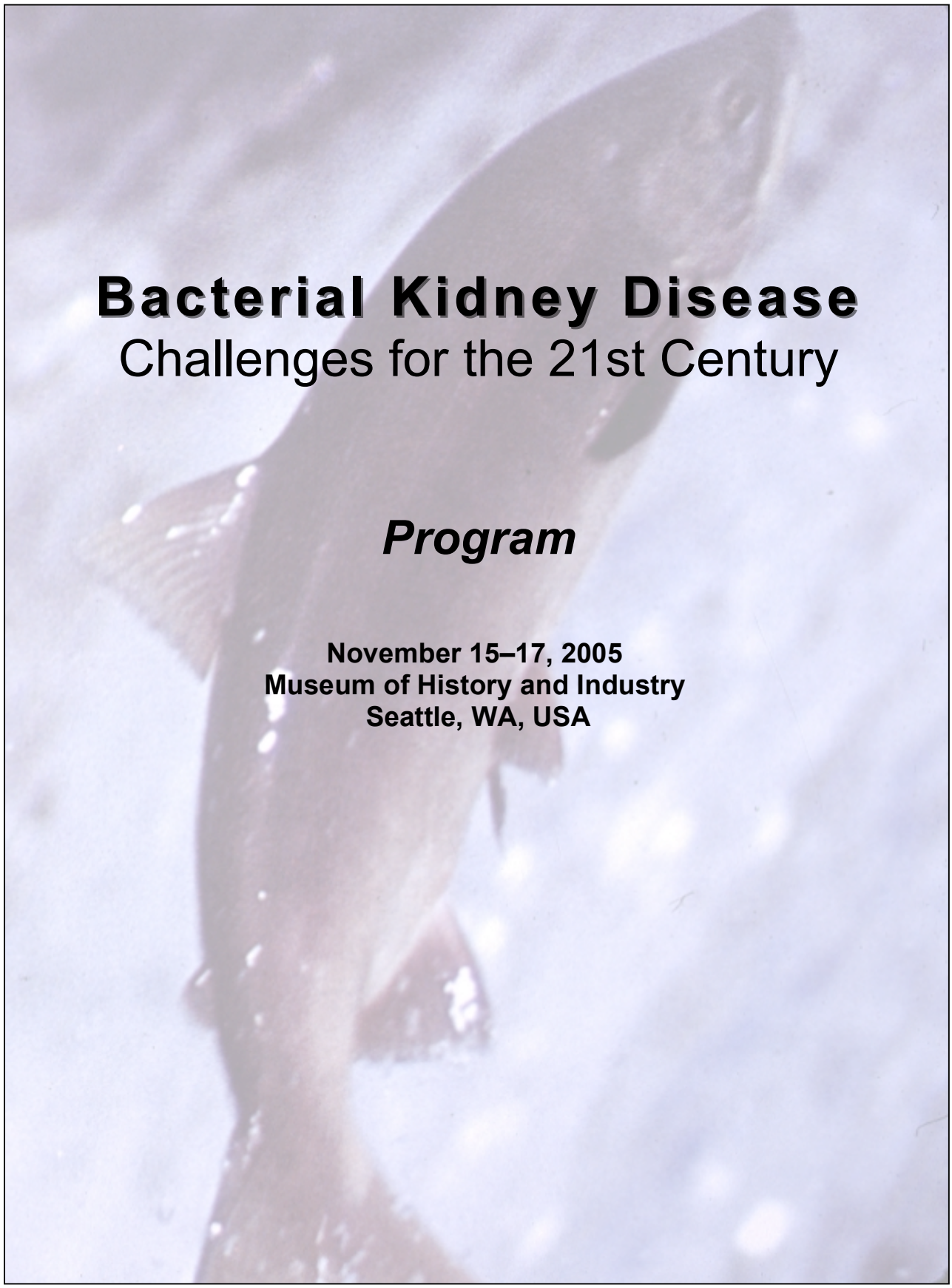

Bacterial Kidney Disease
Challenges for the 21st Century

Program

November 15–17, 2005
Museum of History and Industry
Seattle, WA, USA

Sponsors

National Marine Fisheries Service
and the
Northwest Fisheries Science Center,
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National Center for Cool and Cold
Water Aquaculture,
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Renibacterium Salmoninarum Genome
Sequencing Project
(Funded by USDA CSREES/NSF
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American Fisheries Society,
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Pacific Northwest Fish Health
Protection Committee

Northwest Marine Technology

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USGS and USFWS,
Department of the Interior

Bacterial Kidney Disease - Challenges for the 21st Century

November 15-17, 2005
Museum of History and Industry
Seattle, WA

Welcome to the 2005 Bacterial Kidney Disease Workshop, Challenges for the 21st Century. We are pleased at this large gathering of scientists, fish health practitioners, fish culturists, and fisheries resource managers representing national and international institutions, tribal, federal and state agencies, and the private sector, to discuss the biology of *Renibacterium salmoninarum*, the causal bacterial agent of BKD. BKD has been and continues to be a high priority for hatcheries, public and private aquaculture, and programs designed to maintain salmon stocks listed under the Endangered Species Act.

This workshop will include presentations on the ecology and distribution of *R. salmoninarum*, mechanisms of virulence utilized by this pathogen, and treatment and management of the disease. In addition, we will present the complete sequencing of the *R. salmoninarum* genome. We hope that the availability of the genome sequence will stimulate further research aimed at defining mechanisms of virulence, rational antigen discovery for vaccine design, and the development of novel therapeutic strategies.

We are fortunate to host two renowned Keynote Speakers at the workshop. During the genomics session, Dr. Laura Brown of the National Research Council of Canada, Institute for Marine Biosciences will address the integrated approach being taken to solve the impacts of another aquatic animal pathogen, *Aeromonas salmonicida*. This approach is a model for what can be accomplished with *R. salmoninarum,* involves the application of genomic and molecular studies of both pathogen and host to better understand host-pathogen interactions, and should lead to better vaccine design or other prophylactic or preventative treatments. Dr. Trevor Evelyn, Scientist Emeritus with the Department Fisheries & Oceans, Canada, will open the second day of the conference and will present a talk that will encompass his broad and long-time interest in the ecology and biology of *R. salmoninarum* and BKD, and views on the chances of reducing the impacts of this disease on salmonid stocks.

We look forward to a valuable exchange of scientific ideas and hope that this workshop helps set the stage for a renaissance of research on BKD.

BKD Workshop Steering Committee

Kevin Amos
Walt Dickhoff
Per Heggelund
Mark Strom
Greg Wiens
Jim Winton

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Posters

Modeling BKD-related mortality: The effect of antigen and temperature related suppression of cell mediated immunity, and other factors
Owen Hamel, Northwest Fisheries Science Center, NOAA Fisheries

Potential for Transmission of *Renibacterium salmoninarum* during Coded Wire Tagging: A Comparison of Conventional and Automated Marking Methods
C.L. McKibben, Western Fisheries Research Center, USGS

Renibacterium salmoninarum genome sequencing project: Summary of findings
Donald S Chen, Samuel Crane, Mark Strom, Northwest Fisheries Science Center, NOAA Fisheries

Both msa Genes in *Renibacterium salmoninarum* Are Needed for Full Virulence in Bacterial Kidney Disease
Alison M. Coady, Anthony L. Murray, Diane G. Elliott, and Linda D. Rhodes, Northwest Fisheries Science Center, NOAA Fisheries, and Western Fisheries Research Center, U.S. Geological Survey

Prevalence of *Renibacterium salmoninarum* Infection Among Juvenile Chinook Salmon in North Puget Sound and Implications for Disease Interactions
Shelly Nance, Colleen Durkin, Casimir Rice, and Linda Rhodes Northwest Fisheries Science Center, NOAA Fisheries

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9:10–9:30 AM	OIE and EU perspective on regulatory considerations or *R. salmoninarum* for intra and international commerce *Jim Winton, Western Fisheries Research Center, USGS and OIE Reference Laboratory for BKD*
9:30–10:15 AM	Panel discussion on de-listing of BKD by OIE and direction where the international and domestic regulators need to move. *Jim Winton (Western Fisheries Research Center, USGS), Ole Bendik Dale (Veterinary Institute, Norway), Ken Stepushyn (DFO, Canada). Facilitator - Kevin Amos*
10:15–10:30 AM	**Break**
Session 2	***Roundtable Discussion:*** **Where do we go next?** *Each speaker will have no more than 8 minutes to present their opinion and then the session will be opened up to dialogue between the audience and panel*
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11:45–12:00 PM	**Closing Remarks**

Presentation Abstracts

From Sequence to Sickness: Using genomics and biotechnology to understand aquatic animal pathogens.

Laura L. Brown

National Research Council of Canada, Institute for Marine Biosciences
Halifax, Nova Scotia, B3H 3Z1

Whole genome sequencing is an important first step in understanding aquatic animal pathogens and interactions with their hosts. We are studying the host-pathogen interactions between Atlantic salmon (*Salmo salar*) and *Aeromonas salmonicida*, the causative agent of furunculosis. We have sequenced the genome of the bacterium and information gained has enabled us to investigate virulence factors and other gene products that may have application as vaccines or immunomodulatory candidates. Using knockout mutants of *A. salmonicida*, we are identifying key virulence factors by *in vitro* and *in vivo* assays. Proteomics studies of bacterial cells grown in a variety of media as well as in an *in vivo* implant system have revealed differential protein production and have shed new light on bacterial proteins such as superoxide dismutase, pili and flagellar proteins, type three secretion systems, and their roles in *A. salmonicida* pathogenicity. We have constructed a DNA microarray of *A. salmonicida* genes and the microarray was used in comparative genomic hybridizations (M-CGH) whereby genomic DNA from selected *A. salmonicida salmonicida* isolates, as well as other *Aeromonas* species and subspecies was compared with that of the sequenced strain. Results showed that variation among the virulence associated genes increased across sub-species and species boundaries. We are also investigating the host. Salmon were challenged with *A. salmonicida* and samples of the bacterium and the host tissues were taken at selected times post-infection. From the host, EST libraries were made from mRNA and suppressive subtractive hybridization revealed differential gene expression. These data were used to select genes for an Atlantic salmon cDNA microarray that we use to investigate the functional genomics of the host response to *A. salmonicida*. Genes that are up- and down-regulated during the infection process have been identified. By linking genome sequencing, functional genomics, proteomics, carbohydrate analysis, metabonomics/metabolomics, and immunological assays, we are taking an integrated and innovative approach to pathogenesis research.

Renibacterium salmoninarum: Genome Sequencing, Finishing Strategies and Assembly validation

Rajinder Kaul

Department of Medicine and University of Washington Genome Center, Seattle, WA 98195

Renibacterium salmoninarum is the causative agent for bacterial kidney disease in salmonid species, particularly in the pacific region. The infection is one of the major hurdles faced for culturing the salmon species, and significant resources have been spent in curtailing the infection and the infectious agent. Availability of genomics technologies has made it possible to fully characterize microbial genomes at the sequence level. Such understanding is critical for developing more rational preventive and therapeutic strategies in controlling the bacterial kidney disease and thus reduce the economic losses due to the infectious agent.

The *Renibacterium salmoninarum* genome sequence assembly was initially derived from end sequencing randomly picked small insert plasmid clones created from sheared genomic fragments of about 3 Kb in size. The initial shotgun sequencing provided about 9 X sequence coverage, and accounted for better than 99% of the genome. Following shotgun sequencing, genome was assembled using PHRED/PHRAP/CONSED software tools. The initial assembly had 271 contigs with 197 contigs of >2 Kb in size. AUTOFINISH utility in CONSED allowed improving the sequence quality and closed some of the gaps without any human interventions. Following four rounds of AUTOFINISH runs, an expert human finisher utilized a repertoire of experimental tools to finish the genome assembly. The final genome of Renibacterium salmoninarum strain 33209 is a single circular chromosome of 3,155,260 bps, with 52,686 reads in the final genome assembly. The genome is 56.27% GC rich, and contains 70 IS994 and 10 novel ISRsa2 insertional sequence elements. Gross genome validation was provided by comparing the virtual fingerprint pattern with that of the large fragments derived from pulse field gel electrophoresis of the rare cutting restriction enzymes. The final genome assembly was validated using end sequences and fingerprint data from independently derived fosmid library and comparing it to the virtual fingerprint pattern derived from the assembled sequence using the SEQTILE software tool. Genome annotation and characterization of the assembly will facilitate understand the *Renibacterium* biology, and devise preventive and therapeutic modalities against bacterial kidney disease in salmonid species.

Genome annotation – the ERGO bioinformatics platform

Anamitra Bhattacharyya

Integrated Genomics, Chicago, IL

The ERGO™ Bioinformatics Suite provides a multi-dimensional environment supporting both automatic and manual genome-wide curation. ERGO™ integrates genomic information with biochemical data, literature, protein sequence similarity, gene context clustering, occurrence profiles, regulatory and expression data, as well as functional hierarchies in order to achieve the best possible functional predictions. The functional ORF assignments are used to create a network of metabolic and non-metabolic pathways and subsystems. Using the ERGO™ system, a major part of the metabolism of an organism can be reconstructed almost entirely *in silico*. ERGO™ currently contains 908 genomes from all domains of life, either as complete or draft versions. Case studies of the utility of ERGO in microbial genome analysis will be presented including some initial analysis of the bacterial fish pathogen, *Renibacterium salmoninarum*.

First Findings: Overview of the *Renibacterium salmoninarum* Genome and Examination of Antibiotic Resistance Genes and Cell Surface Proteins

Mark S. Strom, Sudheesh Ponnerassery, Samuel Crane, and Donald Chen

NOAA Fisheries, Northwest Fisheries Science Center, Seattle WA 98112

Bacterial Kidney Disease (BKD) remains a high priority for hatcheries, aquaculture, and conservation programs designed to maintain salmon stocks listed under the Endangered Species Act. Treatment of BKD remains problematic, and currently available antibiotics and vaccines are not completely efficacious. While great strides have been made in recent years in many laboratories in the study of the molecular mechanisms of pathogenesis of *R. salmoninarum*, this bacterium remains difficult to work with due to its slow growth and the lack of easily applicable genetic and molecular tools. The *Renibacterium salmoninarum* Genome Sequencing Project was initiated after successfully receiving funding from the Microbial Genome Sequencing Program jointly administered by the National Science Foundation and the USDA Cooperative State Research, Education, and Extension Service. The project has been accomplished through a unique partnership between federal (NOAA Fisheries Northwest Fisheries Science Center and USDA/ARS National Center for Cool and Cold Water Aquaculture), academic (Oregon State University and the University of Washington Genome Center), and private (Integrated Genomics, Chicago) laboratories. The goal of this project is to completely sequence and annotate the *R. salmoninarum* genome, and to make it available to all interested scientists in the hope that research on understanding the pathogen and mitigating its effects will be stimulated. Initially, *R. salmoninarum* was the first member of the Micrococcaceae to be sequenced. However, the genome of a member of the *Arthrobacter* genus (sp. FB24) has recently become available in draft form, which will aid future comparative genomics studies.

The genome of *R. salmoninarum* is a single, circular chromosome of 3,155,294 base pairs with 56.27% G+C content that encodes 3,667 open reading frames (ORFs). Analysis carried out through the Integrated Genomics ERGO bioinformatics platform resulted in assignments of function to 2,333 of these ORFs (63.62%). Manual annotation initially carried out on the draft sequence first made available in August 2004 and more recently on the completed genome, have increased the number of ORFs with a functional designation to ~88%. In our laboratory we have focused on an examination of genes encoding potential antibiotic resistance factors since these have implications for current BKD treatment regimens. The *R. salmoninarum* genome contains several macrolide-resistance factors including transporters or efflux proteins, macrolide glycosyltransferases, and ribosomal RNA methyltranferases. Using reverse-transcription (RT)-PCR, we have shown that transcription of a 23S rRNA m(1)G 748 methyltransferase (*rlmA*II) and a macrolide efflux pump (*mef*) are induced during growth of *R. salmoninarum* 33209 in sub-clinical concentrations of erythromycin. In addition, transcription of a gene encoding a dimethyladenosine transferase is likewise induced in a clinical isolate of *R. salmoninarum* during growth in sub-clinical concentrations of azithromycin. This particular strain was originally isolated from a Chinook salmon previously treated with azithromycin. While preliminary, these data suggest possible reasons for the inability of macrolides to completely eliminate the bacterium in infected fish.

We are also examining factors involved in protein secretion or surface protein expression, since the future selection of potential vaccine candidates or therapeutics will involve proteins accessible to the fish immune system. We have therefore begun to focus our work towards characterization of a sortase enzyme and a prepilin peptidase, and this will also be discussed.

Novel vaccine targets and assessment of cellular immunity.

Gregory D. Wiens

USDA-ARS National Center for Cool and Cold Water Aquaculture, Kearneysville, WV 25430

Bacterial kidney disease vaccine research has been limited by lack of information about the salmonid host response and the etiological agent, *Renibacterium salmoninarum*. The recent completion of the *R. salmoninarum* ATCC 33209 genome sequence has allowed genome-scale comparisons to other pathogenic, and non-pathogenic, high G+C bacteria and the identification of novel vaccine targets. A total of 3667 open reading frames were identified by Integrated Genomics ORF-Calling software. Using the program PSORTb v2.0, ORFs were classified as putative cytoplasmic (n=1626), cytoplasmic membrane (n=625), cell wall (n=16), extracellular (n=132), or unknown localization (n=1268). These results were within the range of other Gram-positive proteome profiles. We have combined these data with additional motif searches and immunogenicity profiles to develop a list of potential vaccine candidates for further examination. Additionally, we have begun to analyze trout immune gene expression in response to *R. salmoninarum* ATCC 33209 infection. Using semi-quantitative and real-time PCR, mRNA expression patterns of known inflammatory cytokines and novel putative-immune genes have been determined. Robust INF-γ, TNF-α, IL1-β1, IL1-β2 and CXCd gene expression was induced in the spleen and/or anterior kidney of rainbow trout in response to sub-lethal infection. These immune gene assays in combination with detailed *R. salmoninarum* proteome and polysaccharide analysis will provide new resources for understanding salmonid-*R. salmoninarum* interactions.

Training Undergraduates in Genome Analysis and Bioinformatics

Daniel D. Rockey

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Oregon State University, Corvallis, OR 97331-4804

The science of bioinformatics is becoming an integral aspect of every field of biological research. One significant issue associated with the growth of this field is the lack of junior level researcher scientists with bioinformatics experience. We used the *Renibacterium salmoninarum* Genome Sequencing Project as a focal point for training undergraduate students in genomics studies. Our interactions with students was manifested in two different arenas. First, we formed teams of undergraduates to conduct preliminary annotation of open reading frames within the *R. salmoninarum* genome that were not previously annotated through the Ergo annotation system. A team of four Oregon State University students was assigned this project, and completed a preliminary annotation of approximately 1600 orfs during winter and spring terms of 2005. These efforts were mirrored by students enrolled at the University of Washington. Gene assignments by each group were compared and resolved. These students are expanding these studies to involve solving the metabolic pathway map resulting from the completely annotated genome. We determined that such teams can be immediately useful for generation of primary research data relevant to the genome project, particularly if the scope of the work is relatively limited and repetitive. The second arm of this training was the development, at Oregon State University, of a course in Microbial Genomics. This course addressed a broad range of subjects and included online computer laboratories supervised by graduate students working in bioinformatics. It was our experience that teaching the quantitative aspects of this subject was a major challenge and it is recommended that a textbook be included that has clear presentations of quantitative bioinformatics. Another significant challenge in delivering a course in this area is the rapid expansion of both the technical tools and theoretical perspectives in this subject. Thus, an individual teaching a genomics course needs to actively modify the material each year or it is likely the field will proceed beyond the information presented. Collectively, our experiences demonstrated that undergraduate students represent an abundant, inexpensive, and computer-savvy group of individuals who can be successfully trained and quickly be useful in bioinformatics research.

Identification of the sortase enzyme and its substrates in Renibacterium salmoninarum: Solving problems with bioinformatics.

Samuel Crane and Mark S. Strom

Northwest Fisheries Science Center, NOAA Fisheries, 2725 Montlake Blvd. E., Seattle, WA 98112

Sortase enzymes covalently attach virulence-associated proteins to the cell wall peptidoglycan of Gram-positive bacteria. Sortase proteins are therefore of interest in infection models and as potential therapy targets. We have performed various *in silico* analyses of the recently sequenced *Renibacterium salmoninarum* genome in search of sortase homologs and the cell wall proteins anchored by the enzyme. A single, intact sortase enzyme has been characterized; it appears to recognize an LAxTG cleavage motif. Ten putative sortase substrates have been identified, although their status as functional genes is in doubt and awaits further examination. This work lays the foundation for investigations into the effects of these sortase substrates in salmonid infections of this pathogen and the targeting of the sortase enzyme for therapeutics.

Renibacterium salmoninarum: an uncommon "bug," all too common in salmonids

Trevor P.T. Evelyn

Scientist Emeritus, Department Fisheries & Oceans, Canada

Renibacterium salmoninarum (Rs), the causative agent of bacterial kidney disease (BKD) in salmonids, is wide-spread and difficult-to-control. Control of Rs using anti-microbial agents yields only temporary relief, and treatments usually have to be repeated. Also, control by vaccination has thus far yielded disappointing protection, particularly in Pacific salmon. Rs is considered an obligate pathogen as survival outside of the host is limited. It is fastidious in its growth requirements and slow growing. In addition, the disease it causes is chronic rather than acute. These factors have impeded studies on the organism. Nonetheless, considerable progress has been made in understanding why Rs is such a successful pathogen. This paper briefly reviews the what is known about this very unique intracellular pathogen: its distribution and the disease it causes; the methods for its growth, identification and detection; the means by which it spreads and persists in salmonid populations; and the strategies it employs in evading the hosts' defences. In addition, methods for reducing the impact of the disease in salmonid hatcheries and sea farms are discussed.

Challenges Associated with Assessing BKD Status and Interactions among Wild and Hatchery Stocks in the Pacific Northwest: Initial Results of Field Studies

Sonia L. Mumford and Chris Patterson

Olympia Fish Health Center, US Fish and Wildlife Service, 3859 Martin Way E., Suite 101, Olympia, WA 98506

The question, "What disease interactions (in this case, specifically Bacterial Kidney Disease) occur between wild and hatchery fish?" is a hot topic in wild fish conservation. Challenges associated with answering this question include the misuse of the terminology pertinent to the discussion, inherent sampling biases, and the range of utility and limitations of the assays currently used to detect *R. salmoninarum*. Despite these challenges, field studies continue to be conducted and the results of published and new preliminary data from NOAA and USFWS will be presented.

The terms "disease" and "infection" have been used interchangeably. Particularly with *R. salmoninarum*, the two terms are not synonymous and could have very different implications. In addition, a positive signal for *R. salmoninarum* antigen by from Enzyme Linked Immunosorbent Assay (ELISA) is not equivalent to infection. Pascho (1997) showed antigen persisted in rainbow trout for 3 months after killed *R. salmoninarum* was injected intracoelomically. Though this experiment is unlikely to occur in nature, it appears possible that a fish could become infected, clear the infection, and still have a positive signal by ELISA for months. Several papers in the literature present "prevalence" data of antigen signal by ELISA in wild and/or hatchery populations. Although some authors accurately use the terms "antigen prevalence," or explicitly state ELISA values are not equivalent to disease, it is important for the reader not to translate the results of these or other studies as "disease" prevalence. ELISA is a valuable tool in assessing antigen load in hatchery and wild populations, but an in-depth understanding of what the assay is actually testing and what its limitations are is crucial to making sound conclusions from the results. Other detection methods will also be discussed.

In addition to limitations of assays, inherent sampling biases hinder our ability to gather prevalence data in wild populations. Few, if any, fish collection techniques yield a random sample. Electrofishing, seining, fish traps, and "hook and line" have inherent biases, which could have a greater impact on our results than any other factor. Unfortunately, we have no reliable way of estimating the magnitude of this bias. In addition, water temperature, season, and other environmental factors may influence results when making comparisons if the fish are not commingled at the time of collection.

Findings from our studies are consistent with previously published reports. Hatchery and wild fish were sampled by ELISA or Quantitative Fluorescent Antibody Testing (QFAT) within a specific geographical area. Of the areas sampled thus far, there does not appear to be a significant difference (p values ranging from 0.2- 0.7) between the number of fish testing positive from a sample of wild versus hatchery fish when testing by ELISA or QFAT at a given location at the time of fish collection.

This presentation will discuss the challenges in assessing BKD status in wild and hatchery populations. Perhaps the more precise questions are: "How can we measure the degree to which pathogens are being transmitted between wild and hatchery stocks?" and "What is the biological significance of these measurements?"

BKD in the Laurentian Great Lakes Basin: Historical Perspectives and Current Challenges

Mohamed Faisal[1] , Susan V. Marcquenski[2], and John Hnath [1]

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Since the first discovery of Bacterial kidney disease (BKD) in the Spey and Dee rivers of Scotland in 1933, the disease has spread through Europe, North and South America, and Japan. In 1952, BKD emerged in the Laurentian Great Lakes Basin and within five decades, the disease became enzootic affecting native and introduced,, propagated and wild fish of the genera *Onchorhynchus*, *Salvelinus*, *Salmo*, and *Coregonus*. BKD is believed to contribute to natural mortalities of wild and feral fish stocks in the basin. In the late 1980s, BKD is believed to have contributed in the widespread mortalities of chinook salmon in Lake Michigan that was associated with dramatic declines in salmon fisheries.

The Great Lakes Fishery Commission, through its Fish Health Committee, developed policies and protocols to minimize losses of diseases including BKD. The combination of infected broodstock culling, reduced stocking levels, and improved rearing practices in hatcheries has had an apparent positive effect in reducing BKD-mortalities in hatchery production of both coho and Chinook salmon, and may have contributed to increased survival of stocked fish in the Great Lakes.

Despite considerable research on BKD, we continue to lack the basic understanding of how its causative agent, *Renibacterium salmoninarum,* spreads throughout the Great Lakes basin. We also lack knowledge about reservoirs of *R. salmoninarum* within the basin, and the biotic and abiotic factors related to *R. salmoninarum* survival and BKD prevalence. Filling these gaps of knowledge is essential for the design and development of strategies for the effective control and prevention of BKD. Recent surveys demonstrated that a number of non-salmonids, wild fish species harbor *Renibacterium salmoninarum* and may contribute to its spread basin wide.

Given the complexity of Great Lakes systems, current research utilizes a reductionist approach to disentangle ecosystem-level processes of importance to disease prevalence and persistence. In particular, host-pathogen-environment interactions involved in BKD are being studied through a large-scale analysis of altered gene expression profiles. Host genes associated with disease resistance are being sequenced, and their degree of expression, in existing natural stocks and hatchery strains determined. The ultimate goal is to develop a protocol for broodstock selection utilizing among others, genetic markers of resistance to BKD.

The BKD situation in Northern Europe

OB Dale

Fish Health Section, National Veterinary Institute, Oslo Norway

A brief history of BKD in the North Sea and Baltic Sea regions is given, starting with the first report from 1930 of feral Atlantic salmon in Scottish rivers to the BKD problems during the 1980-90'is in both feral and farmed salmonids in the whole region. The disparate development in the various countries is discussed with respect to possible determinants, such as the structure of the farming industry, geography, fish species and the relationship between farmed and feral fish. The main trends seems to be a very low prevalence of BKD in feral fish, but at the same time severe disease problems have occurred among wild broodfish kept under poor condition and in hatcheries involved in restocking. In farming a clear trend is increased disease problems, both in Atlantic salmon and rainbow trout, when volume is growing rapidly. The immediate cause of such epidemics then appears to be one or several farms distributing live material. The origin of the infection is less clear and the role of wild fish is debated. The first cases seen in Norway appear closely linked to the use of roe from infected, feral Atlantic salmon in both restocking operations and commercial farms. On the other hand extensive screening of feral fish in Sweden and Finland is largely negative, and thus the feral fish is not considered to be the source of infection to the rainbow trout farming industry.

The development once BKD is introduced into the farming industry appears to be dependent on the overall health management to stop horizontal transmission, and a careful selection of brood fish to stop vertical transmission. Two approaches to selection are in use. When feral fish is used for sea ranching in Iceland, an individual testing and selection is carried out, apparently with good success. In Norwegian Atlantic salmon farming a selection is done on population level after a close disease monitoring during the whole production cycle of the brood fish population. Also large sister groups are looked at during slaughter. In the maturation period kidneys from all diseased fish are tested for BKD. If no signs of BKD are found in the chosen brood stock, no individual test is performed on the stripped fish, but all fish are autopsied. So far two valuable brood stocks have been discarded due to BKD. The development of BKD problems indicates that this approach is efficient. The sad BKD development in Atlantic salmon farming in the Faroe Islands seems to confirm such a view.

Detection of *Renibacterium salmoninarum* in kidney samples

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In Iceland, BKD was diagnosed for the first time in farmed Atlantic salmon in 1968. The causative agent, *Renibacterium salmoninarum*, has since been detected sporadically in farmed, ranched and wild populations of salmonids. A major outbreak, involving sea ranches started in 1985. Brood stock culling was applied successfully, as was establish by subsequent testing of the progeny and the homing ranched fish. In 1986, an official regulation was issued, demanding a screening for *R. salmoninarum* in all brood fish of wild and ranched origin as well as a minimum of 60 farmed brood fish from each farm. This was followed by culling of eggs from infected parents. Since 1993, only female brood fish have been screened.

Recently, *R. salmoninarum* has been detected in Atlantic salmon and arctic char fingerlings on a few farms. As there is always some trade of eggs and/or fish between farms, it is highly important to be able to detect the bacterium at an early stage of infection, since there may be months between the first positive tests until the first clinical signs of disease emerge.

Since 1992, the main screening method utilized in Iceland has been a sandwich ELISA using polyclonal antibodies to detect *R. salmoninarum* antigens in kidney samples. When developed, this method was shown to be more sensitive than bacterial cultivation on selective medium, SKDM. Additionally, direct immunofluoresence (DFAT) has been applied and recently samples positive in an ELISA test have been analyzed in a PCR test. Results from recent ELISA tests using polyclonal antibodies, displaying different levels of sensitivity, will be presented and compared to DFAT and PCR.

A retrospective look at *Renibacterium salmoninarum*: research from the 20th century on relatives, pathogenicity and antibiotics

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Renibacterium salmoninarum was established as a unique genus and species by sequencing and comparison of its 16S rRNA to those of seventeen genera. A nearly complete sequence (97%) of 1475 nucleotides allowed phylogenetic comparison to 17 genera, including its apparent closest relative, *Arthrobacter globiformis*. This soil bacterium has characteristics that might prove beneficial in understanding the mechanisms of pathogenicity of *R. salmoninarum*.

Examination of its virulence factors, following infection of cells derived from rainbow trout anterior kidney, revealed that *R. salmoninarum* was rapidly phagocytosed by mononuclear phagocytes (MP). As revealed by electron microscopy and culture, the long-term intracellular persistence of *R. salmoninarum* was facilitated by its durable cell wall and ability to escape from the phagosome into the cytoplasm where it could avoid destruction by the MP.

Research on the intracellular aspects of *R. salmoninarum* provided some other highlights. Over 100 experimental antimicrobial compounds were tested on four strains of the bacterium, showing that it was susceptible to 51 of the compounds. Nineteen of these effectively inhibited *in vitro* growth at 1 mg/L or less. In addition, another antibiotic study brought forth the discovery of a bacteriophage that could lyse and kill *R. salmoninarum*, a probable reason for the periodic observations of its demise in broth cultures.

Antigenic variation in *Renibacterium salmoninarum* p57: functional and diagnostic implications.

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The 57-kDa protein (p57) is an important diagnostic antigen and is also implicated in the pathogenesis of salmonid bacterial kidney disease. Little is known about the nature and extent of antigenic variation in p57. Previously, we reported that p57 produced by *R. salmoninarum* strain 684 contains a mutation that disrupts monoclonal antibody 4C11 binding. Sequence analysis of the 5' and coding regions of the 684 *msa*1 and *msa*2 genes identified a single C-to-A nucleotide mutation in both genes as compared to the ATCC 33209 strain. This mutation creates an Ala(139)-to-Glu substitution in the amino-terminal region of p57. Purified p57 from strain 684 displayed enhanced binding for Chinook salmon leukocytes suggesting that the mutation may confer a selective advantage. Here, we examine antigenic variation in a panel of 24 *R. salmoninarum* isolates obtained from diverse geographic locations world-wide. Six p57-specific monoclonal antibodies (4C11, 4D3, 3H1, 4H8, 4D10 and 1A1) were used to probe dot and western blots to determine the relative expression, size and cellular association of p57. Full length p57 was produced by all 24 isolates and for each isolate, the protein was associated with the bacterial cell surface. The epitopes recognized by four Mabs, 4D3, 4H8, 3H1 and 1A1, were conserved among all strains tested. The 4D10 epitope was disrupted in one isolate from British Columbia, while the 4C11 epitope was lost in 5 of 8 strains isolated from Norway. The 5 Norwegian isolates were genetically similar sharing the following traits: one tandem repeat in the ETRA locus, a sequovar-four 16-23S rRNA intervening sequence, an alter msa1 5' region, and they lack additional *msa*1-like genes. These results suggest that while p57 is not highly polymorphic, some antigenic variation exists that may be geographically restricted in distribution.

Genetic manipulation and genotyping of *Renibacterium salmoninarum*

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Molecular genetics is a relatively young discipline in *Renibacterium salmoninarum* research. Over the past five years, we have focused on two topics for molecular genetic studies: genetic transformation (gene introduction) and isolate genotyping. Transformation is a powerful tool for determining gene function and mechanisms of pathogenesis, while isolate genotyping is typically used for epidemiological studies. We have used insertion-duplication mutagenesis (IDM) to create mutant strains with disruptions in either *msa1* or *msa2*, chromosomal genes that encode a highly expressed, immunodominant surface protein called major soluble antigen (MSA; p57). These strains display severely reduced ability to cause mortality in Chinook salmon, and they are the first attenuated strains of *R. salmoninarum* generated through genetic transformation. In contrast, genotype screening of isolates from infected fish throughout the Pacific Northwest has found that the presence of a third locus of *msa* is associated with greater virulence. Taken together, these results strongly implicate MSA as a significant virulence factor. Therefore, genotyping isolates of *R. salmoninarum* can present hypotheses on phenotypic variation in addition to serving as a tool for epidemiological analyses.

Regional Prevalences of *Renibacterium salmoninarum* Among Juvenile Chinook Salmon in the Northeast Pacific Ocean

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The survival of juvenile salmon in the first year at sea is believed to be an important factor in the survival and return of adults; for this reason the debate over "ocean conditions" has been renewed. However, a number of other factors have been identified which may determine salmon survival rates, including the prevalence and severity of infection by parasites and pathogens (which may also be influenced by ocean conditions). One emphasis of our work over the last five years has been on *Renibacterium salmoninarum*, the cause of bacterial kidney disease (BKD) in salmonids. Although the infection has been well studied in fresh water, its effects during the marine phase are not well understood. In 2000, 53% (n=233) of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) caught off the Washington Coast and tested by the polymerase chain reaction (PCR) were positive. There were significant inter-annual fluctuations in prevalence, as 26.3% (n=171) and 28.2% (n=483) of juvenile Chinook were infected in this region in 2001 and 2002, respectively. However, *R. salmoninarum* is not equally distributed in the marine environment; among Chinook salmon caught off the southern Oregon and northern Californian coasts, the prevalence was 3.2% (n=186) in 2000 and 15.8% (n=101) in 2002. These fish appear to be of separate (but still susceptible) stock origin, and the lower level of *R. salmoninarum* infection remains unexplained.

Recently, we analyzed tissue samples from juvenile Chinook salmon captured off of British Columbia and in the Gulf of Alaska in an attempt to gain a broader regional perspective. In 2001, over 40% of the salmon from B.C. were positive for *R. salmoninarum*; in 1998-99, roughly 13% of the salmon captured in Alaskan waters were positive. Although comparisons between the regions are tenuous because of the different years of capture, these results are surprising. The prevalences reported here are for those fish surviving to reach the ocean; by this time, some level of mortality due to *R. salmoninarum* infection has already occurred. Given this information and the chronic nature of bacterial kidney disease, it is plausible that this pathogen may play a significant role in limiting salmon populations in the Northeast Pacific.

BKD Control Begins with Diagnosis: Comparison of *Renibacterium salmoninarum* Detection Methods

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Critical to the success of any control program for bacterial kidney disease (BKD) is the application of reliable diagnostic methods that can detect low levels of *Renibacterium salmoninarum* in a variety of sample types. Control of BKD by avoidance requires the use of specific and sensitive methods to identify pathogen-free fish for propagative purposes. Because of the chronic or subclinical nature of R. salmoninarum infections in many fish, evaluation of the success of any control measure requires, in addition to mortality comparisons, the use of diagnostic tests adequate to detect such infections. Diagnosticians and researchers have long been interested in developing methods for more rapid and reliable detection of *R. salmoninarum* infections. As each new method is developed, there is a tendency to reject older techniques. Nevertheless, no single ideal diagnostic technique has yet been developed. Several criteria have been used to evaluate diagnostic tests. Some of the more important criteria include: 1) specificity for *R. salmoninarum*, 2) sensitivity (ability to detect subclinical infections), 3) ability to quantify infection levels, 4) ability to distinguish live from dead bacteria, 5) ability to detect infections in tissues remote from those sampled, and 6) provision of time and cost savings for evaluation of multiple samples. These factors are considered for the principal diagnostic tests in use today, including bacteriological culture, the fluorescent antibody technique (FAT), the enzyme-linked immunosorbent assay (ELISA), and the polymerase chain reaction (PCR).

Integrated Management of BKD Using ELISA-based Culling

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The Idaho Department of Fish and Game (IDFG) Chinook salmon program has a history of managing Renibacterium salmoninarum, the causative agent of bacterial kidney disease (BKD), dating back to 1969 at Rapid River Hatchery and 1980 for McCall Hatchery. The chronological events described in annual and brood year reports from these facilities document the progression of events that led IDFG into developing an integrated BKD management program for hatchery reared Chinook salmon. The cornerstone of this management strategy was use of the enzyme linked immuno-sorbent assay (ELISA) based culling of eggs from females with high optical density values for Renibacterium. The success of this program has been documented by mortality records, routine inspection, and diagnostic sampling during the 18 month period of hatchery rearing.

Early attempts to control this disease were limited to injection of erythromycin for returning adult salmon and erythromycin medicated feed treatments after clinical signs had been observed. Fertilized eggs were water hardened in 1 mg/l erythromycin. Prophylactic feeding of erythromycin medicated feed began in 1977 in the juvenile salmon at Rapid River Hatchery. The treatments became a standard operating procedure for rearing Chinook salmon at Idaho Department of Fish and Game facilities in 1984.

An integrated program of treating hatchery reared salmon for BKD at each life stage was implemented in 1993. This program consists of the following: (1) 20 mg/kg intra-peritoneal injection of erythromycin to returning adult salmon; (2) iodophor disinfection of fertilized eggs in 100 mg/l iodine for 30 minutes during water hardening; (3) testing of kidney tissue from all spawned females by ELISA. To limit risk of vertical transmission and subsequent horizontal transmission, the eggs from brood females with ELISA optical densities of 0.25 or greater were usually culled. In years with low adult numbers, a high BKD segregation group consisting of progeny of females with ELISA optical densities of 0.25 to 0.6 has been reared in isolation. This has been considered the high BKD segregation group since 1998; and (4) usually each brood year receives two prophylactic regimens of erythromycin feed. The high BKD segregation group received an additional erythromycin medicated feed application.

Pre-spawning mortality of Chinook salmon adults due to BKD averaged 37.3% before the implementation of the strategy described above. Average pre-spawning mortality has decreased to an average of 5% per year, since the implementation of this integrated strategy. The IDFG Chinook salmon hatchery program has not experienced an epizootic due to BKD since 1993. Although clinical signs of BKD have been noticed after stressful events, minor outbreaks have been controlled with medicated feed. Cumulative mortality from ponding to release, from all causes, has decreased from an average of 4.2% to 0.6% per year at Rapid River, and an average of 6.8% to 1.7% per year at McCall, since implementation of this strategy. This strategy has been instrumental in reducing the risk of vertical and horizontal transmission of BKD in the hatchery and subsequently to wild/natural stocks after release. We recommend implementation of this BKD management strategy if the salmon hatchery program is considered risk intolerant.

Controlling BKD in the 'real world'; what has been done and where should it go

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Renibacterium salmoninarum infections in salmonid aquaculture have been difficult to prevent or control. Research to limit the impact of bacterial kidney disease (BKD) has separately focused on hatchery practices (environment), antimicrobials (pathogen) and vaccine development (host response). A variety of hatchery practices are used by the Washington Department of Fish and Wildlife (WDFW) with variable success rates at controlling BKD. Fish husbandry should be based on those methods which reduce fish stress. When hatchery practices fail to prevent an outbreak, several antibiotics have been partially successful at killing the bacterium. However, bacterial drug resistance, environmental protection, and legal issues are a concern in the use of antimicrobials. Research efforts to develop *R. salmoninarum* vaccines have shown modest progress. Researchers at the Western Fisheries Research Center (WFRC) and Northwest Fisheries Science Center (NWFSC) have tested the efficacy of several whole cell vaccines, including the commercial vaccine Renogen, with differential success. NWFSC researchers have evaluated a combinatorial whole cell vaccine which may have prophylactic and therapeutic value for controlling BKD in Chinook salmon. In the future, research into the control of BKD needs to investigate therapies which affect multiple areas of the host pathogen relationship. Appropriately timed antibiotic treatment and vaccination in a low-stress hatchery environment may provide our best chances to limit the damage of BKD.

Genetic Variation in Disease Resistance of Chinook Salmon (*Oncorhynchus tshawytscha*) Exposed to Two Bacterial Pathogens

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Bacterial kidney disease (BKD), caused by the bacterium *Renibacterium salmoninarum*, is a chronic disease of salmonids that can be transmitted both horizontally between cohabiting individuals and vertically from mothers to their progeny via infected oocytes. The disease is considered more severe in chinook salmon than in many other salmonids and is particularly widespread among chinook salmon hatcheries in the Pacific Northwest. BKD is difficult to control because effective vaccines have not yet been developed; antibiotic therapy is only partially effective. Oral chemotherapy of salmonids with erythromycin can reduce mortality but does not eliminate infections from all treated fish. Moreover, juvenile anadromous salmonids infected with *R. salmoninarum*, while in fresh water, may subsequently die of BKD upon entry into seawater as smolts. Many hatchery programs cull from their broodstocks progeny of adults that exhibit high antigen titers for *R. salmoninarum* to minimize risk of BKD outbreaks in their facilities. In this study we employ a quantitative genetic breeding design to characterize genetic and phenotypic variation in chinook salmon ability to survive exposure to two bacterial pathogens: *Renibacterium salmoninarum* and *Listonella* (formerly *Vibrio*) *anguillarum*, a causative agent of vibriosis. After measuring levels of *R. salmoninarum* antigen in 415 male and 84 female adult chinook salmon returning to the Carson National Fish Hatchery, we mated each of 24 males (12 with high and 12 with low bacterial antigen loads) to two females with low to moderate bacterial antigen loads. We then exposed samples of their juvenile progeny to both pathogens in independent experimental trials. Patterns of family variation in mortality, time to death, and bacterial loads of survivors indicate the potential for rapid evolution of the host population to BKD but not to vibriosis. A negative genetic correlation between resistances (as measured by survival) to the two pathogens indicates that broodstock selection based on levels of *R. salmoninarum* in adults could alter susceptibilities to both diseases. Genetic correlations between survival and days to death in the BKD and vibriosis trials indicate that mortalities in families experiencing higher survival died sooner than those in families experiencing lower survival for both pathogens. Families in which fish died more quickly in the BKD trial tended to include fish that lived longer in the vibriosis trial. The phenotypic and genetic relationships between survival rate in the BKD trial and bacterial antigen loads in trial survivors were weak, providing no evidence that variation in antigen load is linked to resistance. The study results underscore the complexity of resistance of salmonids to bacterial pathogens and raise some doubt over the efficacy and long-term consequences of culling hatchery broodstock based on bacterial antigen loads.

A U.S. perspective on regulatory considerations for *Renibacterium salmoninarum* for intra and international commerce.

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The causative agent of bacterial kidney disease (BKD), *Renibacterium salmoninarum* (Rs), is enzootic to North America and occurs in many, but not all, cultured and wild populations of salmonids. Beyond North America, this pathogen is widely distributed in Europe, South America, and Asia. Due to the fact that this pathogen may be spread by either horizontal (fish-to-fish) or vertical (from infected parent to offspring via the egg) routes, careful consideration must be given when determining the risk of spreading Rs via commerce of live fish or eggs.

In 1991, the U.S. Fish and Wildlife Service conducted a workshop to evaluate the status and risk of Rs to state natural resources. While most States recognized the presence of this pathogen within their borders, few had conducted a valid risk assessment to determine if and how the import of infected live product might impact existing fish populations -cultured and wild, infected and Rs-free. With few exceptions, most State officials declared they would not allow the import of any Rs-infected salmon or trout into their State, regardless of the species and/or destination. Little has changed since 1991. Recognizing that it is under the purview of each State and Tribe to manage aquatic animal health issues within their legal jurisdiction, it is also important that their chosen "appropriate level of protection (ALOP)" is valid and functions under a science-based disease management and control program.

The competent Federal authorities of the United States, which include the U.S. Departments of Agriculture, Commerce, and Interior, are drafting in cooperation with our many public, private, and tribal stakeholders, a comprehensive and consistent national aquatic animal health plan which will make recommendations on how to address "program" and "non-program" diseases. While it appears at this time that BKD does not meet the suggested criteria for a program disease, it still is of great local and regional concern. A desirable output of the national plan could be a consistent approach on how States regulate Rs with the understanding that regulations above and beyond a "base level" would be established on a case-by-case basis and when a State or Tribe have their own disease control program that justify their regulations.

Poster Abstracts

Modeling BKD-related mortality: The effect of antigen and temperature related suppression of cell mediated immunity, and other factors.

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Modeling of disease dynamics often takes the form of epidemiological modeling, with a focus on transmission dynamics. However, for chronic diseases such as BKD for which the outcome for each infected individual depends upon the internal dynamics of the pathogen and the influence of environmental co-factors, modeling the course of the infection is perhaps more important. This is especially true for a host species with as complex a life history as Pacific salmon. Appropriate and adequate data for such modeling may be hard to come by, especially if the desire is to model the disease in the natural setting. Controlled experiments can provide good data for modeling, but may leave one with only qualitative conclusions about the natural world or even qualitative predictions about a repeated experiment if not all factors can be controlled.

A 1988 hatchery brood stock segregation experiment, coupled with other research results, provides a basis for modeling which can provide qualitative conclusions about factors affecting the progression and outcome of infection with *Renibacterium salmoninarum* in juvenile spring chinook salmon. Results of this experiment have been interpreted to show that segregation reduces both in-hatchery and post-release BKD-related mortality rates. A re-analysis of these data indicates that other hypotheses, supported by published studies, can explain the observed data without concluding that segregation necessarily improves overall survivorship. The major soluble antigen of *Renibacterium salmoninarum*, p57, is an important factor in the success and virulence of the pathogen. The presence of this antigen in sufficient quantities in the egg before fertilization has been shown to result in reduced immune system functioning and increased mortality in infected offspring. Low temperatures may impair the functioning of the cellular immune system. Immunosuppression due to these two factors can explain the divergent pattern of infection levels and mortality and return rates observed for the six raceways in the segregation experiment. These results are compared with models of temporal dynamics of bacterial levels and related mortality rates in more controlled environments.

Potential for Transmission of *Renibacterium salmoninarum* during Coded Wire Tagging: A Comparison of Conventional and Automated Marking Methods

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Previous research has shown that *Renibacterium salmoninarum (Rs)* and other bacteria can become concentrated in the recirculating water of fish marking facilities. In addition, *Rs* infections can become established in sites of coded-wire-tag (CWT) implantation. This study compared the potential for Rs transmission and the establishment of infections in groups of juvenile Chinook salmon *Oncorhynchus tshawytscha* marked with adipose fin clips and CWT in a conventional marking trailer and in a new automated trailer. The conventional trailer uses recirculating water to deliver anesthetic, and fish are handled by humans for fin clipping and placement in CWT machines. The automated trailer uses specially designed trays that encourage fish to enter marking lines volitionally, a gating system, and gentle mechanical holding, to accomplish fin clipping and tagging of fish without anesthetic in single-pass water, and with minimal handling by humans. During marking of hatchery Chinook salmon, water samples were collected from the holding tanks and tagging stations of the conventional and automated marking trailers, and evaluated by culture for the total number of bacteria, and by the membrane filtration-fluorescent antibody technique (MF-FAT) for the number of *Rs* cells present. Results showed the highest counts of total bacteria and *Rs* in the recirculating water of the conventional marking trailer. However, higher concentrations of *Rs* were measured in both tagging systems compared to the holding raceways. Both tagging systems showed increased Rs concentrations in the water at the clipping and tagging stations. In addition to water samples, we tested two groups of juvenile Chinook salmon that had been separated before hatch on the basis of *Rs* testing of female parents by the enzyme-linked immunosorbent assay (ELISA). We took kidney tissue samples at the time of tagging and 4 months after tagging to determine the prevalence and severity of *Rs* infection by ELISA testing. The progeny of high-*Rs* female parents showed an increased prevalence of *Rs* 4 months after marking in both tagging systems. Fish marked by the conventional method showed higher levels of *Rs* antigen ($P<0.05$), indicating a greater severity of infection, than those marked by the automated system. Fish from low-*Rs* female parents did not show an increase in prevalence or levels of *Rs* ($P>0.05$) after marking by either method.

Renibacterium salmoninarum Genome Sequencing Project: Summary of Findings

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Renibacterium salmoninarum is the causative agent of bacterial kidney disease (BKD) in salmonids. Treatment of BKD remains problematic, and currently available antibiotics and vaccines are not completely efficacious. The aim of the *Renibacterium salmoninarum* Genome Sequencing project is to completely sequence and annotate the genome of *Renibacterium salmoninarum* and to identify potential targets for novel therapeutics that will attenuate the role of this pathogen as a limiting factor in salmonid culturing. Here we summarize the process and findings of our manual *in silico* analysis of the draft genome of *R. salmoninarum*. A map of the circular genome is provided. Various issues are explored in depth, including transposases, sortase enzymes, and the lipid and amino acid biosynthesis pathways.

Both *msa* Genes in *Renibacterium salmoninarum* Are Needed for Full Virulence in Bacterial Kidney Disease

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Renibacterium salmoninarum, a gram-positive diplococcobacillus that causes bacterial kidney disease (BKD) in salmonids, produces abundant amounts of a 57-kDa protein that is associated with the bacterial surface and is released into surrounding tissues. This protein, called p57 or major soluble antigen (MSA), has been previously implicated as an important virulence factor in BKD. *R. salmoninarum* has two chromosomal loci encoding MSA, *msa1* and *msa2*. We generated clones of *R. salmoninarum* bearing disruptions of either *msa1* or *msa2* and characterized them *in vitro* and *in vivo*. Surprisingly, expression of MSA in broth cultures appeared unaffected in the single mutants. However, the virulence of either mutant in juvenile Chinook salmon (*Oncorhynchus tshawytscha*) by intraperitoneal challenge was severely attenuated. This finding provides functional evidence for MSA as a significant virulence factor.

Prevalence of *Renibacterium salmoninarum* Infection Among Juvenile Chinook Salmon in North Puget Sound and Implications for Disease Interactions

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Renibacterium salmoninarum causes bacterial kidney disease (BKD), a chronic and sometimes fatal condition of salmon and trout. This bacterium is transmitted both horizontally and vertically, and high prevalence in a population can lower overall fitness. The purpose of this study was to determine infection prevalences of *R. salmoninarum* among nearshore juvenile chinook salmon in North Puget Sound. The central questions were whether the prevalence rates would vary by fish origin (marked or hatchery vs. unmarked or feral), by season, or by geographical area. Juvenile chinook salmon were collected between April and October in 2002 and 2003 in 32 nearshore habitat sites by surface trawl (townet). Kidney tissue samples were collected and analyzed microscopically by counting bacterial cells stained with an anti-*R. salmoninarum* polyclonal antibody conjugated to fluorescein isothiocyanate. Differences in infection prevalence were observed by geographical area and by season. Although seasonal differences were observed among the distinct geographical areas, no overall seasonal trend was found throughout the North Puget Sound. There was no difference in infection prevalence between marked and unmarked fish in all regions in the North Puget Sound. Furthermore, the probability of infection was associated with capture location, rather than stock origin. Our findings suggest potential disease interactions, possibly through horizontal transmission, between the feral and hatchery fish.