characteristic of a population that has found optimal living conditions within this habitat and suggests that the habitat is spatiotemporally stable.

Other factors such as certain microhabitats influence distribution of the polychaete. A mat forming, epilithic algae (*Cladophora* spp.) provide a stable habitat suitable for the polychaete and fine sand with organic matter were also productive

microhabitats. High nutrient availability in river systems such as the Klamath River will be key basin-scale attributes determining distribution of the polychaete. For example, populations of *M. speciosa* were more clustered above the Trinity River confluence, where nutrient concentrations are higher than below the confluence. The concentration of nutrients in the Upper Klamath River is unique in this system because other river systems (e.g. Columbia River) have highest concentrations near the estuary. Flow velocity and habitat stability (resilience to catastrophic flow events) are small-scale attributes that limit to the distribution and abundance of *M. speciosa*. The combined effects of the hydroelectric projects, nutrient loading and the severe drought conditions may have created conditions conducive to polychaete population growth.

Prevalence of *C. shasta* infection in two populations of *M. speciosa* below Iron Gate Dam were found to be significantly higher than all other populations tested. This may be due to the proximity of these populations to spawning grounds. A conceptual framework provides a likely scenario where returning adults, spawn, die and release large quantities of myxospores that infect nearby populations of *M. speciosa*. These heavily infected populations now provide the principal source of infectious actinospores in the Lower Klamath River. This scenario may be an artifact of Iron Gate Dam by concentrating runs of infected salmon into a smaller area thus increasing the exposure dose to polychaete populations. For a river system with a very limited water budget, the question of how to minimize the impacts of *C. shasta* on Klamath River salmonids should consider the following suggestions.

RECOMMENDATIONS TO MANAGERS

High pulsing flows or flushing flows may be used to remove polychaete populations, however, success will be low to moderate due to stable *Cladophora* beds present throughout the river. *Cladophora* is extremely resilient to high flow events as witnessed at one site located near the I-5 Bridge. High flow events were observed to remove one polychaete population from sand-silt habitat located at the Tree of Heaven campground. Fluctuating high (1d) and low flow (3d) events may be beneficial by exposing *Cladophora* to drying events and then to flushing flows. This regime could be coordinated with sustained high flow events to assist migrating juveniles out of the areas of highest infectivity and should not be conducted at the larval or fry stage. The ability to significantly reduce mortality of juvenile salmon with flows is speculative and should be considered for investigation in conjunction with research objectives recommended below.

RESEARCH RECOMMENDATIONS

Population dynamics and life-history traits are useful parameters in the study of any organism's ecology and are not well known for *M. speciosa*. According to ecological models, stable populations will exhibit alterations of life history characteristics different from unstable or meta-stable populations. The age structure of *M. speciosa* appears to follow a stable age pattern for populations occurring in the Boyle Reservoir and in several of the *Cladophora* beds, indicating that these are stable habitats. Populations with poor reproduction will demonstrate a low ratio of juveniles to older (e.g. larger) age

classes. These populations are considered to be in a state of decline as a result of unknown factors.

- At least two lentic polychaete populations and three or four lotic populations should be selected for a 1 or 2 year study. Populations should be sampled once every month and focus on various aspects of the polychaetes life history traits such as age-structure, birthrates and fecundity. The population size (total number of polychaetes) and density (number per unit area) of those populations should be quantified. Percent organic matter and the composition and abundance of various diatom species should be measured at each site and may be correlated with population changes.

The ability of *M. speciosa* to regain pre-disturbance population density can be estimated with a simple experiment.

- Using one of the populations where size and density is known, manually remove some proportion of the population and then monitor over time. It would be important to enumerate the changes in species composition and abundance between the cleared area(s) and control areas. For example, *M. speciosa* may not be the first to inhabit the disturbed area and other species may actually "prepare" the area to be colonized by the polychaete. The investigator should be conscious of the fact that specimens are being removed without replacement during the monitoring process, thus changing densities.

Infection prevalence of *C. shasta* in polychaete populations would provide data on when peak infection prevalence occurs and the environmental factors of importance. An understanding of when peak spore production and peak spore release(s) occur might provide useful information to managers so they can coordinate release of hatchery salmonids and alternating flow regimes to assist Klamath River stocks.

- Using polychaetes collected during the population studies outlined above, sort the specimens into pools of variable sizes and assay using Polymerase Chain Reaction (PCR) as shown in Appendix A.

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APPENDICES

APPENDIX A: PROTOCOL FOR ESTIMATING THE APPARENT PREVALENCE OF *Ceratomyxa shasta* IN POLYCHAETE POPULATIONS

MATERIALS**:**

Ceratomyxa shasta positive and negative fish tissue (pre-digested and boiled)
Pipettors (P-10, P-20, P-200, and P-1000) and tips
Gridded Petri dish
0.5mL tubes
HPLC grade water
Repeat pipettor
Heat blocks
Fisher Vortex
Incubator/rocker
Centrifuge
Tube racks
0.2mL PCR tubes
bucket of ice
Gel loading pipet tips
Agarose
Gel electrophoresis apparatus
    TAE (1X) buffer
Ethidium bromide
1Kb ladder (GIBCO)
Gel photo imager
PCR thermocycler
Reagents for PCR:
    Master mix:
        CS 1 & 3 – *C. shasta*-specific primers (Invitrogen)
        Protienase-K (PK) from Qiagen
    RNAse-A (Qiagen)

RATIONALE:

To determine the apparent prevalence of the *Ceratomyxa shasta* actinospore in selected polychaete populations. Polychaetes either fresh or preserved in 80 – 100% ethanol can be used for PCR analysis.

Approximately 500 polychaetes should be randomly selected using a method where all worms within the selected population are placed on a gridded Petri dish, the grids are randomly selected and all worms within the grid are collected until the number of worms desired has been obtained.

- Using a 1000µL pipette or extra-fine forceps, select ~500 worms (number and scheme can vary) and sort them into 23 (1.5mL) tubes based on the scheme below.

Number of worms per tube	Number of tubes	Total worms
5	10	50
10	5	50
25	4	100
50	4	200
Total =	23	400

- Label each tube so it corresponds with samples in logbook
- Pipette out excess ethanol from each tube

Controls:

- Select and transfer 5µL of digested and boiled *C. shasta* positive fish tissue into a 1.5mL tube containing 500µL of 90% ethanol. Do the same with negative fish tissue and label the tubes as (+) or (-).
- Place controls with samples

- With the caps open, set the samples in a 37˚C incubator room to evaporate ethanol for approximately 3 – 6hrs. Cover the samples with clean Kim-wipes.
- Remove samples from incubator and spike with 250µL DNA extraction buffer (lysis buffer).
- Spike each sample with 10mL of Proteinase-K using a repeat pipettor and close caps
- Vortex and spin-down samples
- Put samples in a box that holds tubes firmly and put the box in a rocker set at 37˚C for ~3hrs.
- Remove the box once an hour and shake firmly
- After 3 hrs remove box and can be frozen and this point

- Take samples out of rocker or freezer (thaw if necessary)
- Spin down samples at 14,000 rpm for ~10 sec
- Spike sample with 5mL of RNAse (change tips each time)
- Cap the tubes, put in box and shake firmly
- Put the box of samples into the rocker @ 37˚C for 30 min.
- Start heat blocks at 70 to 100˚C
- Remove samples and spin them down as before
- Boil for 5 min

- Samples can be frozen at this point

DILUTION 1:100

- Thaw and spin down samples
- Add 495µL of HPLC grade water to 25 (23 samples plus positive and negative controls) clean 1.5mL tubes using a repeat pipettor.
- Label each tube to match its corresponding sample
- Add 5µL of sample to its dilution tube and change tips each time
- Return original samples to box and freeze
- Vortex and spin down diluted samples
- Diluted samples can be frozen at this point

MASTER MIX: **CS 1&3**

- Mix the following under sterile conditions
    - 7.5ml molecular grade water
    - 1ml Taq 10x PCR buffer
    - 800 µl $MgCl_2$ 25mM (Promega)
    - 20µl of each dNTP (at 100mM; Promega)
    - 10µl of **CS1** at 500µM
    - 10µl of **CS3** at 500µM
- Aliquot into 1.5ml tubes and keep frozen until needed

POLYMERASE CHAIN REACTION

- Label 0.2mL PCR tubes with corresponding numbers from dilution tubes
- Fill an ice bucket and place the racks of PCR tubes in it
- Get out the CS 1 & 3 master mix and let thaw
- Get out the Taq polymerase and put on ice
- Make up PCR mix in a 2mL tube by figuring 20µL per sample tube (will need to figure the total amount to make, for example 52 samples = 1040mL)
- Add 1/100 of Taq Polymerase to the mixture (so for 1040µL add 10.4µL of Taq) and be sure to mix the Taq in by drawing the entire mix up and down with the P-1000 pipettor (this makes the reaction mix that will be used to go into each PCR sample tube).
- Add 1µl RediLoad (Invitrogen) for every 19µl master mix.
- Immediately put the Taq Polymerase and master mix back in the freezer when done.
- Transfer 19µL of the mixture to each 0.2mL PCR tube using the same pipette tip for each row.
- Add 1µL of diluted sample to the (corresponding with number on dilution tube)

0.2mL tube containing the transferred PCR mix.
- Add one drop of oil to each tube (optional)
- The PCR should take ~ 2.5 hrs, remove samples when done and freeze samples until the gel electrophoreses step

GEL ELECTROPHORESIS AND VISUALIZATION

- Make 1.0% (w/v) agarose gel by adding 0.9 g agarose to 90 ml TAE buffer (1X)
- Mix thoroughly and heat (microwave) until agarose dissolves
- Allow to cool until just warm to the touch
- Add 0.001% (v/v) ethidium bromide to solution and mix
- Pour contents into gel electrophoresis tray (sides closed with tape) and insert combs for gel lanes
- Remove any bubbles with pipette tip and allow to cool until hardened
- After gel has solidified, carefully remove combs and tape from sides of tray
- Place tray with gel into the electrophoresis apparatus with wells towards the anode (negative end).
- Make sure the TAE buffer (1X) just barely covers the gel.
- Add 12µl of sample to each corresponding well and 2.5ml of 1kb ladder working solution (20µl of 1kb DNA ladder stock to 20µl 6X loading dye and 80µl of ddH_2O) to outer wells.
- Cover electrophoresis apparatus, insert anode and cathode and turn power supply to 90v and allow to run for ~35min.

- Turn off power, remove gel and transfer to a gel photo imager.

ANALYSIS OF RESULTS

- Compare the bands to the corresponding PCR tubes to determine the positive groups (positive bands will appear at 638bp marker)
- Record the positives from each group and log into the Pooled Prevalence Estimator at http://www.ausvet.com.au/pprev/
- Click on **Pooled prevalence for variable pool size and perfect tests**
- Set Confidence level at 0.95
- Enter the size of each pool as shown above, the number of pools tested and the number of positive pools based on the results from the gel and click on “submit”
- Record the results in notebook

APPENDIX B: KLAMATH RIVER SAMPLE SITES

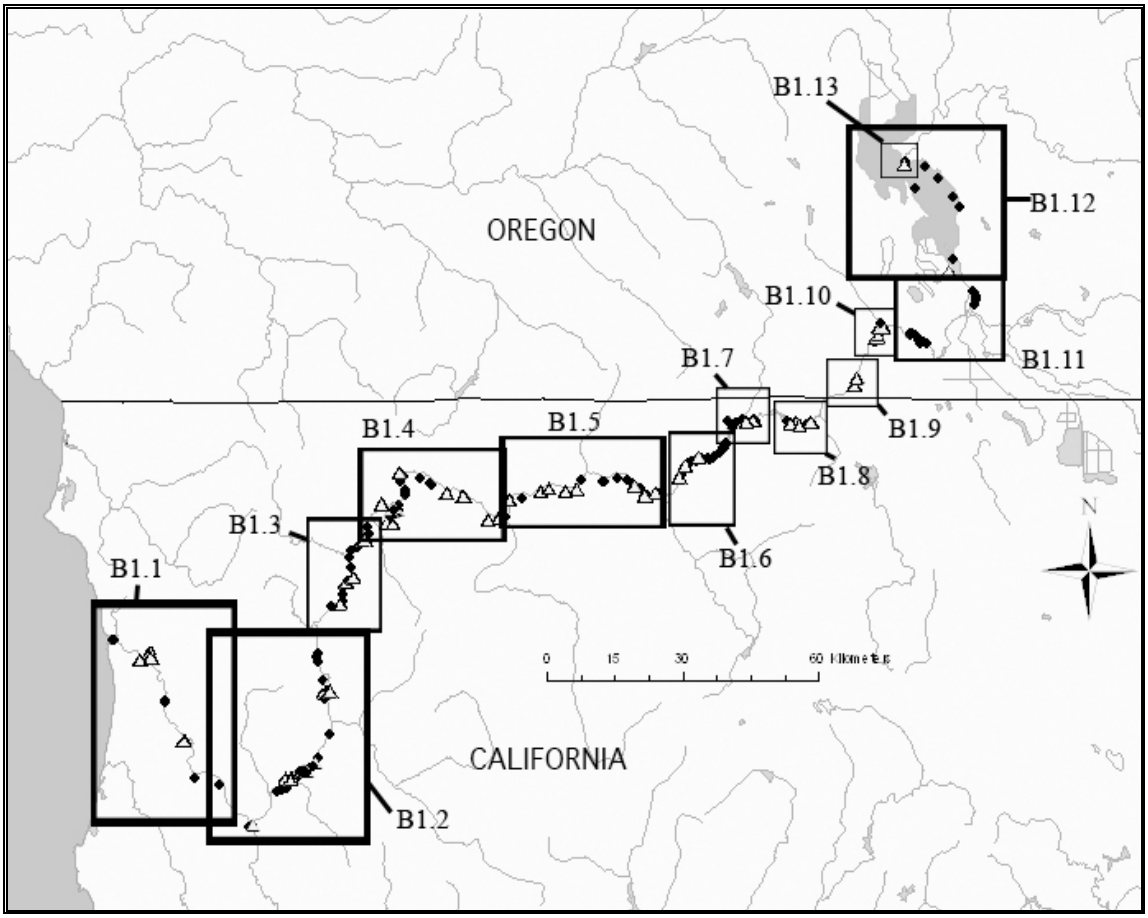

FIGURE B1.0. Map of 2003-2005 Klamath River *Manayunkia speciosa* sampling sites by sections. Individual frames are labeled and presented separately from as Figures B1.1 to B1.13 in the following pages.

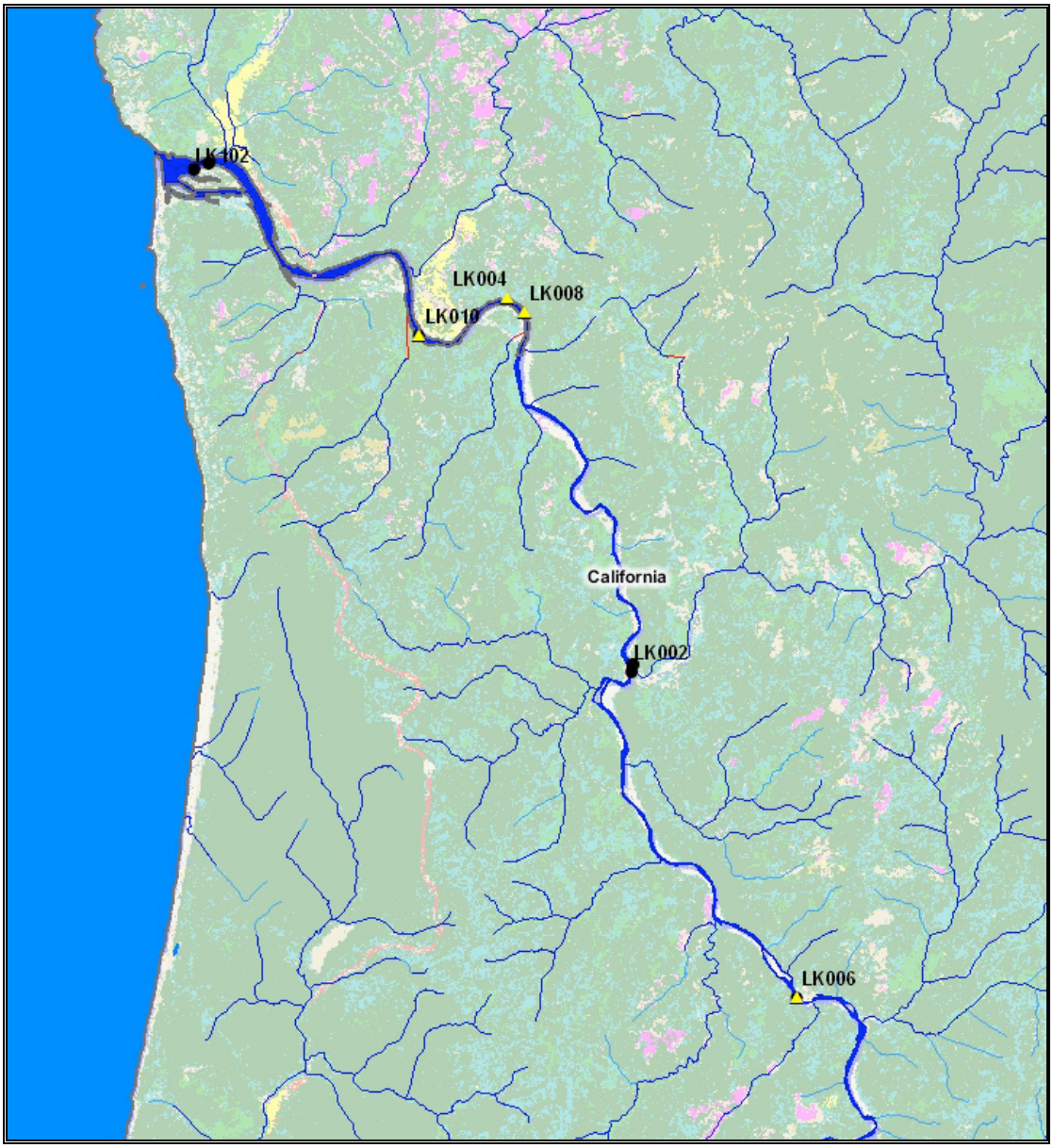

FIGURE B1.1. Map frame (below Trinity River to estuary) from FIGURE B1.0 showing habitat sample sites to detect presence of *Manayunkia speciosa*. Black dots indicate that *M. speciosa* was not identified and yellow triangles indicate that presence was confirmed.

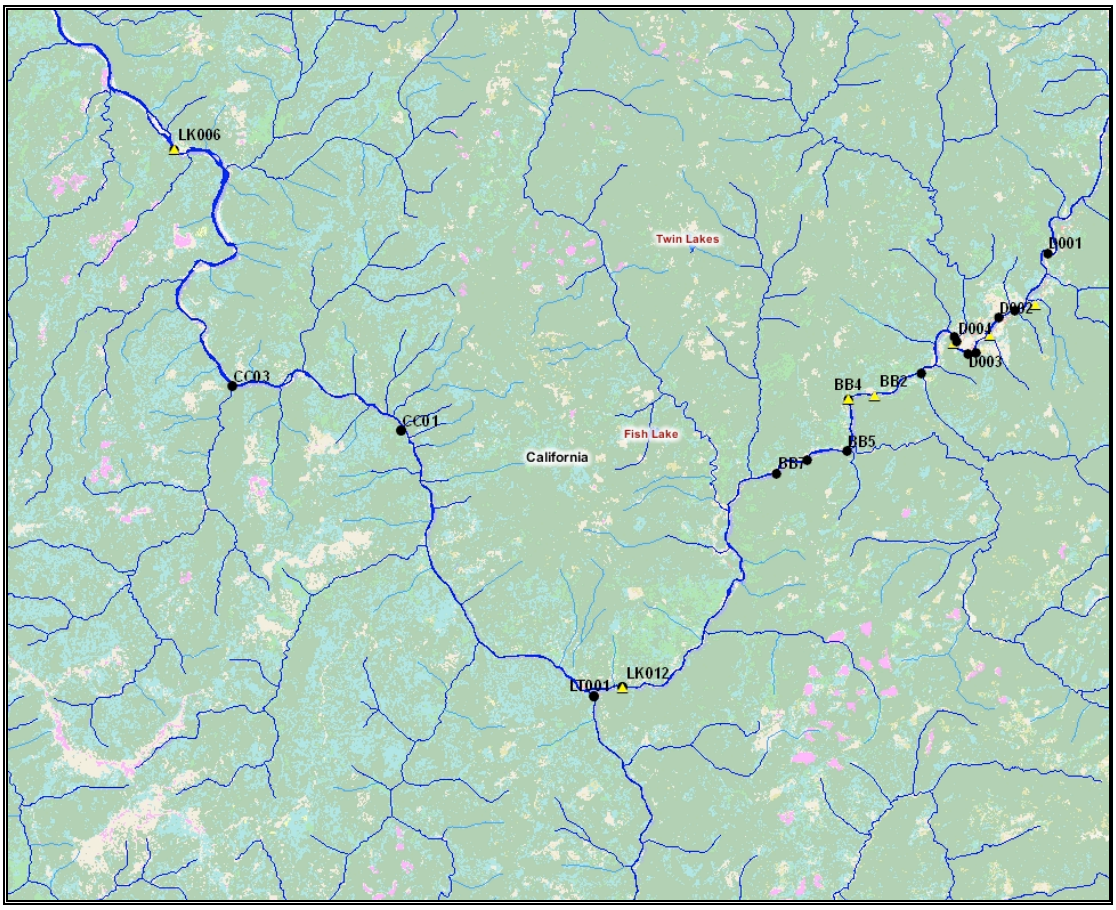

FIGURE B1.2. Map frame (Orleans to Lower Klamath) from FIGURE B1.0 showing habitat sample sites to detect presence of *Manayunkia speciosa*. Black dots indicate that *M. speciosa* was not identified and yellow triangles indicate that presence was confirmed.

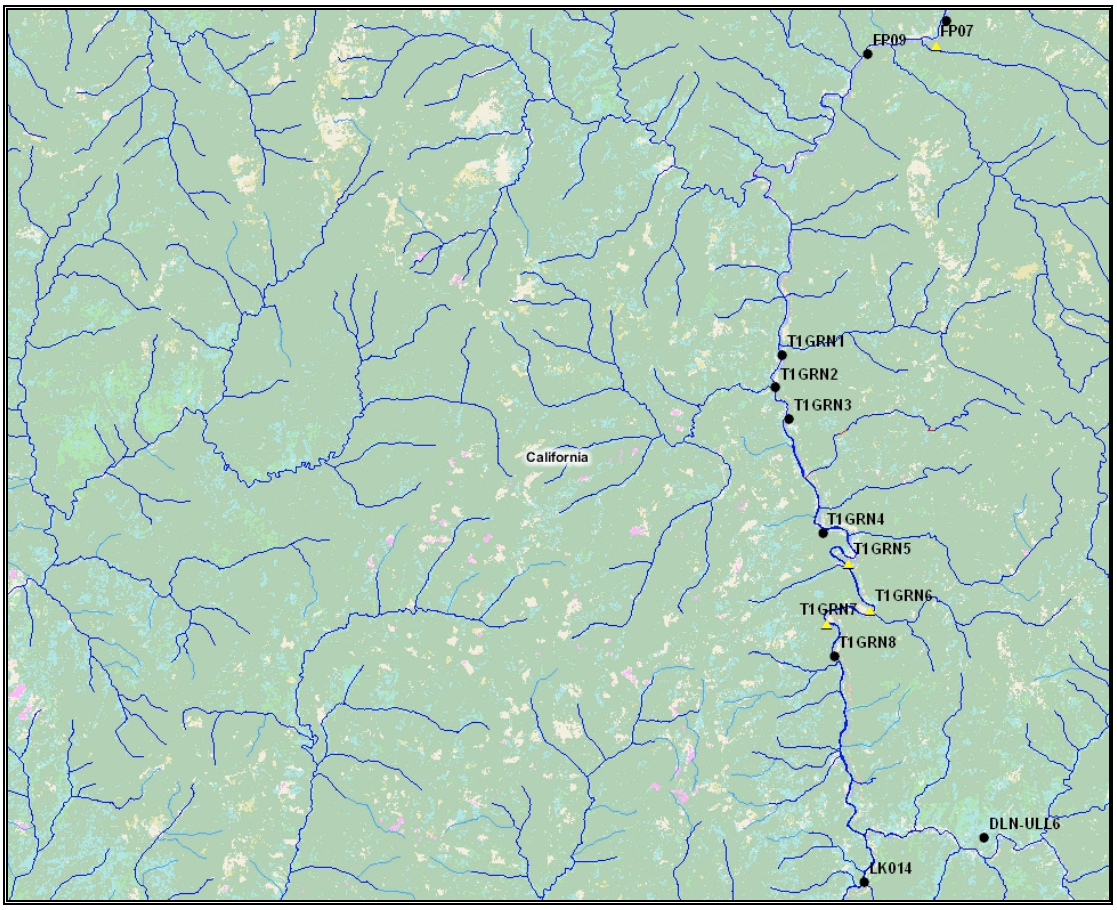

FIGURE B1.3. Map frame (above Salmon River to Bluff Creek) from FIGURE B1.0 showing habitat sample sites to detect presence of *Manayunkia speciosa*. Black dots indicate that *M. speciosa* was not identified and yellow triangles indicate that presence was confirmed.

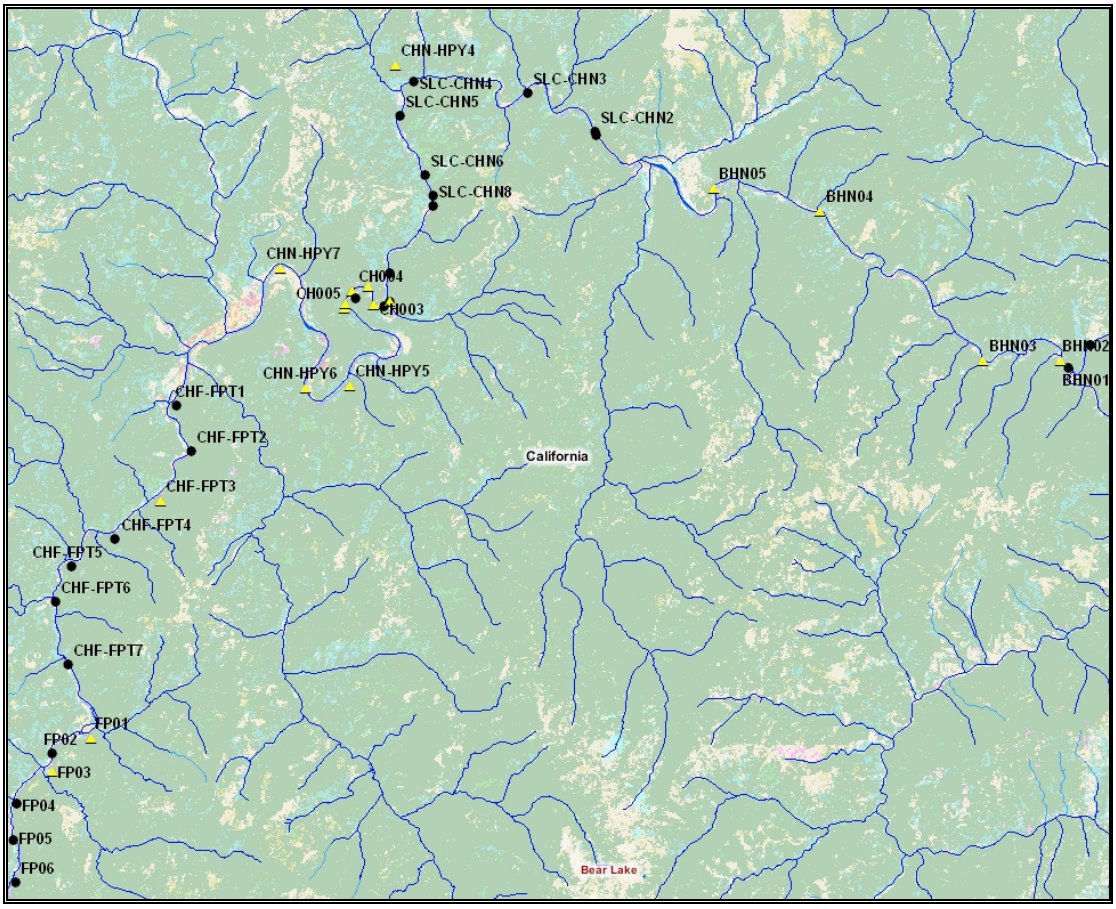

FIGURE B1.4. Map frame (below Scott River to above Salmon River) from FIGURE B1.0 showing habitat sample sites to detect presence of *Manayunkia speciosa*. Black dots indicate that *M. speciosa* was not identified and yellow triangles indicate that presence was confirmed.

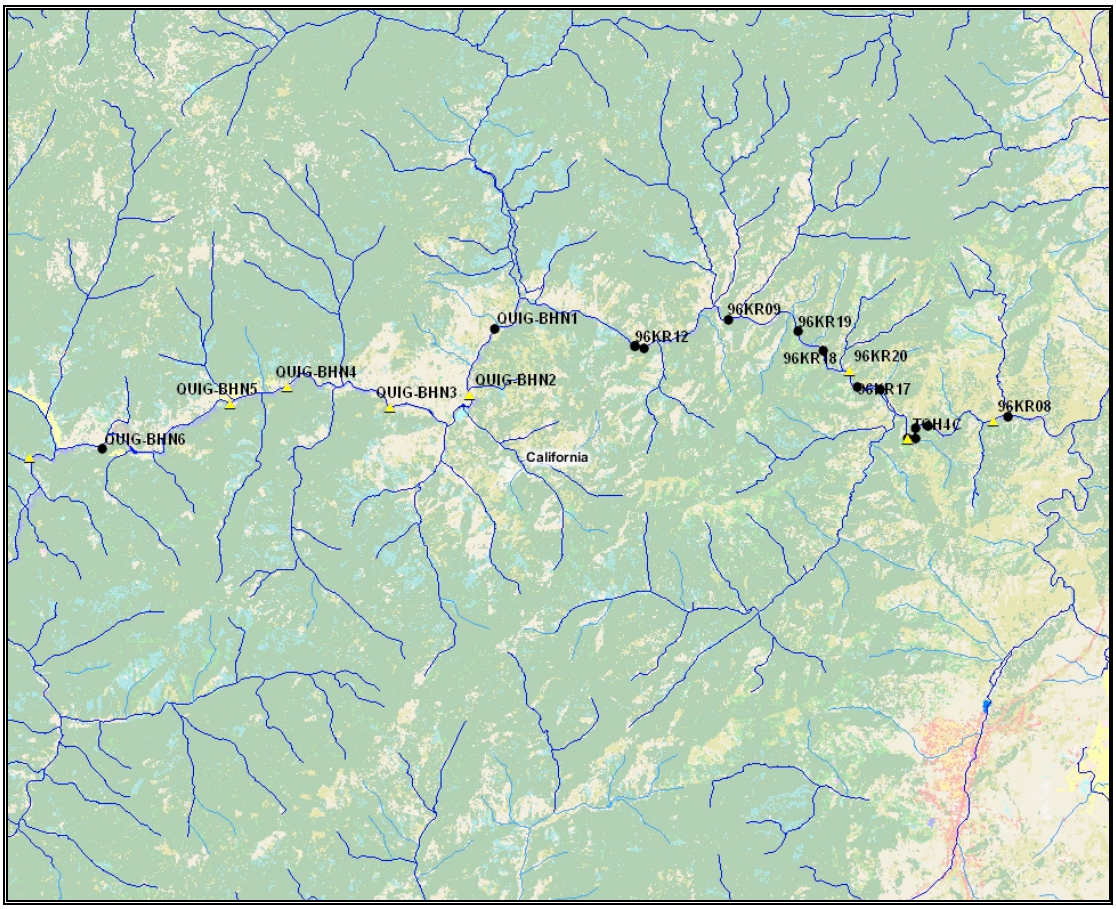

FIGURE B1.5. Map frame (Shasta River to above Scott River) from FIGURE B1.0 showing habitat sample sites to detect presence of *Manayunkia speciosa*. Black dots indicate that *M. speciosa* was not identified and yellow triangles indicate that presence was confirmed.

FIGURE B1.6. Map frame (Iron Gate Dam to I-5) from FIGURE B1.0 showing habitat sample sites to detect presence of *Manayunkia speciosa*. Black dots indicate that *M. speciosa* was not identified and yellow triangles indicate that presence was confirmed.

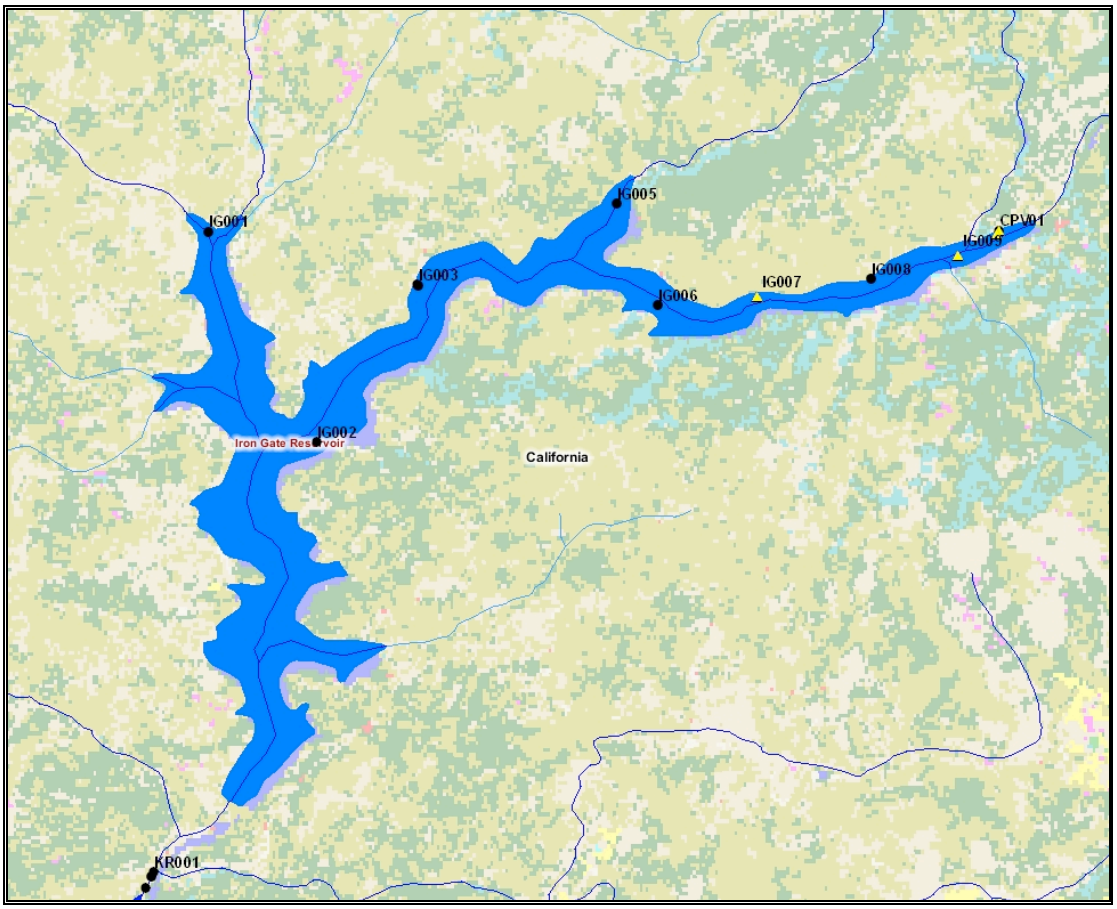

FIGURE B1.7. Map frame (Iron Gate Reservoir) from FIGURE B1.0 showing habitat sample sites to detect presence of *Manayunkia speciosa*. Black dots indicate that *M. speciosa* was not identified and yellow triangles indicate that presence was confirmed.

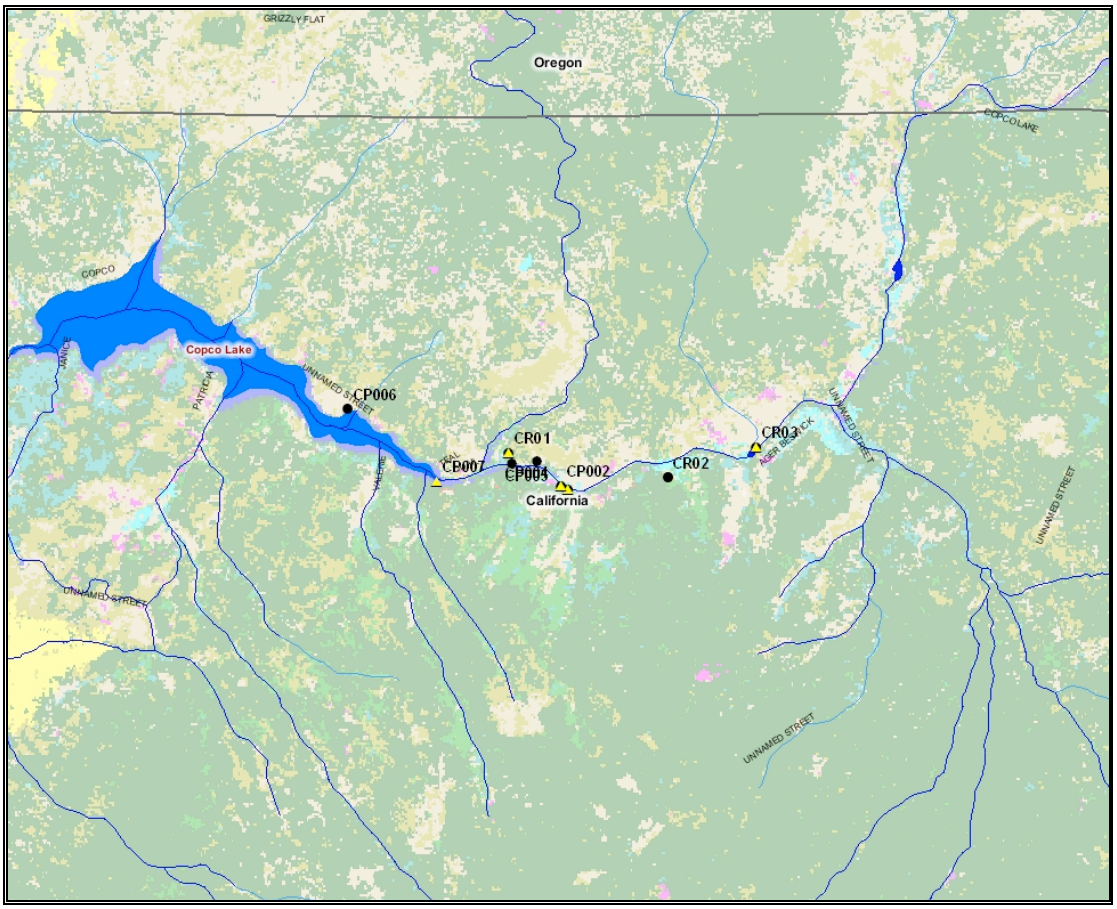

FIGURE B1.8. Map frame (Copco Reservoir) from FIGURE B1.0 showing habitat sample sites to detect presence of *Manayunkia speciosa*. Black dots indicate that *M. speciosa* was not identified and yellow triangles indicate that presence was confirmed.

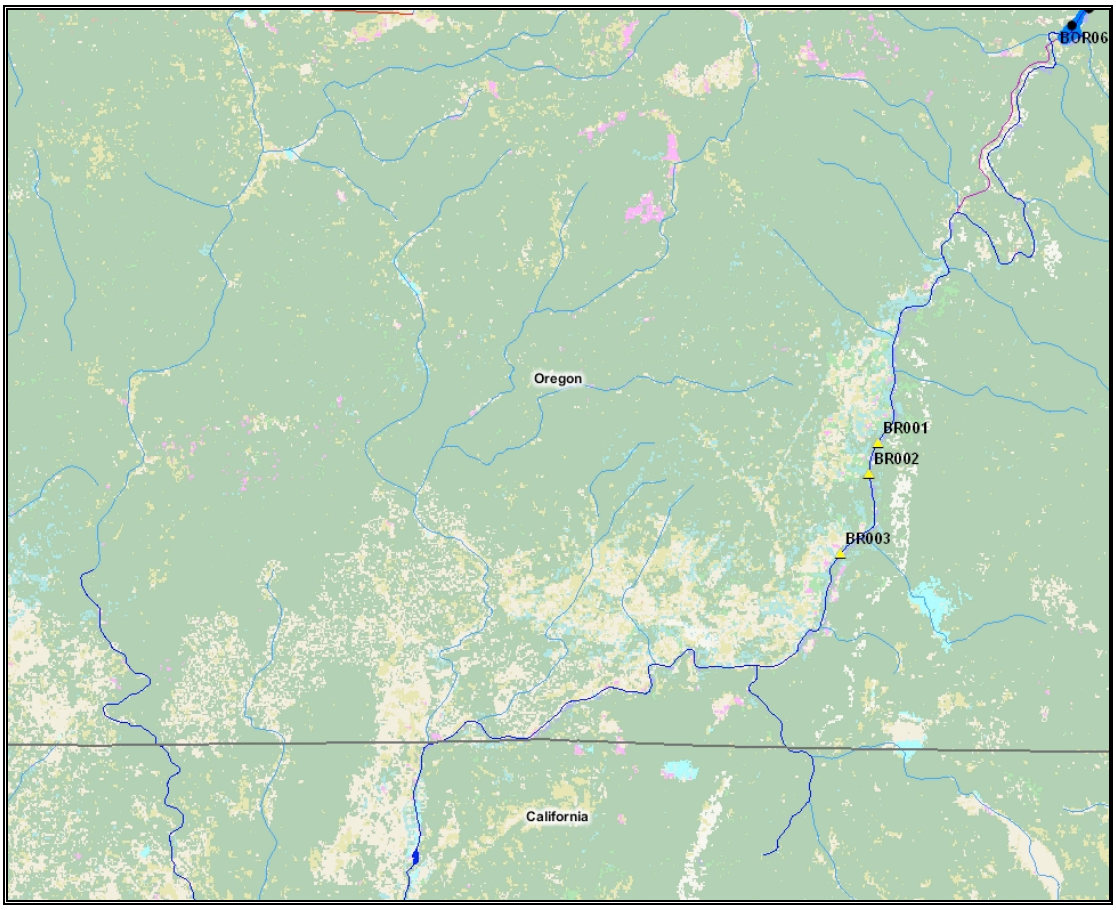

FIGURE B1.9. Map frame (Boyle bypass reach) from FIGURE B1.0 showing habitat sample sites to detect presence of *Manayunkia speciosa*. Black dots indicate that *M. speciosa* was not identified and yellow triangles indicate that presence was confirmed.

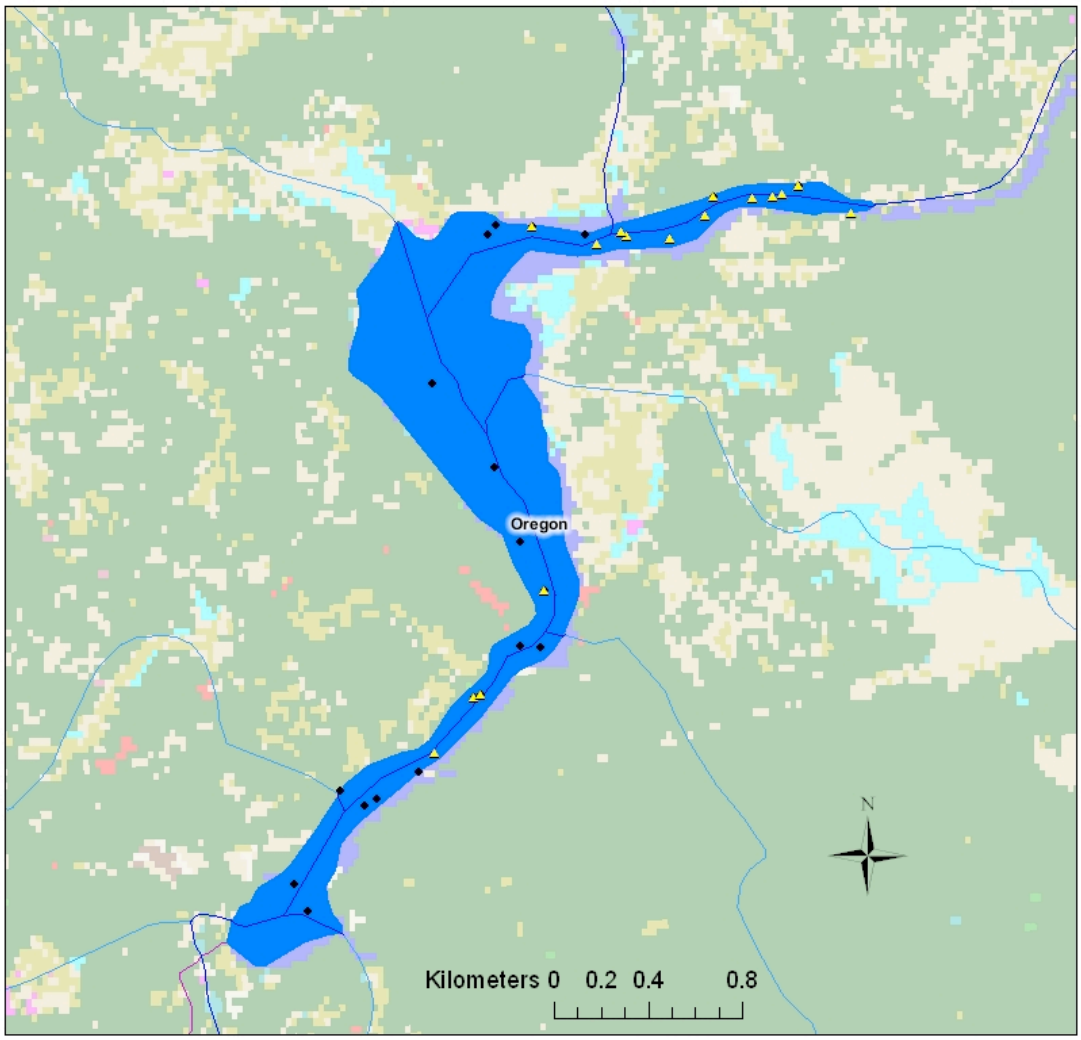

FIGURE B1.10. Map frame (Boyle Reservoir) from FIGURE B1.0 showing habitat sample sites to detect presence of *Manayunkia speciosa*. Black dots indicate that *M. speciosa* was not identified and yellow triangles indicate that presence was confirmed.

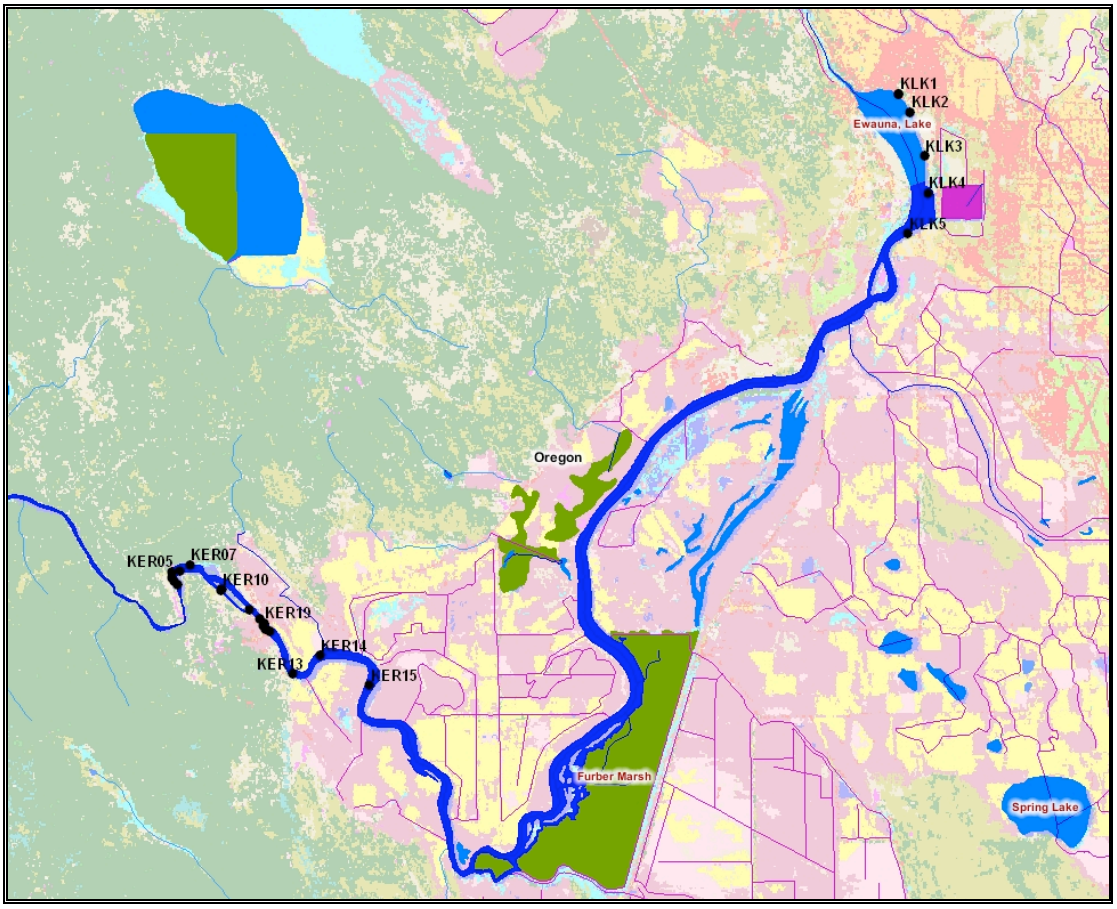

FIGURE B1.11. Map frame (Keno Reservoir/Lake Ewuana) from FIGURE B1.0 showing habitat sample sites to detect presence of *Manayunkia speciosa*. Black dots indicate that *M. speciosa* was not identified and yellow triangles indicate that presence was confirmed.

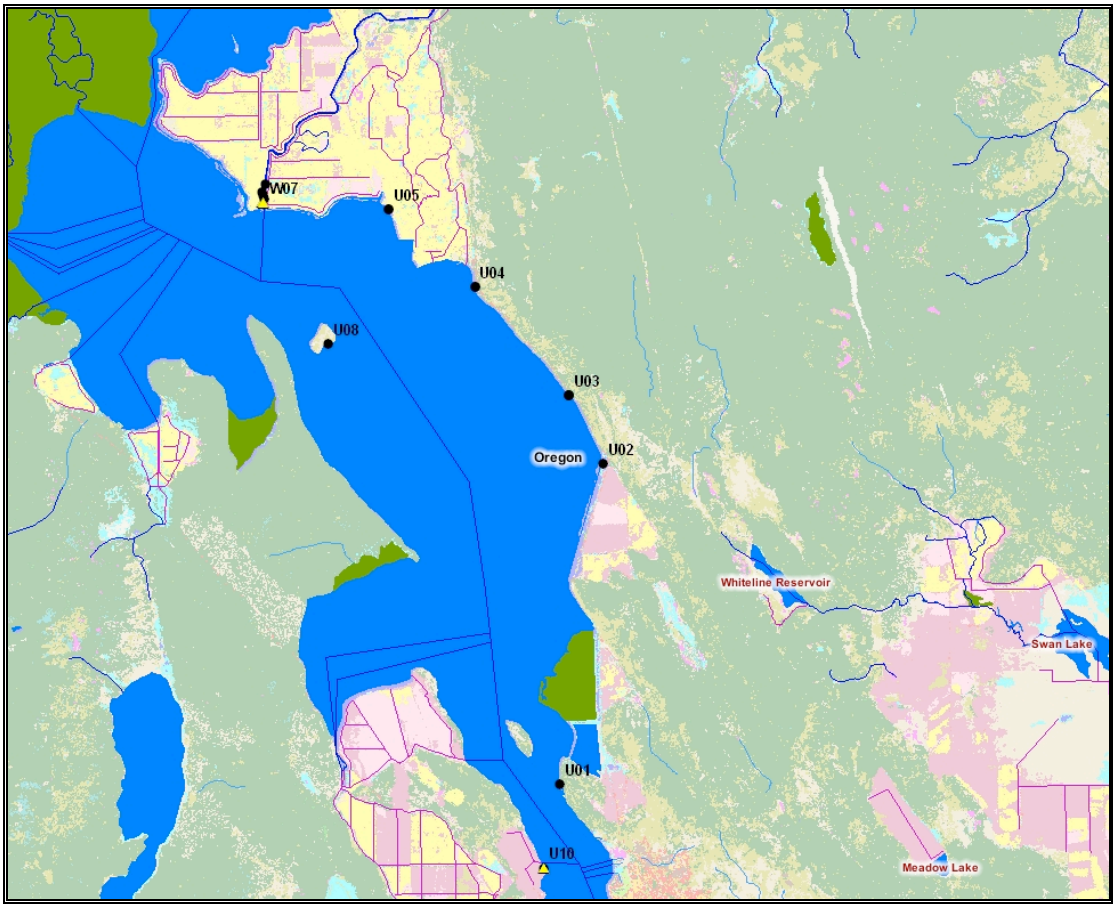

FIGURE B1.12. Map frame (Klamath Lake) from FIGURE B1.0 showing habitat sample sites to detect presence of *Manayunkia speciosa*. Black dots indicate that *M. speciosa* was not identified and yellow triangles indicate that presence was confirmed.

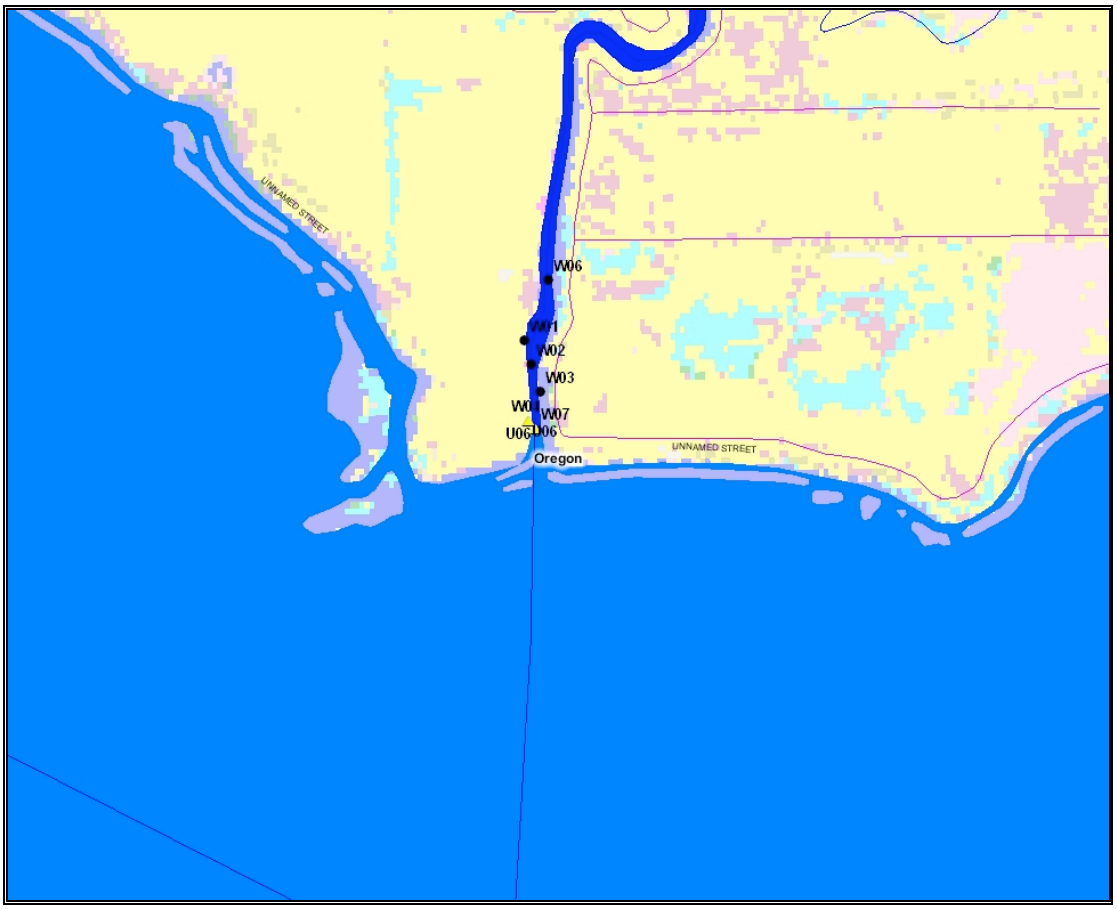

FIGURE B1.13. Map frame (Williamson River inflow) from FIGURE B1.0 showing habitat sample sites to detect presence of *Manayunkia speciosa*. Black dots indicate that *M. speciosa* was not identified and yellow triangles indicate that presence was confirmed.