Report for 2002WA15B: Development of a Comprehensive Monitoring Protocol to Characterize the Concentration and Associated Health Risk of Salmonid Pathogens Suspended in Water

- Water Resources Research Institute Reports:
  - Call, Douglas R., Rollin H. Hotchkiss, Kenneth D. Cain, and Frank J. Loge, 2003, Development of a Comprehensive Monitoring Protocol to Characterize the Concentration and Associated Health Risks of Salmonid Pathogens Suspended in Water, State of Washington Water Research Center, Washington State University, Pullman, Washington, State of Washington Water Research Center Report WRR-14, 9 pages.
- Dissertations:
  - Wallace, Brandi J., 2002, Assessment of the occurrence and relative concentrations of salmonid pathogens in fish hatcheries from Washington state. MS Thesis, Department of Civil and Environmental Engineering, Washington State University, Pullman, Washington, 16 pp.

Report Follows

Problem and Research Objectives

Fisheries management involves activities such as propagation of hatchery fish and collection, transport, and release of indigenous and hatchery fish to bypass water control structures. Propagation and transport practices place a high density of fish in a relatively small volume of water, an environment conducive to reproduction and dissemination of etiological agents responsible for a range of fish diseases. The occurrence of salmonid pathogens in hatcheries, in transport vehicles (e.g., barges and trucks) and in waters receiving fish supplementation is rarely evaluated. The subsequent fate and transport of pathogens in downstream receiving waters is poorly understood.

While fisheries biologists have always been cognizant of the potential detrimental effects of fish pathogens on salmonid populations, there has been no comprehensive monitoring protocol suitable for detecting a broad range of fish pathogens suspended in the water column. Consequently, it has been virtually impossible to evaluate how disease agents are impacted by management practices. The development of a comprehensive monitoring protocol would aid a variety of local, state, and federal agencies and organizations, including the Army Corps of Engineers, the Bonneville Power Administration, the Washington Department of Fish and Wildlife, state and national fish hatcheries, and organizations such as the Whirling Disease Foundation. With suitable monitoring methods, these regulatory and management entities would be able to consider prevalence, incidence and disease outcome when developing and implementing management and control practices to insure the health and maintenance of indigenous fish populations in Washington State.

Molecular techniques have been employed in a broad range of environmental habitats to monitor the occurrence of specific organisms. Molecular techniques have included polymerase chain reaction (PCR), nucleic acid probes (e.g., gene probes), and fluorescent antibodies. When these techniques are used to identify pathogens within aquatic environments, generally a large quantity of water (e.g., 1,000 L) must be concentrated to a relatively small volume (e.g., 0.1 mL) because pathogens typically occur at low concentrations on an intermittent basis. The most common method to concentrate a large quantity of water is filtration. The targeted organisms are then eluted from the filter, the nucleic acid is extracted, and an appropriate molecular technique is used to identify the presence or absence of the target sequence. The above steps constitute a monitoring protocol.

Two principal factors must be accounted for in a molecular-based monitoring protocol before using the results in management and control of engineered and natural systems. First, the detection limit of the molecular-based monitoring protocol must be established. The detection limit is influenced by the percent recovery of organisms off the filter, the extent of PCR-inhibition associated with compounds co-eluted from the filter (e.g., humic acids), and the overall sensitivity of the PCR assay (e.g., number of organisms necessary to produce a positive result). Second, an explicit statement of the health risks associated with positive or negative PCR results must be developed. The health risks should then be characterized with a dose-response model, a mathematical expression that relates the probability of death (infection and illness can also be used as endpoints) to a specified level of exposure to an etiological agent. All previous studies that used molecular-based protocols to monitor environmental samples for specific organisms report results in a +/- reporting scheme. There have been no explicit statements of detection limits, sources of variation, or the health risks associated with a positive or negative result (3, 5). Recently, a comprehensive framework was developed for quantifying the detection limit of PCR-based

monitoring protocol and the health risks associated with positive and negative results (6). The framework was used to evaluate the human health risks associated with recreational contact in waters polluted with non-point source runoff.

We proposed to adapt this risk-based framework to a novel methodology designed to simultaneously detect multiple fish pathogens in the water column. This framework would permit evaluation of the health risks associated with positive and negative assay results. The specific objectives were to: (1) develop a DNA microarray to be coupled with PCR for multiplex detection of salmonid pathogens; (2) quantify the detection limit of the PCR-based monitoring protocol, and (3) perform a cursory evaluation of the occurrence and associated health risks of fish pathogens in hatcheries, transport vehicles, and surface waters within Washington State. A fourth objective involved developing and instructing a new course on the ecological aspects of fish management and control in the Pacific Northwest, but administratively imposed limitations on grant duration eliminated this objective.

Methodology

Sample Collection and Preservation. Water samples were collected from 21 fish hatcheries in Washington State during the summer of 2002. As per sampling agreement, the hatcheries are referenced herein with alphabetical letters A-U to maintain anonymity. At each location, water samples were collected from the inlet and outlet for analyses of hardness, suspended solids, total and fecal coliforms, and enterococci as per Standard Methods, 20th edition (2), methods 2340C, 2540D, 9221B, 9221E, and 9230B, respectively. In addition, 4-204 L were filtered from the hatchery influent and effluent for analysis of selected pathogens. These samples were concentrated on-site by pumping the water (stainless steel progressive cavity pump, Ryan-Herco Products Corp, Sacramento, CA) through a 0.45 μm pore size, 293 mm FALP filter (Millipore, Bedford, MA). The 1-L water samples and filters were transported on ice to Washington State University for processing. Filters were eluted and concentrated immediately upon arrival and the resulting solutions stored at –20 °C. The 1-L water samples were processed within 24 hours for the specified water quality characteristics.

Filter Elution, Nucleic Acid Extraction, and Purification. The 293 mm FALP filters was folded in half and laid flat in a sterile Pyrex glass dish containing 200 mL of elution buffer: 1.5% (w/v) beef extract in 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). The filter was eluted by scrubbing the surface with a sterile nylon bristle brush for 10 minutes. Extracts were collected in 250 mL centrifuge tubes; the brush was rinsed with elution buffer and the liquid pooled with the extracts. The tubes were centrifuged at 6,000 x g for 10 minutes. After removing the supernatant, the pellet was weighed and distributed to 10 mL screw-capped microcentrifuge tubes. The maximum mass allowed per tube was 1g; nucleic acid was extracted from the FALP pellet and purified using a commercially available Ultraclean™ Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Solana Beach, CA).

Pathogen Detection. We screened samples for 15 bacterial pathogens using a combination of PCR and microarray detection. Primers were designed to target conserved sequences from the 16S rDNA gene (179 bp total length). Target sequences were amplified using a standard protocol (initial denaturation at 95°C for 5 min; 30 cycles consisting of 95°C for 30 sec, 60°C for 60 sec, and 72°C for 60 sec; and a final extension step at 72°C for 10 min). Reaction constituents included 1X reaction buffer, 5 mM DNTPs, 25 mM $MgCl_2$, 2 U AmpliTaq Gold (Applied Biosystems, Foster City, CA) and 20 uM 16srDNA primers. For each PCR assay, positive and negative controls were included. The PCR products were identified on a DNA microarray as follows. PCR products were

automatically tagged with biotin by including a 5′ biotin conjugate on each PCR primer. The 179 bp products from each sample were verified by agarose gel electrophoresis before adding 5 µl of PCR product to 35 µl hybridization buffer (4X SSC and 5X Denharts). Each PCR product was heat denatured by boiling 2 min and then aliquoted into two wells of a 12-well Teflon masked microarray slide. Each well included oligonucleotide probes complementary to DNA sequences for 15 pathogenic fish bacteria and 3 controls (*E.coli*, *S. aureus*, and *V. phosphoreum*). After overnight hybridization at 60°C, PCR products were removed and the arrays were washed and detected as described by Call et al. (2003). Microarrays were then scanned images processed using an ArrayWoRx scanner (Applied Precision, Issaquah, WA). "Spots" on the array signified hybridization of a PCR product with a specific pathogen probe. Spots with intensity values greater than non-hybridizing probes were considered positive for this analysis.

Baseline Sensitivity and inhibition of PCR. The sensitivity of the PCR/microarray assay was shown to be equivalent to less than 10 colony forming units. The relative impact of inhibitory substances on PCR was evaluated for each location. An aliquot of the filter extract obtained from the FALP filter was spiked with ca. 100 cells of *S. aureus* prior to purification. The level of inhibition was determined by amplifying 10-fold serial dilutions of the purified samples and visualized by gel electrophoresis. For example at positive PCR band at the second 10-fold dilution would be have a concentration of 1,000 cfu in the original undiluted filter extract.

Recovery from FALP Filters. An overnight culture of *E. coli* K-12, grown in nutrient broth (Difco), was prepared in dechlorinated tap water. The initial titer of viable *E. coli* was enumerated on nutrient agar plates incubated overnight at 37°C. The solution was filtered through three separate 293 mm FALP filters pre-wetted with methanol. The concentration of viable *E. coli* cells was enumerated in the filter eluate to assess bacterial recovery and the experiment was replicated three times. Based on findings from a previous study (1) the mean recovery established in this *E. coli* recovery experiment was assumed to correspond to all field samples.

Quantification of the detection limit. The detection limit of the overall assay was quantified using the following equation (1):

$$\text{Detection limit}\left(\frac{\text{cfu}}{\text{L filtered}}\right) = \frac{(\text{I})(S)}{(V_f)(\%V_p)(R_e)} = \frac{I}{(R_e)(V_f)} \cdot \frac{S}{\%V_p} = \frac{RI}{V_f} \cdot \frac{S}{\%V_p} \quad (1)$$

where S is the sensitivity of the PCR assay, V_f is the volume of sample processed through the filters, R_e is the recovery of organisms from FALP filters, I is inhibition of PCR assays, and $\%V_p$ is the fraction of concentrated sample analyzed with PCR.

Principal Findings and Significance

Standard Water Quality Indices. The concentration of total suspended solids was below the state regulatory requirement of 15 mg/L in all 21 hatchery effluents sampled in this study. With the exception of one hatchery, the total suspended solids concentration was below the EPA recommended value of 6 mg/L for hatcheries with an offline sedimentation basin. The influent concentration of fecal coliform and enterococci at each hatchery varied considerably, with values ranging from <2 to 300 and <2 to 49 MPN/100mL, respectively. Relative to influent concentrations, 30% of the hatcheries had a statistically significant increase in the concentration of fecal coliform in the effluent, and 40% had a similar increase in the concentration of enterococci. Conversely, 24% of the hatcheries produced a net reduction in the concentration of fecal coliform, and 19% had a similar decrease in the concentration of enterococci. Hence based on a single sampling event the

hatchery environment does not appear to constitute a significant reservoir for the propagation of standard indicator organisms.

Detection and Implication of Salmonid Pathogens. Assay sensitivity and inhibition are important considerations when detecting pathogens. If a pathogen is detected, then some quantitative estimate is needed to understand the potential biological significance. If, however, no pathogens are detected, we need to know what the minimum sensitivity of the assay is before we can draw any conclusions. That is, if a sample is negative but has very low sensitivity, then there is little confidence in the negative result. The sampling strategy described here provides a means to estimate the minimum concentration that could be detected when all of the variables in equation 1 are considered. Overall 23 of 38 (60%) of the water samples had detectable concentrations of fish pathogens. In eight cases (21%) we detected no pathogens and there was no inhibition detected for the assay suggesting that these samples were truly negative within the constraints of entire assay. The remaining 19% of samples were also negative, but some inhibition was present that reduced assay sensitivity. In general, the water samples from the fish hatcheries were amenable to our sampling strategy when compared with other environmental samples (1).

Both influent and effluent samples were available for 15 of the 21 sampled hatcheries. Six of the 15 bacterial species were detected in the influent and effluent of the hatcheries. There was no correlation between influent water source, whether it was river, well, or a spring, and the detected pathogens. The predominant organism was *Aeromonas* spp.; 79% of the influent samples contained a detectable concentration suggesting that these organisms are fairly ubiquitous in the aquatic environment. Motile aeromonad septicaemia causes haemorrhagic ulceration and lesions on and around the fins, and haemorrhaging of internal tissues leading ultimately to the demise of the infected fish (4).

Within the constraints of this study (only one sampling visit per hatchery), the hatcheries do not appear to serve as a reservoir to further propagate pathogens in the effluent. This conclusion is based on the observation that in 46% of hatcheries where both influent and effluent samples were available, one or more pathogens was detected in the influent, but not the effluent and there was no relationship with assay inhibition that could otherwise explain this result. In only one case were pathogens detected in effluent, but not the influent.

In three cases, *Mycobacterium* (*M. chelonae* or *M. fortuitum*) was detected in effluent samples. Mycobacteriosis is a disease that affects a large number of fish species and is manifested by chronic systemic disease with granulomas forming throughout the internal organs. *M. chelonae* and *M. fortuitum* are both capable of causing disease in humans (4). *Yersini ruckeri* was detected in both the influent and effluent of one hatchery, which was the only acclimation pond sampled in the study. This organism causes enteric redmouth disease in trout that is manifest by severe haemorrhagic septicaemia and high mortality (4). Several hatcheries reported active cases of cold water disease (*Flavobacterium psychrophilum*) during the time that samples were collected, but no species from this genus were detected in the hatchery water. Failure to detect any *Flavobacterium* despite good assay performance suggests that either the microarray detector needs to be improved or that we sampled at an inopportune time to detect these organisms. It would be reasonable to expect all three *Flavobacterium* species to be found in a planktonic state. *S. aureus* was also detected in some samples, but this latter organism is not a fish pathogen and may represent a nonspecific detection of a closely related organism.

The health risks associated with the relative concentrations of pathogens detected in this study are not readily quantifiable due to the lack of dose/response data. The occurrence of salmonid

pathogens in the hatchery influent, while not surprising does raise potential health concerns for both hatchery stock and potentially for native stocks receiving effluent waters. Findings from this study support the need to further study the ecology of planktonic salmonid pathogens. Bacterial salmonid pathogens can clearly survive within the aquatic environment, but the factors influencing the rates of growth and decay, and the corresponding health risks, are poorly understood. The fact that the majority of effluent samples were biologically "cleaner" than the influent samples suggests that there are effective management practices already in use at most facilities. Further work is needed to determine if these encouraging results are applicable at other sampling times and to identify which management practices are most effective for controlling biological quality of hatchery effluent.

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