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Nanophyetus salmincola infection and toxic contaminant exposure in outmigrating Steelhead Trout from Puget Sound, Washington: implications for early marine survival

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Running title: *Nanophyetus salmincola* and toxic contaminants in Steelhead Trout

Abstract

Outmigrating Steelhead Trout *Oncorhynchus mykiss* from four Puget Sound rivers, and associated marine basins of Puget Sound in Washington State were examined for the parasite

Nanophyetus salmincola in 2014 to determine whether recent trends in reduced marine survival are associated with the presence of this pathogen. A subset of Steelhead Trout from three of these river-marine basin combinations was analyzed for the presence of persistent organic pollutants (POPs) to assess whether exposure to these contaminants is a contributing factor to their reduced marine survival. The prevalence and parasite load of *N. salmincola* were significantly higher in fish from central and southern Puget Sound than fish from river systems in northern Puget Sound. The proportion of Steelhead Trout samples with concentrations of POPs higher than adverse effects thresholds (AETs) or concentrations known to cause adverse effects was also greater for fish from the central and southern regions of Puget Sound than the northern region. Polybrominated diphenyl ether concentrations associated with increased disease susceptibility were observed for 10% and 40% of the Steelhead Trout sampled from central and southern Puget Sound regions, respectively, but none of the fish sampled from the northern region. The AET for polychlorinated biphenyls was exceeded in Steelhead Trout collected from marine habitats: 25% of the samples in the marine basins in the central and southern regions of Puget Sound, and 17% of samples from northern Puget Sound region. Both *N. salmincola* and POP levels suggest adverse health effects on outmigrating steelhead from one southern and one central Puget Sound River that have lower early marine survival than a river system in northern Puget Sound.

Introduction

Steelhead Trout *Oncorhynchus mykiss* populations in Puget Sound, WA are currently less than 4% of historical abundance (Gayeski et al. 2011), and are listed as threatened under the U.S. Endangered Species Act (NOAA 2007). From 1999 to 2004, adult Steelhead Trout populations in Washington State increased 48% overall, but populations within Puget Sound decreased 23% (Scott and Gill 2008). Hatchery Steelhead Trout populations in Puget Sound have smolt to adult returns (SAR) of 0.4% compared to 4.4% for hatchery steelhead returns from the adjacent Olympic Peninsula (Scott and Gill 2008), suggesting that factors influencing low marine survival are specific to this region. The SAR is a result of combined ocean and early marine survival (EMS). Early marine survival is defined as survival over the distance from river mouth to open ocean entry (Moore et al. 2012). Acoustic telemetry studies show that hatchery Steelhead Trout outmigrating from two rivers in the central portion of Puget Sound (Puyallup and Green-

Duwamish rivers) have an EMS rate of 5%, compared to the 20% EMS rate for hatchery steelhead from the Skagit River in the northern region of Puget Sound (Moore et al. 2015), despite use of a similar, non-native broodstock at the three locations. This implied that a freshwater rearing factor specific to certain regions or rivers could contribute lower EMS.

Nanophyetus salmincola is a parasitic trematode that infects salmonid fishes in fresh waters of the Pacific Northwest (Millemann and Knapp 1970). The adult worm lives in the intestine of fish-eating birds and mammals. Eggs shed into the water by the host hatch into miracidia which penetrate the first intermediate host, one of two species of snail *Juga plicifera* or *J. silicula*. Asexual reproduction occurs within the snail, resulting in the development of miracidia into redia and then cercaria. Developed cercaria emerge from the snail, and penetrate the piscine secondary intermediate host. The cercaria then encyst as metacercariae in various organs of the fish, including gills, muscle, and heart, but predominantly the posterior kidney (Millemann and Knapp 1970). Once encysted, metacercariae typically survive the ocean phase of salmonid life cycle (Weiseth et al. 1974). Because *N. salmincola* does not replicate within the fish host, any host tissue damage occurs typically during the early penetration and tissue migration stages when infections can be lethal to young salmonids (Baldwin et al. 1967). Under controlled conditions, salmonids with early *N. salmincola* infections demonstrate reduced swimming performance (Butler and Milleman 1971). Penetration and migration through the fish tissues causes damage to nearly every organ system (Wood and Yasutake 1956). *Nanophyetus salmincola* has been shown to reduce resistance of Chinook Salmon *Oncorhynchus tshawytscha* to *Flavobacterium columnare* (Roon et al. 2015), and *Vibrio anguillarum* (Jacobsen et al. 2003), two pathogenic bacteria that would be encountered by Steelhead Trout in fresh water and saltwater. *Nanophyetus salmincola* is a likely cause of mortality to juvenile Coho Salmon *Oncorhynchus kisutch* during the early ocean rearing phase (Jacobsen et al. 2008), and it is one of the most prevalent pathogens of outmigrating Chinook Salmon in estuaries throughout the Pacific Northwest (Arkoosh et al. 2004).

Juvenile Pacific salmon migrating through urbanized estuaries are exposed to toxic contaminants at concentrations at which adverse effects are known to occur (Arkoosh et al.1998, Stehr et al., 2000, Johnson et al. 2007a, b,, Johnson et al. 2013, Olson et al. 2008, Meador et al. 2010, Sloan et al. 2010, O'Neill et al. 2015, Yanagida et al. 2012), however, such information is

lacking for Steelhead Trout. Salmonids exposed to environmentally relevant concentrations of toxic contaminants may experience poor growth and metabolic dysfunction (Meador et al. 2002, 2006), and reduced immune function, rendering them more vulnerable to naturally occurring pathogens (Arkoosh and Collier, 2002; Arkoosh et al. 1994, 1998, 2001, 2010, 2013; Bravo et al. 2006), either alone or in conjunction with other stressors such as parasites (Jacobsen et al. 2003), which may ultimately reduce their marine survival (Johnson et al. 2013, Meador et al. 2014). Within Puget Sound, juvenile Chinook Salmon migrating from urban rivers and estuaries are exposed to higher toxic contaminants than those from non-urban estuaries (Arkoosh et al.1998, Johnson et al. 2007a, Sloan et al. 2010, O'Neill et al. 2015), including persistent organic pollutant (POPs) and polycyclic aromatic hydrocarbons (PAHs), with approximately one third of the fish sampled having concentrations of POPs high enough to potentially reduce their early marine survival (O'Neill et al. 2015). Although juvenile Steelhead Trout spend less time in estuaries than juvenile Chinook Salmon (Quinn 2005), accumulation of contaminants leading to the loss of disease resistance could be a causal factor in low early marine survival for this species.

This study asks: are juvenile Steelhead Trout exposed to *N. salmincola* and POPs during freshwater rearing or during migration from fresh water to salt water in Puget Sound? And if exposure exists, does it coincide with lower survival rates occurring in some Puget Sound rivers? The specific objectives were to determine: 1) the prevalence and intensity of *N. salmincola* in juvenile fish during outmigration from representative river systems, 2) whether POPs accumulate in outmigrating fish, and 3) compare our findings to previously published studies showing harmful effect levels of *N. salmincola* and POPs in salmonid fish.

<A>Methods

<B>Sampling design and fish collections

Puget Sound field assessment - Steelhead Trout smolts were collected during a field survey implemented from March – June 2014. Sampling locations were chosen to represent the northern, central, and southern regions of Puget Sound. Fish were collected from the in-river and estuary habitats of the Skagit, Snohomish, Green-Duwamish, and Nisqually Rivers and the associated marine habitat of the north (Whidbey), central and south basins of Puget Sound

(Moore et al. 2008), hereafter referred to as river systems (Figure 1). Sampling methods in each river system consisted of screw traps at in-river locations, fyke net or beach seines in estuaries, and purse seine in marine habitats. For each river system, multiple efforts were made to collect 30 fish each of wild- and hatchery-origin (if present) from the in-river, estuary and the marine habitats, however logistical constraints prevented a full complement of samples from each sampling location. As Steelhead Trout hatchery programs do not exist on the Nisqually River, only wild fish were collected from this river system. Hatchery-origin Steelhead Trout were identified by adipose clips or coded wire tags. All sampled fish were euthanized with an overdose (100-200 mg/l) of tricaine methanesulfonate.

Hatchery sampling- Brood Year (BY) 2013 Steelhead Trout (n=30 per hatchery) were sampled by dip or cast-net in April 2014 prior to the scheduled release in May from Marblemount Hatchery (Skagit River), Wallace River Hatchery (Snohomish River), and Soos Creek Hatchery [Green River; (Figure 1)]. A legal challenge prevented the release of Steelhead Trout from Marblemount and Soos Creek hatcheries in 2014 however, the data from these two locations is relevant to other portions of the study. Additionally, the seasonal accumulation of *N. salmincola* in Steelhead Trout was assessed by collecting monthly samples (March - December) young-of-the-year (BY 2014) fish (*n*=15-30) that were reared on surface water at the Soos Creek hatchery

Progress of infection after release- The known rearing locations and time of release and recapture of hatchery-origin steelhead in the Green-Duwamish watershed were used to investigate the progress of infection during their downstream migration. The Green-Duwamish watershed was described in Goetz et al. (2015), and summarized briefly here. The Duwamish River is the lower 16 River Kilometer (RK) estuarine section, and the Green River is the 126 RK freshwater section. The portion of the river most altered by human development is the lower Green River, RK 16-51. Although no BY 2013 Steelhead Trout were released directly from Soos Creek Hatchery, located at RK 55, some cohorts (adipose clipped), reared on surface water at Soos Creek Hatchery until July 2013, were transferred upstream to Icy Creek Ponds (RK 78), and reared in spring water until release on March 25, 2014. Additional BY 2013 were reared entirely in spring water until release at Flaming Geyser State Park (RK 71).

<B>Sample Processing

Nanophyetus salmincola- Processing of captured fish for *N. salmincola* was standardized throughout this study. Each fish was assigned a unique number, and examined for lesions or deformities. Fork length, weight, and presence/absence of adipose fin marks and coded wired tags were recorded for each fish. *Nanphyetus salmincola* infection prevalence and intensity were determined by counting metacercariae in the posterior kidney, and our methods were designed to permit direct comparison of counts with Jacobsen et al. 2003, Jacobsen et al. 2008, and Roon et al 2015. The posterior half of each kidney was placed in a labeled Whirlpak sample bag (60 ml), and frozen at -20°C until later enumeration of metacercariae. Enumeration consisted of compressing the thawed, bagged samples between two glass plates (100 x 70 x 2.5 mm) that were marked with a counting grid of 100 rectangles. Metacercariae were counted using a dissecting microscope (15 X magnification). When the number of metacercariae exceeded 150 per rectangle, the count in that rectangle was estimated as the percent of the area occupied by metacercariae, multiplied by 600 (the estimated number of that would solidly occupy a rectangle, one layer thick). The contents of the entire sample bag were included in the count, which was expressed as metacercariae per kidney (MPK). The MPK was used as a measure of intensity or the number of parasites in an infected host.

Histopathology- Infected and uninfected Steelhead Trout were examined by histological methods to evaluate the relationship between *N. salmincola* infection and tissue damage. The first left gill arch, heart, a five mm^3 piece of liver, and the anterior kidney were fixed in Davidson's solution then transferred to 70% ethanol. The posterior kidney could not be examined in tissue sections due its use in the quantitative *N. salmincola* assay. Muscle, eye, and fin samples were also collected from fish displaying morbidity. Fixed samples were embedded, sectioned, and stained with hematoxylin and eosin by standard histological methods.

Chemical contaminant analyses- Samples for chemical analyses were collected from a subset of the fish analyzed for the presence of *N. salmincola.* For each of the Skagit, Green-Duwamish, and Nisqually River systems, up to 15 wild Steelhead Trout were collected for each habitat type (in-river, estuary, and marine). Whole bodies (less gut contents) of individual fish were ground, placed in pre-cleaned glass jars, and stored at -20°C for subsequent chemical analyses. Samples from each in-river and estuary habitat were later thawed, and equal weights of tissue from individual fish were combined to make three composite samples containing four to five fish each

representing each habitat type within a river system. Fish from marine habitats were analyzed as individuals.

Whole body samples were analyzed for POPs including polychlorinated biphenyls (PCBs), polybrominated diphenylethers (PBDEs) flame retardants, and organochlorine pesticides dichloro-diphenyl-trichloroethanes (DDTs), chlordanes, hexachlorocyclohexanes (HCHs), hexachlorobenzene (HCB), aldrin, dieldrin, mirex, and endosulfan, using established methods (Sloan et al. 2014). This method comprises three steps: (a) extraction, (b) cleanup by gravity flow silica/aluminum columns and size-exclusion high-performance liquid chromatography (HPLC), and (c) quantitation of POPs using gas chromatography /mass spectrometry (GC/MS) with selected-ion monitoring (SIM). Samples were extracted with methylene chloride using an accelerated solvent extractor, which provided an extract that was used for POP analysis and gravimetric lipid determinations. A method blank and a National Institute of Standards and Technology (NIST) Fish Muscle Standard Reference Material (SRM 1946) were analyzed with each sample batch (Sloan et al. 2014). Concentrations of individual analytes measured in SRM 1946 were in excellent agreement with the certified and reference values published by NIST. The method blank and surrogate recovery quality control samples all met established laboratory criteria.

An estimated total PCB concentration was calculated by summing the detected values for 17 commonly detected (and co-eluting) congeners (18, 28, 44, 52, 95, 101(90), 105, 118, 128, 138(163/164), 153(132), 170, 180, 187(159/182), 195, 206, and 209), and multiplying the result by two (Lauenstein and Cantillo 1993). Analyte data are presented as summed values for PBDEs, DDTs, chlordanes, and HCHs. Summed PBDEs were calculated by adding the congeners 28, 47, 49, 66, 85, 99, 100, 153, 154, 155, and 183. Summed DDTs were calculated by summing the concentrations of o,p'-DDD, o,p'-DDE , o,p'-DDT, p,p'-DDD, p,p'-DDE, and p,p'-DDT. The HCHs were measured as α- HCH, β-HCH, and γ-HCH. Summed chlordanes were calculated by summing concentrations of the following 8 analytes: α-chlordane, *cis*-nonachlor, β-chlordane, heptachlor, heptachlor-epoxide, nonachlor III, oxychlordane, and *trans*-nonachlor. The amount of total, nonvolatile extractable lipid (percent lipid) in whole body samples of steelhead were determined gravimetrically (Sloan et al. 2014). Because POP toxicity is inversely dependent on lipid content (Lassiter and Hallam 1990), summed or total POP results

were expressed as nanogram (ng) of contaminant per g of fish lipid (ng/g lipid). To evaluate the potential health effects of contaminant exposure on the marine survival of juvenile Steelhead Trout, POP tissue residues were compared with published adverse effects thresholds (AETs) for salmon exposed to PCBs (Meador et al. 2002) and DDTs (Beckvar et al. 2005) and concentrations known to cause adverse effects for salmon exposed to PBDEs (Arkoosh et al. 2010, 2013), as detailed in the Supplemental material.

<B>Statistical Analysis

Fisher's exact test (Conover 1980) and the chi-square statistic (χ^2) were used to examine the comparisons being conducted and ensure that the level of significance (*P*) across all tests for association between the intensity of infection with tissue pathology and to compare prevalence of infection and tissue pathology at different locations. For the series of pair-wise Fisher's exact tests conducted to compare prevalence between locations, the significance levels of the individual tests were adjusted using the Bonferroni method to account for the multiple each parasite type or pathology was ≤ 0.05.

A General Linear Model (GLM; SYSTAT 2016) was used to measure the statistical significance of differences in POP concentrations in Steelhead Trout among regions and habitat types. Lipid normalized levels of total PCBs, summed PBDEs and summed DDTs were tested for differences among three river systems, one in each of three Puget Sound regions (north, central and south), among sampling habitat types (in-river and estuary pooled vs. marine habitats), and among habitat types within regions. All contaminant data were ln transformed to meet assumptions of normality and homogeneity of group variances. Fish length was included as a covariate as bio-accumulative contaminant can be affected by fish size, an indirect measure of age and duration of exposure. Multiple comparisons testing (Tukey's Honestly-Significant-Difference Test, SYSTAT 2016) was used, when appropriate, to conduct pairwise comparisons of among group means (region and habitat type). Additionally, because lipid-normalized POP concentrations are affected by fish lipid content, we tested for lipid differences in fish among regions using a Kruskal-Wallis (K-W) ANOVA on ranked data and between riverine and marine habitat types using a Mann-Whitney (M-W) Rank Sum Test. Differences in fish length among regions were also tested using a K-W ANOVA on ranked data.

<A>Results

<B>*N. salmincola* in Puget Sound river systems

There was a significant difference (P<0.001) in prevalence of N. salmincola infection in fish from the Skagit, Green-Duwamish and Nisqually river-systems. The prevalence (4.7%) of *N. salmincola* infection in fish from the Skagit River, was significantly lower (P<0.001) than the prevalence found in fish from the Green-Duwamish (74.1%) and Nisqually (98.6%) Rivers (Table 1). *Nanophyetus salmincola* was only found in gill sections (but not the corresponding kidney) from a single wild fish captured at the lower Skagit River (RK=26) smolt trap (n=21). No *N. salmincola* infection was found at the upriver Marblemount Hatchery (RK= 126, n=30). Similarly, *N. salmincola* was not detected in any Steelhead Trout from the Snohomish River watershed, including the Wallace River hatchery (RK=97, n=30), the smolt trap (RK=42.6) on the Skykomish River tributary (n=4), and the Snohomish estuary (RK=0, n=3). An *N. salmincola* prevalence of 7.1%, (n=42) was found in Steelhead Trout collected from marine locations around north Puget Sound, combined as the Whidbey Basin (Table 1), significantly (P<0.05) lower than the prevalence of 93.3% (n=15) steelhead collected from the central and south marine basins combined.

In central Puget Sound, *N. salmincola* infections increased in prevalence and intensity as Steelhead Trout outmigrated through the Green-Duwamish watershed (Table 2). Among wild fish, infection prevalence increased from 13.3% (n=30) at the in-river smolt trap (RK 55) to 86.7% (n=30) at the estuary (RK 9.5). Mean (±SE) intensity also increased from 93 ± 53.2 MPK at the smolt trap to 809 ±218 MPK in the estuary. An analogous progression in infection occurred in hatchery-origin Steelhead Trout released at Icy Creek Ponds; where the mean intensity increased with time in the watershed, from 84 ±8.2 MPK at the in-river trap on March 25, and114 ± 14 MPK in the estuary on April 2 to 734 ±198 MPK in the estuary on April 29 (Table 2). Mean intensity in both wild and hatchery-origin Steelhead Trout was significantly higher in samples from the estuary than in those from the in-river trap (P < 0.001). All of the fish collected from central Puget Sound marine habitat (n=11, combined hatchery and wild) were positive for *N. salmincola*, with mean intensity of 740 ±450 MPK.

In south Puget Sound, *N. salmincola* at levels exceeding 1000 MPK was found in wild Steelhead Trout collected at both trap and estuary locations in the Nisqually River (Table 3). Through a one-month outmigration period, a prevalence of 97.5% and mean (±SE) intensity of 1753 ±309 MPK occurred at the in-river trap (RK=20). High prevalence (100%) and a mean (±SE) intensity of 2545 ±420 MPK was also found in fish collected in the estuary (RK=0.6). The four Steelhead Trout caught in the marine habitat adjacent to the Nisqually estuary had a lower mean (±SE) intensity of 1086 ±734 MPK than those caught at the trap and the estuary but the difference was not significant (P=0.14).

Seasonal accumulation of parasites in hatchery Steelhead Trout. After rearing in surface water for 14 months, BY 2013 fish from the Soos Creek Hatchery demonstrated 100% infection prevalence with mean (±SE) intensity of 3830 ±332 MPK, The difference in parasite intensity in BY2013 fish from the Icy Creek ponds (i.e., reared on surface water at the Soos Creek Hatchery for three months then transferred to spring water and released at Icy Ponds, Table 2) compared to the BY 2013 fish reared entirely at the Soos Creek hatchery led us to monitor temporal changes in infection at the hatchery for BY2014 fish. Steelhead Trout from BY2014 had a 100% prevalence of infection with *N. salmincola* with mean (±SD) intensity of 124 ±53 MPK after three months of rearing on surface water (Figure 2), similar to the intensity of newly released BY 2013 fish (84.1± 8.2 MPK). Beginning in September 2014, the intensity rose sharply until December, reaching a mean of 2800 ±1253 MPK. Signs of recent *N. salmincola* exposures, including exopthalmia and severely eroded fins (Bennington and Pratt 1960), were detected in October and November but were no longer observed in December.

<B>Tissue lesions and association between pathological conditions and parasites

Microscopic lesions were detected in the gill, heart muscle and kidney of fish infected with *N. salmincola*. Gill inflammation (branchitis) was observed, with or without accompanying fibrosis and involvement of histiocytes (Figure 3). Despite not having posterior kidney (the part of the kidney with the highest metacercaria concentration) to examine, anterior kidneys were found with inflammation (nephritis) around embedded metacercariae (Figure 4). Histiocytic myocarditis (Figure 5) was found, with or without fibrosis. Other pathogens such as blood flukes and myxozoans were detected in the histological sections of fish from certain watersheds; however, neither infection prevalence nor microscopic examination of tissues provided any

indication that they adversely affected Steelhead Trout health and survival in this study. On the other hand, *N. salmincola* metacercaria were found in all tissues with branchitis, nephritis or myocarditis.

Microscopic lesions were associated with *N. salmincola*. Nephritis was observed only in fish sampled from Soos Creek Hatchery (Green River drainage), and the three habitat types in the Green-Duwamish and Nisqually River systems. Branchitis and gill fibrosis were more frequently observed in Steelhead Trout from the Nisqually and Green-Duwamish River systems than the South or Central marine areas. Significant associations were found between the percent of Nisqually River fish with 1000 or more MPK and lesions of the gill ($\chi^2 = 10.883$, $P = 0.001$), and heart ($\chi^2 = 16.794$, $P < 0.001$. The presence of 1000 or more MPK was significantly associated with branchitis and gill fibrosis ($\chi^2 = 8.826$, $P = 0.006$), as well as myocarditis and heart fibrosis ($\chi^2 = 15.852$, $P < 0.001$) in fish sampled from the Green-Duwamish River.

The prevalence and severity of tissue lesions was examined during an acute natural exposure to *N. salmincola*. Steelhead Trout (BY 2014) were sampled at Soos Creek Hatchery during the peak fall (October-November) infection period (Figure 2). Branchitis was found in 33.3% of the fish (*n*=9), 77.8% had myocarditis (*n*=9), and moderate to severe nephritis and kidney fibrosis was found in 100% (*n*=10). Additional tissue sampling revealed 44.4% of the fish had *N. salmincola* in the eye (*n*=9), 70.0% had inflammation of fin tissues (*n*=10), and 100.0% had mild to moderate somatic muscle inflammation (*n*=10).

<B>Toxic contaminant exposure and effects

Among the POPs evaluated, PCBs, PBDEs and DDTs were detected in every sample, with mean (±SD) concentrations of 1000 ±690, 480 ±629 and 270 ±200 ng/g lipid, respectively (Table 4). Chlordanes, HCB, and dieldrin were detected less frequently (83, 72, and 28% of the samples, respectively), many at concentrations just above the limits of quantitation, and consequently, had much lower mean concentrations of 58 ±47, 40 ±14, and 29 ±16 ng/g lipid, respectively. No other POPs were detected. Detailed results are only reported here for PCBs, PBDEs and DDTs.

Concentrations of PCBs in Steelhead Trout did not exceed AETs though they were positively affected by fish length, but not region or habitat type where fish were found. Lipid

normalized PCB concentrations ranged from 290 to 3500 ng/g lipid and were 25 – 32% higher in the central region than those from the northern and southern Puget Sound regions, and 33% higher in steelhead collected from marine than river habitats, however, these differences were not significant by region [F (2, 25) = 0.883, P = 0.426], habitat type [F (1, 26) = 0.582, P = 0.452], or habitat type within region [F (2, 25) = 0.173, P = 0.842]. In each of these models Steelhead Trout length was significant [F (1, 25) = 6.039. P = 0.021; F (1, 26) = 6.179, P = 0.020; and F (1, 25) = 4.812, P = 0.038; respectively]. Overall, a model containing only Steelhead Trout length as a factor was the best model to predict lipid normalized PCB concentration [F (1, 27) = 5.849, P = 0.023], accounting for 18% of the observed concentration. Although PCB ng/g lipid concentration was positively correlated with fish length, fish length did not vary among regions (ANOVA on ranked values; H = 0.687, df = 2, P = 0.707). The levels of PCBs levels in Steelhead Trout were below AET concentrations associated with multiple adverse effects for juvenile salmonids, ranging from enzyme induction to mortality ($\geq$ 2400 ng/g lipid, Meador et al. 2002), except for one individual fish sample in each marine habitat (17, 25, and 25% Whidbey, Central and South Sound Basins, respectively; Table 5).

Concentrations of PBDEs in Steelhead Trout in some samples were at concentrations known to adversely affect fish health and were affected by the region of Puget Sound where fish were found, but were not affected by habitat type or fish length. Lipid-normalized PBDE concentrations in Steelhead Trout ranged from 28 to 3200 ng/g lipid and varied significantly by region [F (2, 26) = 10.063, P = 0.001]; PBDE concentrations in fish collected from the southern region of Puget Sound were significantly higher, by approximately three to five fold, than those measured in fish from central and northern regions (mean (± SD) of 920 ±880 vs. 290 ±260 and 190 ±120 ng/g lipid; P = 0.001 and 0.005; Table 4). Regional differences in lipid-normalized PBDE concentrations were not affected by regional lipid differences, as fish from northern, central and southern regions had statistically similar lipid levels (0.97%, 1.2% and 0.84%, respectively; H = 8.125, df = 4, P = 0.087). Furthermore, unadjusted wet weight levels of PBDEs were also highest in fish from the southern region. The lipid-normalized PBDE concentrations in fish from the central region were statistically similar to those in fish from the northern region (P= 0.692). Lipid-normalized PBDE concentrations in Steelhead Trout did not vary by fish length [F (1, 26) = 0.923, P = 0.346] or habitat type [F (1, 26) = 0.012, P = 0.915]. Steelhead Trout from rivers (in-river and estuary pooled) and marine habitats had similar PBDE

concentrations (means of 550 and 400 ng/g lipid), in part due to the lower lipids measured in fish from marine habitats (1.1 vs 0.89%; Table 4). Overall, PBDEs in Steelhead Trout were at concentrations known to increase disease susceptibility (PBDE 47 plus 99 $\geq$ 470 $\leq$ 2500 ng/g lipid estimated from Arkoosh et al. 2010, 2013; see Supplemental material) in 40% of fish Nisqually River system, none of the fish from the Skagit River system and 10% of fish from the Green-Duwamish River system (Table 5). Steelhead Trout had potentially harmful levels of PBDEs in each of the habitat types of the Nisqually River system (33% of samples from each of the in-river and estuary habitats and 50% of samples from the marine habitat), but were limited to 25% of marine habitat samples associated with the Green-Duwamish River system.

Concentration of DDTs in Steelhead Trout did not exceed AETs though it was positively affected by fish length but not region or habitat type where fish were found. Levels of DDTs in Steelhead Trout samples ranged from 75 - 900 ng/g lipids (Table 4), well below AETs for juvenile salmonid fish ($\geq$ 6000 ng/g lipid, Beckvar et al. 2005 and Johnson et al. 2007; Table 5). Similar to PCBs, mean DDT concentrations varied positively with fish length [$F(1, 27) = 7.080$, $P = 0.013$], accounting for 21% of the observed variation, but did not vary significantly among Puget Sound regions [$F(1, 27) = 1.128$, $P = 0.340$], among habitat types [$F(1, 27) = 0.573$, $P = 0.456$] or among habitat types within regions [$F(1, 27) = 0.010$, $P = 0.990$].

Discussion

This study was not designed to determine cause-and-effect relationships between *N. salmincola* infection, toxic contaminants and Steelhead Trout survival, nevertheless several lines of epizootiological evidence support the theory that these factors contribute to the observed downward trends in EMS throughout Puget Sound. Lower EMS for wild Steelhead Trout from the Nisqually River compared with other Puget Sound rivers (Moore et al. 2015) coincides with high *N. salmincola* infection prevalence and intensity, as well as the highest PBDE levels in that population. Moreover, adverse health effects in juvenile salmonids have been linked to *N. salmincola* loads comparable to those we observed in fish from the Green-Duwamish and Nisqually river systems (Baldwin et al. 1967; Butler and Milleman 1971, Jacobsen et al. 2003, and Roon et al. 2015) and to PBDE levels comparable to those we observed in fish from the Nisqually River (Arkoosh et al. 2010 and 2013), potentially lowering their EMS. Additionally,

we found increased gill, kidney, and heart inflammation and fibrosis in fish from Central and South Puget Sound river systems compared to North Puget Sound river systems.

An increasing *N. salmincola* prevalence and intensity occurred along a north to south gradient of Puget Sound rivers and marine basins. The geographic location of *N. salmincola* observed in this study are consistent with previous studies that documented infection in Steelhead Trout from Green-Duwamish, Puyallup, Deschutes, and Nisqually Rivers in central and southern regions of Puget Sound (Wood 1979, and Dalton 1989) and 0 prevalence in wild Steelhead Trout from the Skagit River (Dalton 1989). The north-south gradient of infection found in this study is likely explained by distribution of the snail host. The *Juga* spp. host has been found as far north as the Green-Duwamish watershed (Johannes, 2010), the northern extent of *N. salmincola* prevalence exceeding 10% in this study. The < 10% prevalence of *N. salmincola* in Steelhead Trout collected from marine habitat in northern Puget Sound, in spite of its absence from the associated rivers, indicates that either a narrow *N. salmincola*-positive zone exists below the respective in-river sampling locations in the northern Puget Sound rivers, or that some of the fish collected from the adjacent marine basin originated from *N. salmincola*-positive rivers in the southern region of Puget Sound. Support for the later theory is provided by telemetry studies indicating that some Green River-origin Steelhead Trout pass through northern Puget Sound marine habitat during their seaward migration (Goetz et al. 2015).

Evidence for a *N. salmincola* infection zone occurring in the lower reaches of endemic watersheds was provided in the Green-Duwamish River watershed, where the prevalence and intensity of infections increased as outmigrating Steelhead Trout entered the lower river. Infrequent *N. salmincola* exposure above the in-river sampling location (RK 55) was indicated by in-river infection prevalence of 13.3% and MPK of 93.1 in wild Steelhead Trout that reared in the upper portions of the watershed for 1-2 yr (Table 2). The 100% prevalence but intensity of only 84 MPK (BY 2013) found in hatchery-origin Steelhead Trout from Icy Creek Ponds (RK 78), were most likely acquired during their early rearing phase in the *N. salmincola* infection zone of the lower watershed. Prior to their transfer to Icy Creek Ponds, these fish were reared on surface water at the Soos Creek Hatchery, located in the lower watershed (RK =55), until mid-July, a time when prevalence was expected to reach 100% (Figure 2). An *N. salmincola* infection zone was further indicated by increasing intensity as fish released from Icy Creek

406 Ponds moved through the lower reaches of the watershed. This lower watershed infection zone
407 is also supported by in-river observations from coastal Oregon, where exposure to *N. salmincola*
408 infection increases in the lower reaches of rivers (Ferguson et al. 2010).

409 Extended sampling from the estuary of the Green-Duwamish River indicated that *N.*
410 *salmincola* infections likely increased with transience in the lower portions of the river.
411 Hatchery-origin fish were released from Icy Creek Ponds (RK 78) beginning March 25, and their
412 peak passage through the in-river trap (RK = 55) occurred on March 29 (Topping and Anderson
413 2015). These fish were first detected in the estuary on April 2; however, a prolonged transience
414 in the estuary or lower-river was indicated because Icy Creek Pond fish were still captured in the
415 estuary at 35 d post release. The mean intensity of infection increased six-fold between April 2 –
416 29, indicating exposure to *N. salmincola* continued. Although there is tidal influence at the
417 estuary sampling site (RK 9.5) in this study, at RK 10 or higher the river is primarily fresh water
418 (Goetz et al. 2015) which would permit continued *N. salmincola* infection.

419 The lower-watershed *N. salmincola* zone was less apparent in the Nisqually River trap and
420 estuary habitat, where infection prevalence and intensity were extremely high at both locations.
421 However, any effect of increasing *N. salmincola* infections towards the lower reaches of the
422 watershed was likely masked by the in-river sampling location (RK 20), which was located in the
423 presumptive *N. salmincola* infection zone of the lower watershed. Infection prevalence upstream
424 from this location was not investigated.

425 Intensity of infection by *N. salmincola* in Steelhead Trout from the Green-Duwamish and
426 Nisqually Rivers was sufficient to damage critical fish tissues. We found a strong association
427 between *N. salmincola* infections of over 1000 MPK, and prevalence of fish with gill and heart
428 lesions. We found high prevalence of kidney, muscle and fin lesions, as well as infection of the
429 eye, in sentinel fish with a natural ongoing *N. salmincola* challenge, in agreement with Wood
430 and Yasutake (1956).

431 The intensity of infection by *N. salmincola* found in wild Steelhead Trout from the Green-
432 Duwamish and Nisqually River estuaries (808 and 2545 MPK, respectively), was similar to or
433 greater than biological effects levels reported in laboratory studies. *Nanophyetus salmincola* can
434 cause direct mortality to susceptible salmonid fish, with intensity levels as low as 295 MPK

435 resulting in 50% mortality of Rainbow Trout fry in 24 h (Baldwin et al. 1967). Butler and
436 Milleman (1971) observed mortality, reduced swimming speed and earlier onset of fatigue in
437 Steelhead Trout exposed to 1500 cercaria (survivors of the swim tests had mean intensity of
438 1,013). Infection intensity of 394-430 MPK resulted in reduced immune function and higher
439 mortality of Chinook salmon when challenged by *Listonella (Vibrio) anguillarum* (Jacobsen et
440 al. 2003). *Nanophyetus salmincola* intensity of greater than 200 MPK is sufficient to lower
441 resistance of Chinook Salmon to the common bacterial pathogen *Flavobacterium columnare*
442 (Roon et al. 2015).

443 One field study Jacobsen et al. (2008) found decreasing *N. salmincola* infection prevalence
444 and intensity Coho Salmon during their first summer in the Pacific Ocean, suggesting that
445 differential ocean mortality may occur with intensity of 400 MPK. On the other hand, Romer et
446 al. (2013) reported that outmigrating Steelhead Trout in two Oregon coastal rivers with high
447 prevalence of *N. salmincola* but different intensity levels (Nehalem River: 1345 MPK, Alsea
448 River 279 MPK, metacercaria per gram levels adjusted to be equivalent to our MPK) had
449 roughly equal survival rates to the estuary-ocean boundary ranging from 41-78%. However,
450 these survival rates were higher than those reported for Steelhead Trout migrating through large
451 estuarine environments such as 27% for the Cheakamus River, British Columbia (Melynchuk et
452 al. 2007) and 16% for wild fish in Puget Sound (Moore et al. 2015), and may reflect the different
453 distances to the ocean and conditions encountered.

454 *Nanophyetus salmincola* has been recognized as an important mortality factor in several
455 salmonid fish hatcheries located in south Puget Sound watersheds. Significant mortalities and
456 operating challenges due to *N. salmincola* contributed to prior closure of the McAllister
457 Hatchery, located at the western edge of the Nisqually River delta (Hatchery Reform Committee
458 2003). The severity of *N. salmincola* infection (over 4000 MPK) in the surface water at Soos
459 Creek Hatchery shows the rationale behind moving fish to spring water supplied facilities such
460 as Icy Creek Ponds early in the rearing cycle.

461 Our study is the first to report on biologically significant POP concentrations in wild-origin
462 juvenile Steelhead Trout in their natural habitats. Juvenile Steelhead Trout, (which spend a year
463 or more in fresh water before migrating to salt water), sampled from the heavily industrialized
464 Green-Duwamish River system had PCB concentrations (ng/g lipid) that were 25 – 32% higher

than those from the less developed Nisqually and Skagit River systems. However, PCBs levels were below AET concentrations associated with multiple adverse effects for juvenile salmonids, ranging from enzyme induction to mortality ($\geq$ 2400 ng/g lipid, Meador et al. 2002), except for one individual fish sample in each marine basin. Fish with PCB concentrations above the AET, had low to moderate wet weight concentrations (5.8 – 20 ng/g ww) but very low lipid content (ranging from 0.24 – 0.57%), indicating that decreasing lipid level associated with sea migration increases the potential that some fish will experience biologically significant, sub-lethal adverse effects shortly after they enter the marine environment. Ocean-type juvenile Chinook Salmon, which migrate to sea as sub-yearlings, sampled from the estuary, nearshore and marine habitats associated with the Green-Duwamish River were also reported to have elevated PCB levels compared to those from the Skagit and Nisqually Rivers (Johnson et al. 2007a, O'Neill et al, 2015), however at considerably higher concentrations than we measured in Steelhead Trout. Moreover, in contrast to Steelhead Trout, 79% of the Chinook Salmon samples from the estuary and associated nearshore habitats of the Green-Duwamish River and 50 – 83% of those from the offshore marine habitat of the Whidbey and Central basin had PCB concentrations that exceeded the AET values (O'Neill et al. 2015). These species differences are likely related to habitat use, diet and metabolism as well as sources of PCBs in these systems. Although Steelhead Trout generally reside in fresh water longer than ocean-type juvenile Chinook Salmon, they are distributed in the upstream portions of the freshwater habitat and spend less time in the estuary and nearshore habitats during their seaward migration than juvenile Chinook Salmon (Quinn 2005). Assuming the estuary and nearshore habitats of the Green-Duwamish, which are more developed than upstream freshwater habitat, are important sources of PCBs for out-migrant salmonids, higher contaminant exposures in Chinook Salmon are consistent with their more prolonged period of estuarine/nearshore exposure compared to Steelhead Trout. Coho Salmon, which like Steelhead Trout have a more limited period of estuarine and nearshore residence, generally have lower contaminant concentrations than Chinook Salmon sampled from the same locations (Johnson et al. 2007a).

In contrast to PCBs, PBDE concentrations in Steelhead Trout did not coincide with the degree of river system development, but rather increased along a north to south gradient in Puget Sound, with PBDEs measured in fish from the Nisqually River system at levels known to adversely affect fish health. Elevated PBDE concentrations (ng/g lipid) measured in wild

Steelhead Trout from the Nisqually River system were not solely due to lower lipid levels, which alter the lipid normalized values of POP, because higher unadjusted wet weight levels of PBDEs were also observed in the fish from the southern region of Puget Sound. Based on laboratory exposure studies of Chinook Salmon (Arkoosh et al. 2010, and 2013), 30-50% of the fish sampled from each habitat type in the Nisqually River system had PBDE concentrations high enough to potentially increase their susceptibility to naturally occurring pathogens. At other river systems, only one sample, an individual steelhead collected from the marine habitat offshore of the Green-Duwamish River had potentially harmful levels of PBDEs. We used lipid-normalized PBDE concentrations generated from Arkoosh et al. (2010 and 2013; see details in the Supplemental material) rather than wet weight concentrations because decreases in lipids generally make fish more vulnerable to the effects of POPs (Lassiter and Hallam 1990; van Wezef et al. 1995), especially for migrating salmon that experience rapid lipid reduction during migration (Debruyn et al. 2005 and Meador et al. 2002). However, for PBDEs there is some uncertainty with using lipid weight to normalize and model concentrations of PBDEs in fish (Bethune et al. 2005). In the current study, both lipid normalized and wet weight concentrations of PBDEs indicate that 30% of the fish samples from each of the in-river and estuary habitats of Nisqually River system had PBDE concentrations high enough to potentially increase their susceptibility to naturally occurring pathogens. However, based on wet weight concentrations of PBDEs, all Steelhead Trout sampled from marine habitats had PBDE levels below concentrations known to affect their susceptibility to naturally occurring marine pathogens.

The elevated PBDE levels observed in Steelhead Trout from the Nisqually River system were unexpected, given below those known to affect disease susceptibility (O'Neill et al. 2015). Collectively, these data suggest an upstream source of PBDEs, above the in-river sampling location on the Nisqually, to which Steelhead Trout are disproportionally exposed compared to Chinook Salmon. Sources of PBDE to the Puget Sound include input from wastewater treatment plants, followed by stormwater and then atmospheric deposition (Osterberg and Pelletier 2015) but the relative importance of these sources in the upper Nisqually watershed are unknown.

Although not tested in this study, contaminants and high *N. salmincola* infection prevalence and intensity may contribute to decreased disease resistance, resulting in lower marine survival, than either factor alone. Because PBDEs and *N. salmincola* were detected in the same fish in our

study we can definitely conclude that the potential for synergistic effects on fish health are present in wild Steelhead Trout in the Nisqually River system. The combination of both PCBs and *N. salmincola* were found to cause lower resistance of juvenile Chinook Salmon to the marine bacterial pathogen *Listonella (Vibrio) anguillarum* in a laboratory study than did either factor alone (Jacobsen et al. 2003). Thus, *N. salmincola* infections and toxic contaminants may serve as mortality cofactors, with the proximate causes of death involving bacterial pathogens or selective predation on infected cohorts. The predation risk is likely heightened by the increased regional abundance of certain marine mammal predators such as harbor seals (Jeffries et al. 2003) in recent years. Because restoration options are currently being explored for the recovery of endangered Puget Sound Steelhead Trout stocks, it is recommended that further efforts be employed to understand the impacts of *N. salmincola* and contaminants on their early marine survival.

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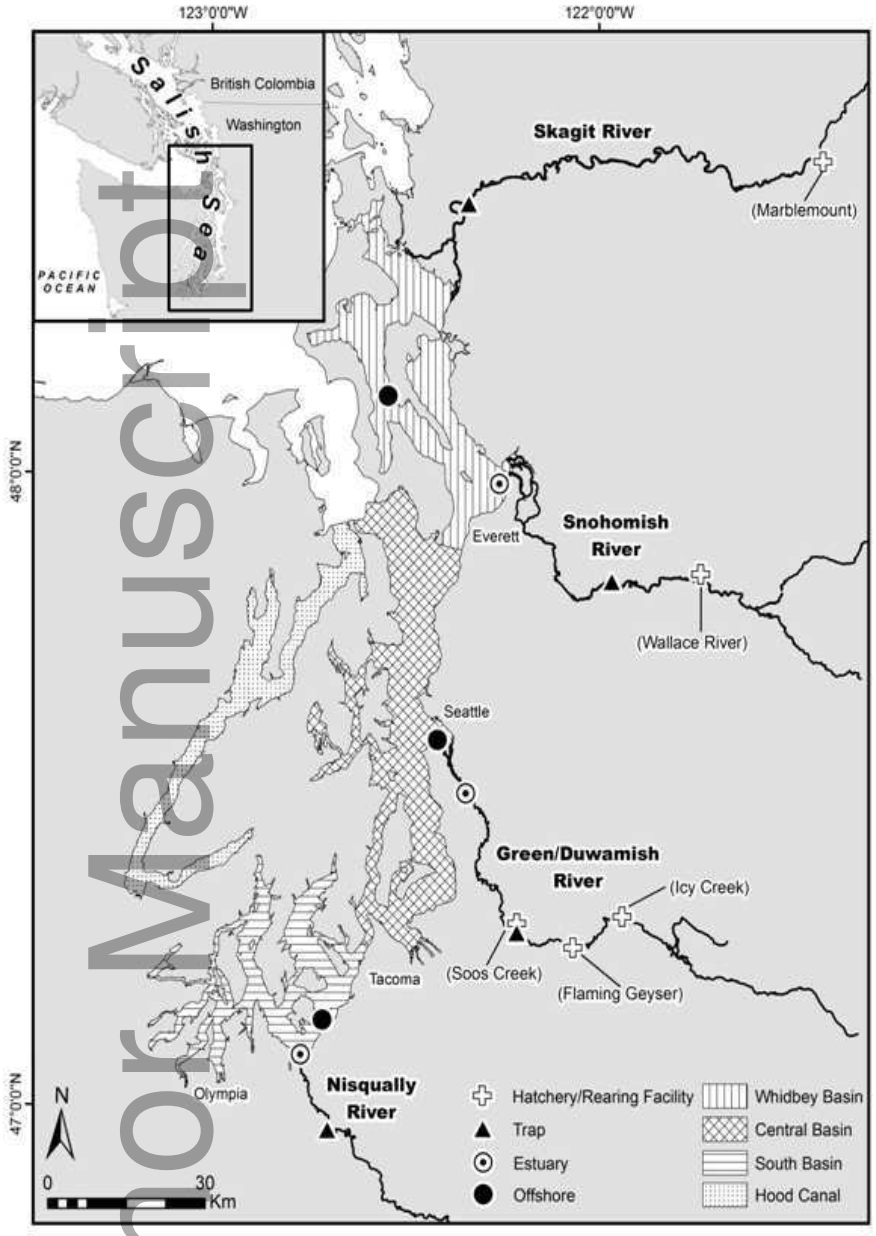


FIGURE 1. - Sampling locations within four Puget Sound rivers and three marine basins where outmigrating Steelhead Trout were collected and examined for *N. salmincola* prevalence and intensity of infection in 2014. Contaminant analyses was completed for a subset of Steelhead Trout collected from the in-river, estuary and marine basin sites in the Skagit, Green-Duwamish, and the Nisqually River systems.

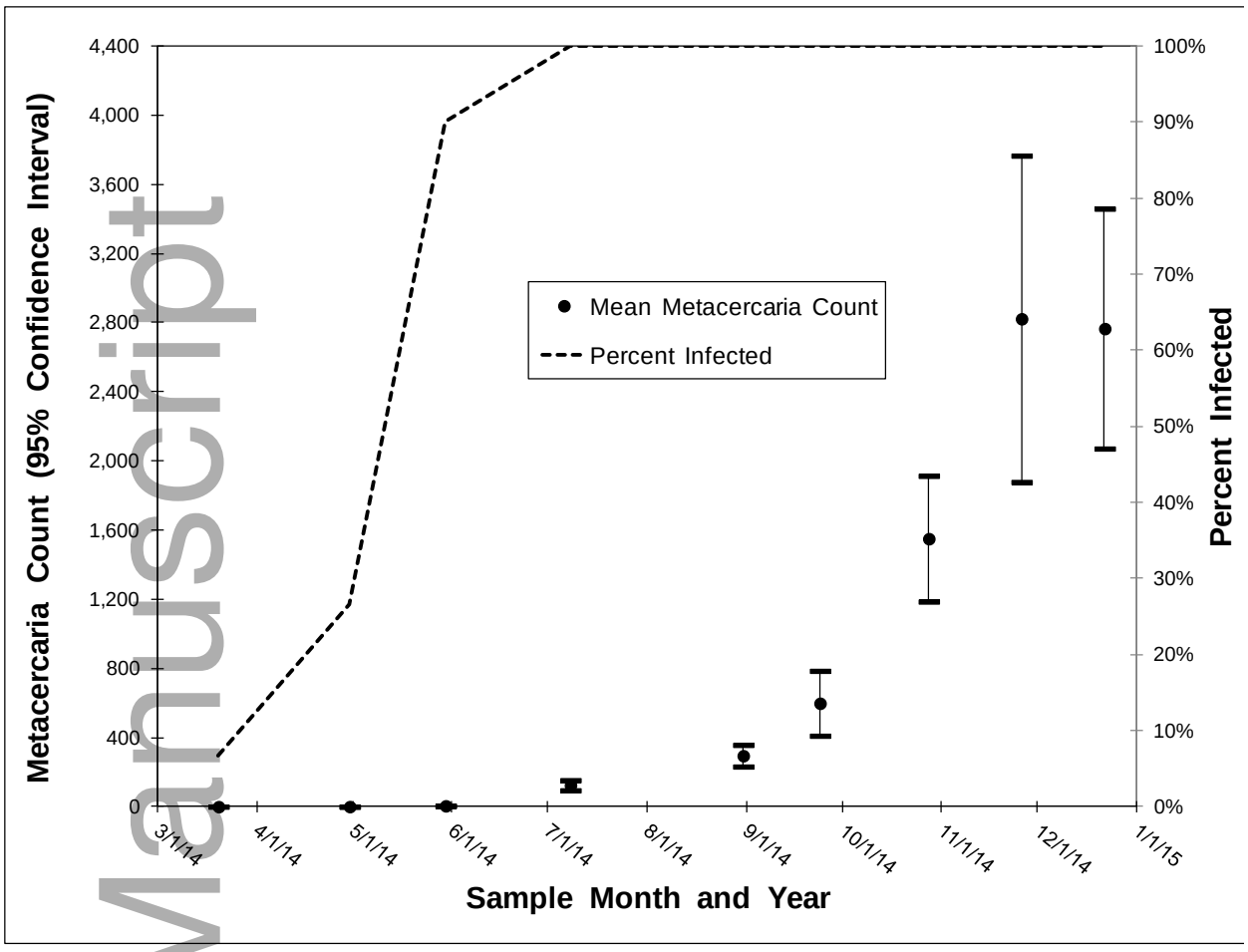
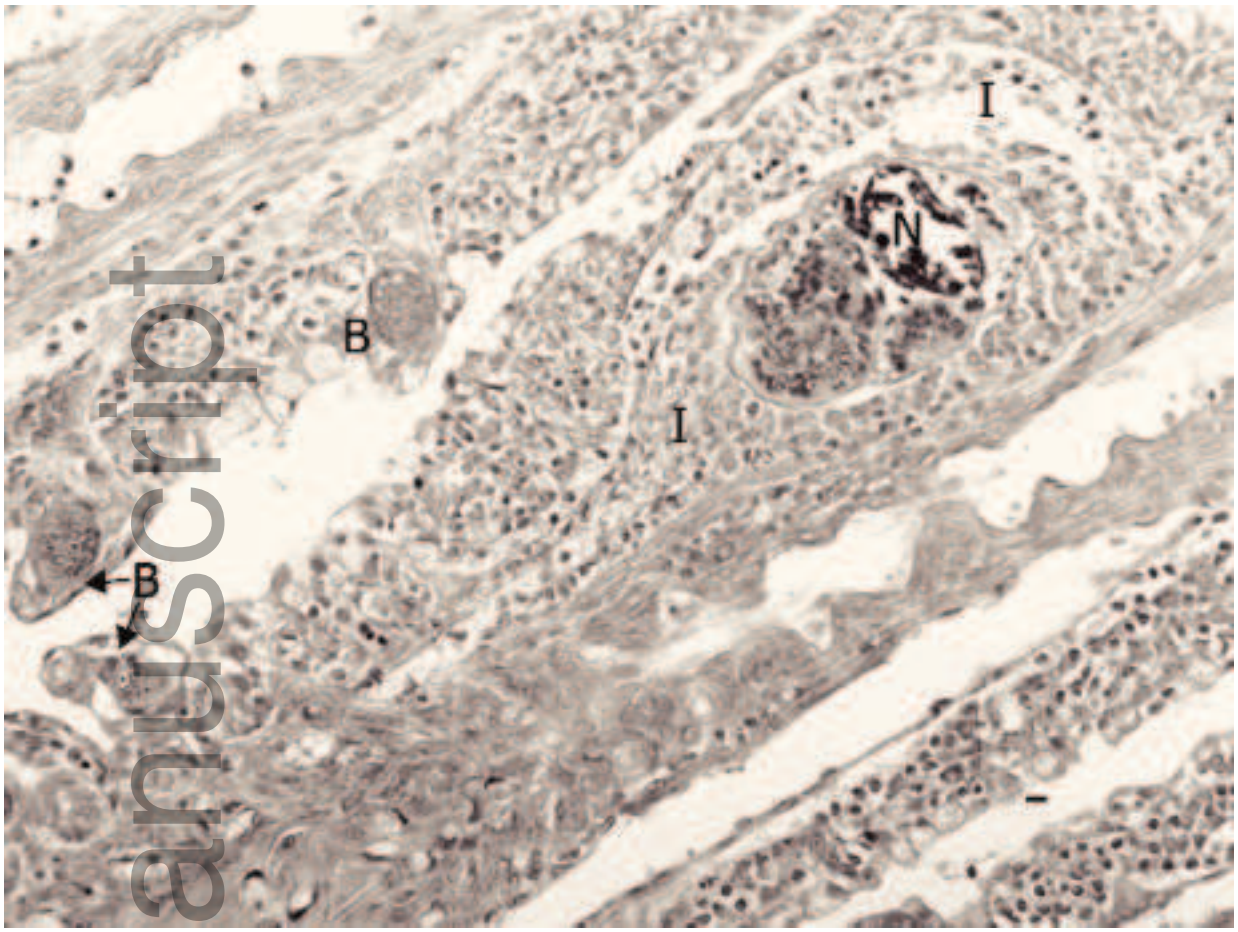


FIGURE 2.- *Nanophyetus salmincola* load and prevalence over time in juvenile Steelhead Trout raised at Soos Creek Hatchery (Brood Year 2014) on unfiltered surface water from March – December 2014; n=30 (March-May_ or 15 (July-December) per month. Prevalence of *N. salmincola* reached 100% in the sample collected July 8. Error bars indicate 95% confidence intervals.



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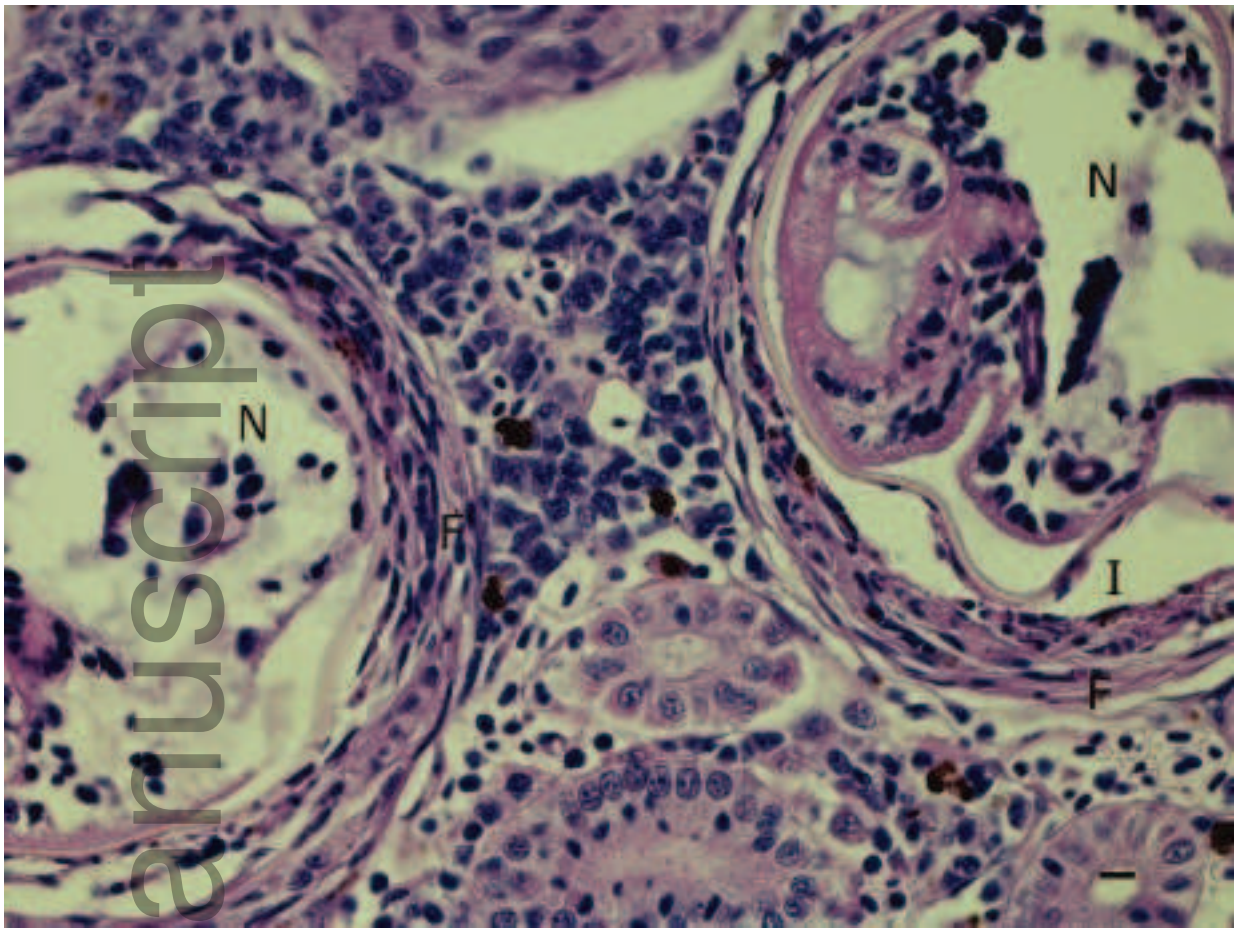

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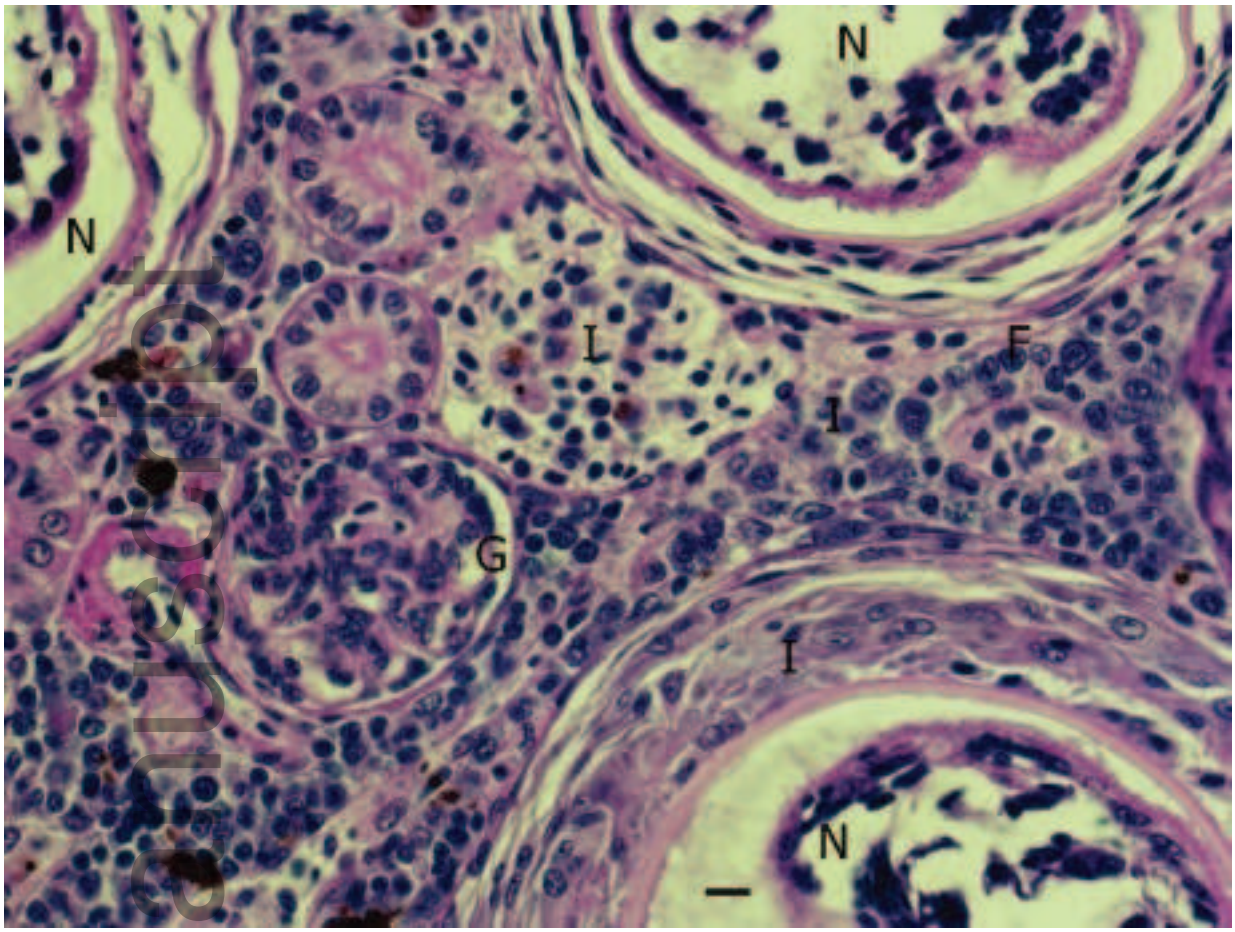

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