

**OBSERVATIONS ON THE EPIZOOTIOLOGY OF BACTERIAL KIDNEY  
DISEASE IN FARMED SALMONIDS**

by

**Shannon K. Balfry**

**B.Sc., Simon Fraser University, 1984**

**THESIS SUBMITTED IN PARTIAL FULFILLMENT OF  
THE REQUIREMENTS FOR THE DEGREE OF  
MASTER OF SCIENCE**

in the Department

of

Biological Sciences

© **Shannon K. Balfry, 1990**

**SIMON FRASER UNIVERSITY**

December 1990

All rights reserved. This work may not be reproduced in whole or in part, by photocopy or other means, without permission of the author.

APPROVAL

Name: SHANNON KATHLEEN BALFRY

Degree: Master of Science

Title of Thesis:

OBSERVATIONS ON THE EPIZOOTIOLOGY OF BACTERIAL KIDNEY DISEASE IN FARMED  
SALMON

Examining Committee:

Chairman: Dr. A.P. Farrell, Professor

Dr. L.J. Albright, Professor, Senior Supervisor,  
Dept. of Biological Sciences, SFU

Dr. T.P.T. Evelyn, Adjunct Professor, Dept. of  
Biological Sciences, SFU

Dr. F.C.P. Law, Professor, Dept. of Biological  
Sciences, SFU, Public Examiner

Date Approved

13<sup>th</sup> December 1990

PARTIAL COPYRIGHT LICENSE

I hereby grant to Simon Fraser University the right to lend my thesis, project or extended essay (the title of which is shown below) to users of the Simon Fraser University Library, and to make partial or single copies only for such users or in response to a request from the library of any other university, or other educational institution, on its own behalf or for one of its users. I further agree that permission for multiple copying of this work for scholarly purposes may be granted by me or the Dean of Graduate Studies. It is understood that copying or publication of this work for financial gain shall not be allowed without my written permission.

Title of Thesis/Project/Extended Essay

Observations on the epizootiology of  
bacterial kidney disease in farmed salmon.  
\_\_\_\_\_  
\_\_\_\_\_

Author: \_\_\_\_\_

(signature)

Shannon Balfry

(name)

DEC. 17, 1990

(date)

## **ABSTRACT**

*Renibacterium salmoninarum* is the causative agent of Bacterial Kidney Disease (BKD), a leading cause of death among farmed salmonids in British Columbia. The pathogen has been shown to be vertically (i.e., from parent to progeny) and horizontally (i.e., from fish to fish) transmitted, although the relative importance of each process is unclear. The purpose of this research was to investigate the role of the fecal-oral route of horizontal transmission among farmed salmon held in sea water.

Populations of farmed salmon were examined during their growth in sea water for trends in the natural prevalence of *R. salmoninarum*. All of the salmon populations examined experienced a significant increase in the prevalence of *R. salmoninarum* over a 12 month period. Horizontal transmission appeared to be responsible for the observed increase in prevalence.

Viable *R. salmoninarum* cells were isolated from seawater samples taken from within a pen of chinook salmon experiencing a BKD epizootic. The feces from these chinook salmon appeared to be the source of *R. salmoninarum* in the sea water.

Experiments were designed to determine the survival of *R. salmoninarum* shed into sea water in the feces of

infected salmon. The survival of *R. salmoninarum* suspended in sea water (and four other test solutions) for 28 d at 10°C was investigated. *R. salmoninarum* cells were detected in the sea water by microscopy over the entire 28 d; however, viable cells were detected for only 7 d.

The fecal-oral route of horizontal transmission was demonstrated by orally intubating *R. salmoninarum*-laden feces into young coho salmon. The prevalence of *R. salmoninarum* in the fecal and kidney samples of the surviving coho (71 d post-challenge) was found to be significantly higher in the experimentally challenged coho than in those receiving *R. salmoninarum*-free feces.

*R. salmoninarum* has been demonstrated in the feces of infected fish, and once shed into sea water can remain viable long enough to be ingested by neighbouring fish. This research, therefore, indicates that the fecal-oral route of horizontal transmission may contribute significantly to the increase in BKD prevalence observed on salmon farms.

## ACKNOWLEDGEMENTS

I would like to thank my senior supervisor, Dr. L.J. Albright, and my co-supervisor, Dr. T.P.T. Evelyn, for their constructive criticisms and comments during this research project.

I am grateful for the tremendous help given to me by the staff of all the participating salmon farm Companies. The technical assistance of L. Prosperi-Porta, and J. Ketcheson of the Pacific Biological Station in Nanaimo, is also appreciated.

Thanks are also expressed to my many friends and colleagues at SFU who have helped me with their useful suggestions and advice. In particular, I would like to thank L. Brown and S. Blundell for their thoughtfulness and friendship.

I would also like to thank J. Tadey for helping me through the preparation of this thesis with his advice and understanding. I am grateful to my parents for their love and encouragement; without it this thesis would not have been possible.

This research was supported by Science Council of B.C. G.R.E.A.T. Awards, and Simon Fraser University Graduate Fellowships.

**DEDICATION**

To my brother, Alfie.

## TABLE OF CONTENTS

	page
TITLE PAGE .....	i
APPROVAL PAGE .....	ii
ABSTRACT .....	iii
ACKNOWLEDGEMENTS .....	v
DEDICATION .....	vi
TABLE OF CONTENTS .....	vii
LIST OF FIGURES .....	ix
LIST OF TABLES .....	x
INTRODUCTION .....	1
MATERIALS AND METHODS .....	7
I. FARM SURVEY .....	7
COMPANY A .....	8
COMPANY B .....	10
COMPANY C .....	10
NET PEN SAMPLING AND NECROPSY PROCEDURE .....	11
DETECTION OF <i>R. salmoninarum</i> BY THE IFAT .....	12
DETECTION OF <i>R. salmoninarum</i> BY CULTURE .....	14
II. WATER SAMPLING EXPERIMENT .....	15
SOURCE OF WATER .....	15
DETECTION OF <i>R. salmoninarum</i> IN WATER AND FISH ..	16
III. <i>R. salmoninarum</i> SURVIVAL EXPERIMENT .....	18
BACTERIAL ISOLATE .....	18
TEST SOLUTIONS .....	19
SAMPLING PROTOCOL .....	21
DIRECT MICROSCOPIC ENUMERATION .....	22
MEMBRANE FILTRATION IFAT ENUMERATION .....	24
ANALYSIS OF DATA .....	25
IV. FECAL CHALLENGE EXPERIMENT .....	26
SOURCE OF FISH TO BE CHALLENGED .....	26
PREPARATION OF INOCULUM .....	27
CHALLENGE PROCEDURE .....	28
SAMPLING AND DATA COLLECTION .....	29



<b>RESULTS</b> .....	32
I. FARM SURVEY .....	32
COMPANY A .....	32
COMPANY B .....	34
COMPANY C .....	34
II. WATER SAMPLING EXPERIMENT .....	36
III. <i>R. salmoninarum</i> SURVIVAL EXPERIMENT .....	39
IV. FECAL CHALLENGE EXPERIMENT .....	44
<b>DISCUSSION</b> .....	51
I. FECES AS A SOURCE OF <i>R. salmoninarum</i> .....	51
II. PRESENCE OF <i>R. salmoninarum</i> IN SEAWATER NET PENS .	54
III. SURVIVAL OF <i>R. salmoninarum</i> IN SEA WATER .....	56
IV. EXPRESSION OF BKD IN SALMON CHALLENGED WITH <i>R.</i> <i>salmoninarum</i> -LADEN FECES .....	60
V. PREVALENCE OF BKD IN FARMED SALMON .....	62
<b>SUMMARY AND CONCLUSIONS</b> .....	66
<b>APPENDICES</b> .....	67
A. COMPANY A SURVEY RESULTS .....	67
B. COMPANY B OLD & NEW SITE SURVEY RESULTS .....	68
C. COMPANY C OLD SITE SURVEY RESULTS .....	70
D. COMPANY C NEW SITE SURVEY RESULTS .....	71
<b>REFERENCES</b> .....	73

LIST OF FIGURES

	<b>page</b>
<b>FIGURE 1.</b> Chart showing the location of Companies A, B, and C's farm sites that participated in the BKD survey .....	9
<b>FIGURE 2.</b> Results from the BKD survey conducted at Companies A, B and C's farm sites .....	33
<b>FIGURE 3.</b> Survival of <i>R. salmoninarum</i> over a period of 28 days (at 10°C) in (A) natural seawater, and (B) filter sterile seawater.....	40
<b>FIGURE 4.</b> Survival of <i>R. salmoninarum</i> over a period of 28 days (at 10°C) in (A) peptone-saline, (B) saline, and (C) SKDM-2 broth .....	41
<b>FIGURE 5.</b> Levels of IFAT detected <i>R. salmoninarum</i> in the feces and kidney material of the experimental and control coho that survived the fecal challenge experiment .....	50

## LIST OF TABLES

	<b>page</b>
<b>TABLE 1.</b> Experimental protocol for the challenge of coho salmon with feces or <i>R. salmoninarum</i> -laden feces .....	28
<b>TABLE 2.</b> Summary of results from the water sampling experiment.....	37
<b>TABLE 3.</b> Number and average weight of coho mortalities collected from the (A) experimental, and (B) control pens.....	45
<b>TABLE 4.</b> Comparison of prevalence of <i>R. salmoninarum</i> in the kidney and feces of coho before and after fecal challenge.....	48

## INTRODUCTION

Bacterial kidney disease (BKD, Corynebacterial kidney disease, salmonid kidney disease, Dee disease) is a systemic, usually chronic disease of wild (feral, free-living) and cultured (farmed, penned) salmonids. The causative organism is *Renibacterium salmoninarum*, a Gram positive rod-shaped bacterium (Sanders and Fryer 1980).

BKD was first described in 1930, from infected Atlantic salmon (*Salmo salar*) in the Dee River system of Scotland (Smith 1964). The first report of BKD in British Columbia, Canada was in wild cut-throat trout (*Salmo clarki*) collected from Cultus Lake (work by D.C.B. Duff cited by Evelyn 1988). Since these initial reports, BKD has been identified in salmonids from most areas of the world (Evelyn 1988). No cases of naturally occurring BKD have been reported to occur in fishes other than salmonids (Paclibare 1989; Fryer and Sanders 1981).

External signs such as exophthalmia, abdominal distension, hemorrhagic areas and superficial blisters or ulcers may be observed (Fryer and Sanders 1981). Even though BKD is a systemic disease, the kidney appears to be the primary target organ. Kidneys typically are swollen and contain greyish-white granulomatous lesions made up of

leucocytes, cellular debris and *R. salmoninarum* cells (Fryer and Sanders 1981). Other organs such as the spleen and liver may also contain lesions.

The causative organism of BKD, *R. salmoninarum* is a small (0.5 x 1.0 um), Gram-positive, non-acid-fast, non-sporulating, non-motile, non-encapsulated, slow-growing and fastidious bacillus (Sanders and Fryer 1980). Six to eight weeks incubation at 15-18°C on growth media are often required for primary isolation from infected tissue (Evelyn 1977; Austin et al. 1983; Daly and Stevenson 1985). *R. salmoninarum* is closely related to the Coryneform Group of Bacteria in which it was once classified (Sanders and Fryer 1980).

BKD causes mortality in salmonids reared in fresh water and in sea water. These mortalities represent a significant economic loss to the salmon farming industry in B.C. Attempts to reduce BKD-related mortalities by chemotherapy and vaccination have met with minimal success (Evelyn 1988; Elliott et al. 1989). The intracellular survival of *R. salmoninarum* (Young and Chapman 1978; Bruno 1986) has been cited as the main reason for the difficulty associated with developing effective control methods (Fryer and Sanders 1981; Elliott et al. 1989; Evelyn 1988).

*R. salmoninarum* is known to be transmitted horizontally between fish and vertically from the female

parent to her progeny via the eggs (Fryer and Sanders 1981; Mitchum and Sherman 1981; Bell et al. 1984; Klontz 1983; Bullock 1980; Evelyn 1988; Elliott et al. 1989). However, the relative importance of each mode of transmission is unknown.

At the time of this research, the salmon farming industry in B.C. was routinely screening potential broodstock in an attempt to reduce the vertical transmission of the pathogen and subsequent BKD-related mortalities. The IFAT (indirect fluorescent antibody technique) was commonly used to detect *R. salmoninarum* in the ovarian fluid, and those eggs from females with heavily infected ovarian fluid were discarded. However, because the IFAT is not a highly sensitive detection method some infected eggs (from females with *R. salmoninarum*-infected ovarian fluid) may pass the screening procedure (Armstrong et al. 1989). Evelyn et al. (1984) have found that up to 15% of the eggs from heavily infected (*R. salmoninarum*-infected ovarian fluid) wild females may be infected with *R. salmoninarum*. The question then arises as to how the prevalence of *R. salmoninarum* in populations of penned salmon can increase to such an extent that some salmon farms experience up to an 80% loss due to BKD.

The most obvious explanation is that *R. salmoninarum* is being transmitted horizontally between salmon reared in

seawater netpens. One could then suggest that the few fish infected at the time of hatch shed the pathogen and initiate the process of horizontal transmission from the freshwater stage onwards.

The way in which *R. salmoninarum* is horizontally transmitted has not been determined. Direct contact with infected salmon may not be the only way for healthy fish to be exposed to pathogens. It is possible that *R. salmoninarum* may occur free in the water, maybe associated with particles such as silt, feed or feces in the water, or it may be present in the fish feed. The primary surfaces for the attachment of pathogens into the fish probably are the gills, the gut, and external lesions or abrasions (Munro 1982). Pathogens can also be shed from point sources such as the gills and external lesions, as well as excreted in the urine or feces from infected fish (Munro 1982).

The ingestion of *R. salmoninarum*-laden fecal material (which has been shed from infected salmon) may be a significant route for the horizontal transmission of BKD. *R. salmoninarum* has been found in the lower gut (Bullock et al. 1978) and feces (Austin and Rayment 1985; Bullock et al. 1980; Embley 1983) of infected salmonids. Evelyn (1988) has shown that *R. salmoninarum* can remain viable in sea water long enough for neighbouring fish to infect one another. Penned salmon commonly take fecal material into

their mouths (and presumably ingest it) during feeding (personal observation). The ability to produce BKD infections by feeding *R. salmoninarum*-contaminated food to salmon, was demonstrated by Wood and Wallis (1955).

The experiments described herein were designed to test the hypothesis that *R. salmoninarum* can be transmitted horizontally among salmon reared in seawater netpens by the fecal-oral route. Specifically, experiments were designed to address the following questions:

1. What are the trends in the natural prevalence of *R. salmoninarum* and BKD in salmon reared in seawater netpens? Do the observed trends indicate that horizontal transmission occurs? What are some of the contributing factors that may influence horizontal transmission?
2. Can *R. salmoninarum* be detected in the sea water of netpens containing salmon actively affected with BKD?
3. Does *R. salmoninarum* shed from infected salmon survive in natural sea water long enough to be transmitted (by ingestion) to neighbouring salmon?



4. Does ingestion of *R. salmoninarum*-laden fecal material result in the infection of previously uninfected salmon?

## MATERIALS AND METHODS

### **I. FARM SURVEY**

A survey of the prevalence of BKD and *R. salmoninarum* in seawater penned chinook (*Oncorhynchus tshawytscha*) and coho (*Oncorhynchus kisutch*) was carried out in cooperation with three salmon farming companies (Companies A, B and C). The progression of BKD in a given population of salmon was determined by sampling a designated group of fish every two months for periods up to 12 months. Details of the sampling and analyses performed are given below.

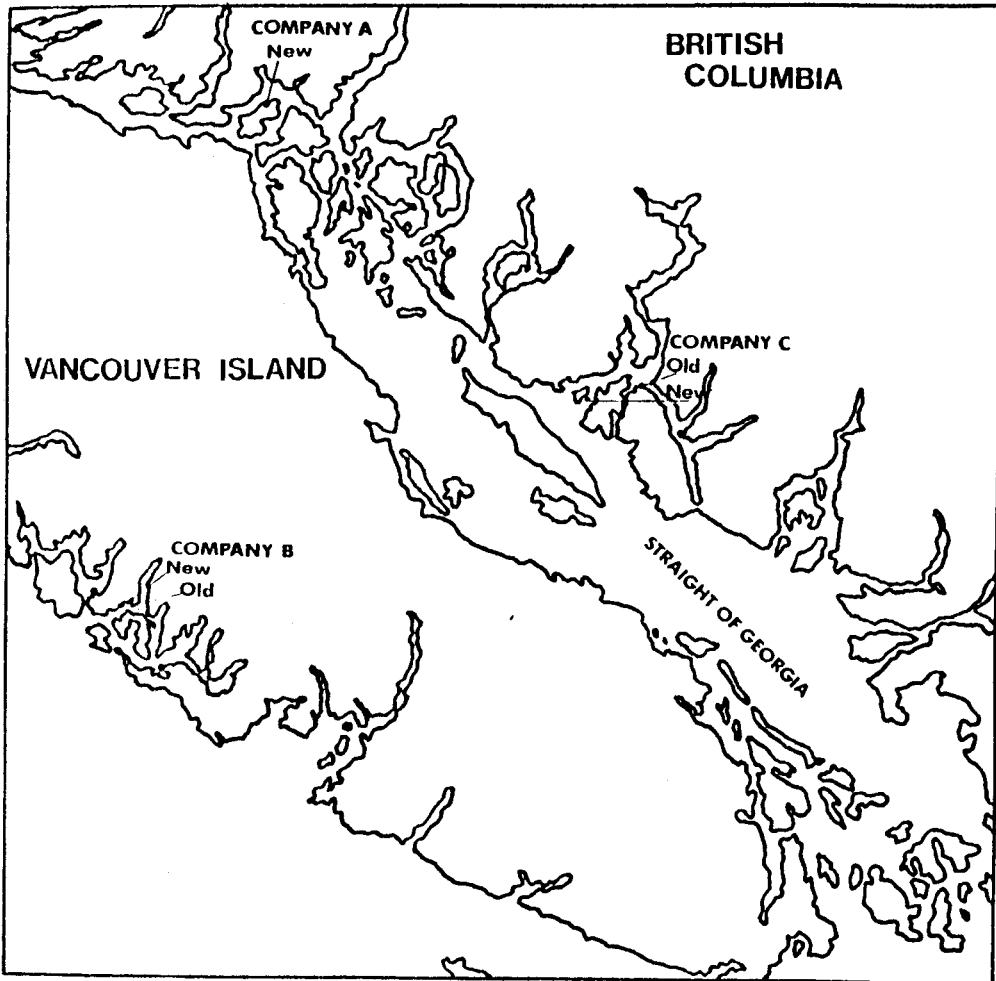
Each of the three Companies operated two sites - an old site and a new site. The effect of site history (i.e., old vs new) on the progression of BKD was examined at Companies B and C by comparing results obtained from their new and old sites. The sampling at Company A was performed only at its new site. The new sites were established in pristine coastal locations, i.e., locations that had never been occupied by penned salmon. The netpens at the new sites contained a single age class of salmon that were smolted there. Old sites had been in operation for one to three years and contained netpens of salmonids as old as the operation (i.e., one to three years old). BKD-related mortalities first occurred at the old sites after the fish had been maintained there for approximately 10 months. These mortalities continued to occur during the time when

the subsequently introduced experimental fish were being sampled. Figure 1 shows the five survey sites belonging to Companies A, B and C were located in three distinctly different areas along the southern coast of British Columbia.

#### COMPANY A

A group of wild and domestic feminized (artificially manipulated to produce all phenotypic females) chinook (73,000) were introduced to sea water at the new site in July 1986. In November of that year a population (59,000) of same-aged coho (many of which showed clinical signs of BKD) was placed in a netpen alongside the netpen of chinook. The coho originated from an old site belonging to Company A - a site in which the penned salmon displayed high, chronic BKD-related mortalities. When the coho at this site began to exhibit high mortalities and clinical signs of BKD, they were moved to the new site. The chinook population at the new site was sampled for the first time in April 1987, at which time it had been next to the diseased coho for five months. In May 1987, the chinook were moved approximately 200 m away from the coho. When the chinook were sampled for the second time (June 1987), the coho had been removed from the site. The chinook at Company A's new site were sampled six times over a period of one year.

**FIGURE 1.** Location of the salmon farm sites belonging to Companies' A, B and C that participated in the BKD survey.



## COMPANY B

Domestic coho were first sampled in July 1987, two days after they were placed into a netpen at the new site. Two weeks after seawater entry, approximately 400 coho were removed from this source pen and 200 placed in each of two - 20' x 20' netpens (200 fish per pen). One netpen of 200 coho was held at the new site, and the second netpen of 200 coho was moved to an old site. The old site contained netpens of chinook, coho, and rainbow trout yearlings, all experiencing BKD-related mortalities. The old and the new sites were located 1 km apart. The coho from each of the two experimental netpens were sampled four times over a period of 10 months.

## COMPANY C

Two groups of chinook (domestic and wild) were sampled from netpens belonging to Company C. The first group was a population (61,000) of domestic chinook. Sampling of this group began when the chinook were introduced to sea water at the Company's new site in August 1987. By February 1988, these chinook had been sampled three times. At this time the pen of chinook was graded and split into two size classes. The smaller fish (under one kg) remained at the new site, and the larger fish (over one kg) were moved to Company C's old site approximately four km away. This old

site contained one to three-year-old chinook, which, in some cases, were experiencing heavy BKD-related mortalities. The two populations of domestic chinook (one at the new site; the other at the old site) were each sampled three times after the grading.

The second group of chinook sampled was a population (11,000) of wild feminized chinook, introduced to sea water (May 1987) netpens at Company C's old site. This was the same old site just mentioned above. Sampling from the chinook at the old site began in October 1987 and continued for 10 months. During this time five samples were taken.

#### NET PEN SAMPLING AND NECROPSY PROCEDURE

Fish were sampled from each netpen using a seine net (1/2" mesh size). Fifty-seven (see Appendices for exceptions) fish were randomly dip-netted out of the seined sample and placed in a bath containing a lethal concentration of anaesthetic (2-phenoxyethanol). The size of the sample permitted the detection (with a 95% confidence limit) of at least one carrier fish in a population where the disease prevalence was  $\geq 5\%$  (Ossiander and Wedemeyer 1983). Due to the small population size in the experimental pens (200 fish pen<sup>-1</sup>) at Company B's new and old sites, only 54 fish were required to be sampled each time to provide the same level of detection

sensitivity. The fish samples were bagged, placed on ice, and immediately transported to the laboratory for necropsy.

Fish were examined by necropsy within 24 h post-mortem. Weights and fork lengths were recorded, as well as any internal or external abnormalities. Those fish that had white granulomatous lesions (typical of BKD) in the kidney were considered to be clinically infected with *R. salmoninarum* (i.e., BKD affected). The prevalence of clinically infected fish was expressed as the percentage of fish with clinical signs of BKD in each sample. Culture and IFAT methods were used to detect *R. salmoninarum* in the kidney tissue of the sampled fish. These detection methods are discussed in detail below.

#### DETECTION OF *R. salmoninarum* BY THE IFAT

Kidney smears were prepared from each fish using a standardized technique: i.e., a sterile loop was inserted into the mid-posterior kidney and pushed forward to the anterior kidney. The contents of the loop were placed on a microscope slide and smeared with a coverslip to ensure even distribution. The smear was heat-fixed, and a circle (approximately 13 mm diameter) was then drawn on the smear using a Mark Tech pen (Fischer Scientific Co.). Two to three drops of rabbit anti-serum, specific for *R. salmoninarum* (Microtek, 1:50 dilution), were placed on the smear within the circle and left for 45 min. The anti-serum

was then rinsed away with phosphate buffered saline (PBS) (0.85% NaCl, 0.12% K<sub>2</sub>PO<sub>4</sub>, 0.34% KH<sub>2</sub>PO<sub>4</sub>, pH 7.2). The smear was then soaked in PBS for 10 min and blotted dry.

The smear was then reacted for 20 min in the dark with fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit globulin (0.1 mL) (Sigma Chemical Co.) containing a rhodamine counterstain (0.1 mL) (BBL Laboratories) and sterile PBS (4.8 mL). The smear was then rinsed in carbonate-bicarbonate buffer (C/BC) (1.06% Na<sub>2</sub>CO<sub>3</sub>, 3.36% NaHCO<sub>3</sub>, pH 9-9.5), soaked for 10 min in the same buffer, and blotted dry.

The stained smears (and a positive control smear containing *R. salmoninarum* cells) were examined under oil immersion at 1000x magnification using a Zeiss epifluorescence microscope. One hundred fields were systematically observed and the number of *R. salmoninarum* cells within each field recorded. Those fish in which one or more *R. salmoninarum* cells were detected in the IFAT-stained kidney smear were considered IFAT-positive. The prevalence of IFAT-positive fish was expressed as the percentage of IFAT-positive fish in each sample.

The IFAT procedure described above was slightly modified for the detection of *R. salmoninarum* in fecal samples and for the identification of *R. salmoninarum* on culture plates. Fecal samples were massaged out of the



posterior intestine into sterile culture tubes. A sterile loop was then dipped into the feces and the contents of the loop smeared on a microscope slide. Colonies on culture plates suspected to be *R. salmoninarum* were removed from culture plates with a sterile loop and smeared onto microscope slides. The smeared slides were heat-fixed and the IFAT performed as described above.

#### DETECTION OF *R. salmoninarum* BY CULTURE

Kidneys were aseptically removed from each fish, weighed, homogenized, washed once in peptone-saline (P-S) (0.10% peptone, 0.85% NaCl), and a 50% homogenate prepared using P-S (Evelyn et al. 1981). Ten-fold serial dilutions (usually to  $10^{-6}$ ) (using sterile, chilled P-S) were made from the 50% homogenate and then five 25 ul drops from each dilution were inoculated onto selective charcoal agar plates (SKDM-C) (Daly & Stevenson 1985; Austin et al. 1983). Plates were wrapped in plastic bags to prevent dehydration and incubated at 15°C for a maximum of 6 weeks.

Any growth observed on the plates was examined by IFAT to determine whether it was *R. salmoninarum*. Samples on plates that failed to yield growth after 6 weeks incubation were considered negative. The concentration of *R. salmoninarum* in the kidney tissue was determined by counting the colonies (IFAT confirmed) present, and calculating the number of colony-forming units (cfu)  $g^{-1}$  of

kidney material. The prevalence of culture-positive fish was expressed as the percentage of culture-positive fish in each sample.

The total prevalence of *R. salmoninarum*-infected fish was determined for each fish sample. Total prevalence was the maximum number of fish positive per number tested (expressed as a percent) using both IFAT and culture methods. The total prevalence was determined because of problems (discussed later) in the sensitivity of these two detection methods.

## II. WATER SAMPLING EXPERIMENT

In the following experiment, sea water was sampled to determine the concentration of *R. salmoninarum* present in a netpen of BKD affected salmon.

### SOURCE OF WATER

Seawater samples were removed from a netpen containing 14,000 domestic feminized chinook salmon (304 g mean weight) that were experiencing high BKD-related mortalities. A 3L Van dorn water sampler was suspended above the center of the netpen. Some feed was sprinkled on the water surface to attract the fish and stimulate feeding (and defecation). The water samples were obtained when the

fish were visible in the netpen. Seawater samples were obtained from depths of 1,2,3 and 4 m, placed in sterile containers, and transported to the laboratory at in-situ temperature (10°C). After the sea water was sampled, 57 chinook (from the same netpen) were collected for necropsy (as described earlier).

#### DETECTION OF *R. salmoninarum* IN WATER AND FISH

Kidney and fecal samples were aseptically removed from 57 chinook and examined for the presence of *R. salmoninarum* using the IFAT and culture methods. The prevalence of IFAT and culture-detected *R. salmoninarum* in the fecal and kidney samples was determined. Total prevalence was also determined.

Within 6 h , a 1 L volume of water from each depth sampled, was centrifuged (at 5°C and 5,000 x g for 30 min). The resulting pellet was resuspended in 10 mL sterile, chilled P-S, mixed, and 0.1 mL portions spread onto SKDM-C agar plates in triplicate. Two ten-fold dilutions of the pellet suspension were similarly plated in triplicate. After 6 weeks incubation (at 15°C), smears from colonies that appeared to be *R. salmoninarum* were confirmed by IFAT. The remaining 1-2 L of the original seawater samples were frozen.

Further quantification of *R. salmoninarum* in the sea water was attempted using the remaining frozen seawater sample. The seawater samples were slowly thawed and kept chilled throughout the procedure. For each sample the entire volume of thawed water was measured and centrifuged as described above. The resulting pellet was resuspended in 10 mL of sterile, chilled P-S. Ten-fold serial dilutions of the concentrate were prepared (to  $10^{-5}$ ) using cold, sterile P-S.

For each depth, the concentrated and serially diluted samples were drop-inoculated onto SKDM-2 plates in triplicate (five 25 uL drops per plate). The agar medium used was changed from SKDM-C to SKDM-2, to ensure that the selective property of the medium (i.e., the effect of the anti-microbials) was not being compromised by the presence of charcoal. The 'nurse-culture' technique (Evelyn et al. 1989) was used to accelerate the growth of *R. salmoninarum*. Smears from growth that appeared to be *R. salmoninarum* were examined by IFAT for confirmation.

After the removal of samples (approximately 0.5 mL) for the culture procedure, the concentrates and their dilutions were fixed with formalin (2% final concentration). The concentration of *R. salmoninarum* in the preserved samples was determined by the membrane filtration-IFAT (MF-IFAT) described later. A second

observer confirmed the *R. salmoninarum* positive cells. The number of *R. salmoninarum* cells mL<sup>-1</sup> of sea water was then determined.

### III. *R. salmoninarum* SURVIVAL EXPERIMENT

An experiment was designed to examine the ability of *R. salmoninarum* to survive in sea water. Survival of the pathogen in natural (non-sterile) sea water (SW) was compared with its survival in four other solutions: filter-sterilized sea water (FSW), saline (SA) (0.85% NaCl), peptone-saline (P-S), and SKDM-2 broth (SKDM-2B) (Evelyn 1977).

#### BACTERIAL ISOLATE

Eighteen-day old *R. salmoninarum* (Pacific Biological Station, Nanaimo #384) growth was harvested from SKDM-C agar plates and transferred into sterile, iced-cold P-S. The suspension was aseptically homogenized with a teflon-tipped homogenizer probe to disrupt aggregates (microscopically confirmed). The concentration of *R. salmoninarum* in the suspension was estimated from absorbance measurements made at 420 nm (using the relationship 2.0 O.D. at 420 nm = 10<sup>8</sup> *R. salmoninarum* cfu mL<sup>-1</sup>). An appropriate volume (20 uL) of the bacterial suspension was then added to each of the test solutions to

give a final concentration of  $10^6$  cfu *R. salmoninarum* mL<sup>-1</sup>. Actual cfu mL<sup>-1</sup> values were determined by diluting the suspension with P-S and drop-plating ten 25 uL aliquots from each dilution onto SKDM-C agar plates. The plates were incubated at 15°C for six weeks and the IFAT confirmed *R. salmoninarum* colonies counted.

#### TEST SOLUTIONS

Sea water (10°C, 22‰ salinity) from Burrard Inlet (a fiord contiguous to the Strait of Georgia), was obtained from a depth of 2 m, using a sterile Niskin sampler. The water sample was stored in a sterile 1 L container and placed on ice for transport to the laboratory. A 100 mL sample of the sea water was fixed with formalin (2% final concentration) at the time of sampling and again just prior to the start of the survival experiment. The seawater samples were examined by the direct microscopic enumeration method (described below) to determine if natural microflora had changed significantly during the six hour interval between sampling and the start of the experiment. The seawater sample was also examined for the presence of *R. salmoninarum* using the MF-IFAT (described below).

Fifteen-125 mL Erlynmeyer flasks were acid-washed and thoroughly rinsed with distilled water. They were fitted with cotton gauze stoppers, wrapped in aluminum foil (to

prevent any interference by light), and autoclaved. Seventy-five mL portions of each of the following solutions were added to each of three flasks:

1. Seawater (SW)
2. Filtered Sterile Seawater (FSW)
3. Filtered & Autoclaved Sterile Saline (SA)
4. Filtered & Autoclaved Sterile Peptone Saline (P-S)
5. Filtered & Autoclaved Sterile SKDM-2 Broth (SKDM-2B)

Filter sterilization was performed by passing the solutions through a 0.2 um sterile Nuclepore filter. The effect of the natural microflora on the survival of *R. salmoninarum* in sea water could therefore be determined by comparing the survival of the pathogen in the SW and FSW solutions. The SA, P-S, and SKDM-2B solutions were filtered to remove particulates and then autoclaved.

It was important to examine the ability of *R. salmoninarum* to survive in P-S because it was used as a diluent. The survival of *R. salmoninarum* in saline was also investigated to determine how *R. salmoninarum* adapts to a nutrient-depleted environment. SKDM-2B was chosen as a control solution because it contains all the nutrients (including L-cysteine) required for optimal growth of *R. salmoninarum*, plus antimicrobials (D-cycloserine, polymyxin-B-sulfate, oxolinic acid, cycloheximide) to prevent growth of bacteria other than *R. salmoninarum*.

Twenty uL aliquots of the chilled *R. salmoninarum* suspension were added to each flask for a final concentration of approximately  $10^6$  cfu mL<sup>-1</sup>. The flasks were incubated in the dark at 10°C (in situ temperature of the sea water at the time of sampling) for 28 d. Flasks were gently swirled by hand several times a day to minimize attachment of *R. salmoninarum* cells to the flask walls.

#### SAMPLING PROTOCOL

Each flask was sampled at time 0 (time immediately after *R. salmoninarum* was added), and then at 8 h, 1, 2, 4, 7, 14, 22 and 28 d. At every sampling time, 3 mL portions were aseptically removed from each flask and placed into sterile culture tubes. In addition, the inside walls of the flasks were swabbed with sterile Q-tips. The swab samples were then inoculated onto individual SKDM-C plates and incubated at 15°C for 6 weeks. This procedure was done to determine if viable *R. salmoninarum* cells were attaching to the inside walls of the flask.

Each 3 mL sample was placed on ice and ten-fold dilutions (to  $10^{-2}$ ) were prepared using P-S. One hundred-fold dilutions (to  $100^{-2}$ ) were prepared from the SKDM-2B and P-S solutions after day 14 to compensate for growth of the pathogen. The number of culturable *R. salmoninarum* cells present at each sampling was determined by drop



plating five 25 uL aliquots of each ice-cold sample and its dilutions onto triplicate SKDM-C agar plates. The 'nurse-culture' technique was used to accelerate the growth of *R. salmoninarum* (Evelyn et al. 1989) in the samples. Plates were incubated at 15°C for 8 weeks. The *R. salmoninarum* colonies were counted (IFAT confirmed) as they appeared, and the average cfu mL<sup>-1</sup> calculated. If no growth was observed after 8 weeks incubation, a negative result was recorded.

The volume remaining from each 3 mL of sample (see above) was fixed in formalin (2% final concentration). The formalin-fixed samples were counted by direct microscopic enumeration (described below). The MF-IFAT method was used to confirm that all the bacteria observed by direct microscopic enumeration were *R. salmoninarum*. The seawater samples, however, were not sterile and contained other bacterial cells in addition to *R. salmoninarum*. In this case, the MF-IFAT was necessary to distinguish between the *R. salmoninarum* and non-*R. salmoninarum* bacterial cells.

#### DIRECT MICROSCOPIC ENUMERATION

Each of the samples taken from the test solutions was stained with DAPI (4'6-diamidino-2-phenylindole) and examined by the direct microscopic enumeration method (Velji and Albright 1985). Each sample was vigorously mixed

and 0.5 mL was transferred to a sterile culture tube. This subsample was diluted with 4.5 mL of filter sterile saline. Filter sterilized sodium pyrophosphate (0.0005 M final concentration) was added to the sample, left without agitation for 30 min, and then sonicated for 20 s at 300 Watts. Following sonication, filter-sterilized DAPI was added to the sample (3.33 ug mL<sup>-1</sup> final concentration). The sample was then placed in the dark for 20 min.

Nucleopore filters (26 mm diameter, 0.2 um pore size) were stained with Irgalin Black (2 g L<sup>-1</sup> in 2% acetic acid), rinsed with distilled water, and mounted on a glass filtration apparatus. The DAPI-stained samples were gently filtered using a vacuum pump (pressure less than 5 mm Hg). Each filter was then air-dried and placed on a microscope slide that had been smeared with immersion oil. A drop of immersion oil was placed on top of the filter followed by a coverslip. The slides were read under oil immersion at 1000x magnification using a Zeiss epifluorescence microscope, fitted with an ocular grid.

The survival of *R. salmoninarum* in each solution was determined in triplicate (i.e., three flasks per solution). Two slides were prepared from each of the three flasks for a total of six slides per solution type per sample time. A total of 100 grids was systematically counted from the six prepared slides.

The counts were converted to total number of cells mL<sup>-1</sup> using the formula:

$$\frac{\text{Ave. Number Cells/Grid} \times \text{Conversion Factor}}{\text{Volume Filtered}}$$

The conversion factor was the ratio of the total filtering surface area divided by the filter surface area viewed using the oil immersion lens. The diameter of each filtration barrel was slightly different thereby affecting the total effective filtering surface area. Consequently, a different conversion factor was calculated for each of the six barrels used.

#### MEMBRANE FILTRATION IFAT ENUMERATION

Membrane filtration IFATs (MF-IFATs) were used to determine the concentration of *R. salmoninarum* in the samples. The MF-IFAT procedure (described by Lee 1989) used here combines the methodologies of the IFAT and DAPI stained direct counting techniques (described earlier).

Each sample was mixed and a subsample (0.5 mL) removed, placed in a sterile culture tube, and diluted with 4.5 mL of filter-sterile PBS. The subsample was mixed and gently filtered through an (0.2 um pore size; 26 mm diameter) unstained Nuclepore filter mounted on the same filtration apparatus as used in the DAPI counting procedure.

*R. salmoninarum*-specific rabbit anti-serum (approximately 0.5 mL) (Microtek, 1:20 dilution) was carefully placed on the filter, left for 45 min and gently filtered. The filters were rinsed with five mL of a filter-sterilized solution of PBS containing 0.5% (v/v) Triton X-100 (PBS-Triton). Goat anti-rabbit FITC/rhodamine counterstain (0.5 mL) was then placed on the filter and left in the dark for 20 min. The stain was then filtered off and the filter rinsed with 5 mL of filter-sterile PBS-Triton. Each filter was air-dried, placed on a microscope slide and mounted with a coverslip.

The slides were read immediately under oil immersion at 1000x magnification using a Zeiss epifluorescence microscope fitted with an ocular counting grid. Two slides were prepared from each of the 3 flasks for a total of 6 slides per solution type per sample time. A total of 100 grids was systematically counted from the 6 prepared slides. The total number of *R. salmoninarum* cells per volume of sample was calculated using same formula described earlier for the direct count enumeration method.

#### ANALYSIS OF DATA

The *R. salmoninarum* counts obtained from the culture and microscopic enumeration procedures were converted to cfu mL<sup>-1</sup> and cells mL<sup>-1</sup>, respectively. For the seawater

sample, the total cells  $\text{mL}^{-1}$  was determined (by the DAPI-stained direct enumeration method) in addition to the number of *R. salmoninarum* (determined by the culture and MF-IFAT methods). The average, standard deviation (SD) and standard error (SE) (95% confidence limit) for the test solutions were calculated at each sample time. Student t-tests (Zar 1974) were performed to see if there was a significant difference between (a) the MF-IFAT counts and the direct (DAPI) counts, and (b) between the culture and the microscope enumeration results. A student t-test was also used to determine if there was a significant change in the number of *R. salmoninarum* over the sampling period.

#### **IV. FECAL CHALLENGE EXPERIMENT**

##### SOURCE OF FISH TO BE CHALLENGED

Domestic coho were used for this experiment. The coho were first sampled at the freshwater stage in May 1989, approximately 3 months after ponding. They were sampled again when they were placed in a seawater netpen at a commercial salmon farm (June 1989). The presence of *R. salmoninarum* in the kidney tissue was determined by the IFAT and culture methods. The coho were sampled again one week before the fecal challenge experiment (August 1989). At this time, both kidney and fecal samples were taken from

the coho, and the IFAT and culture methods used for the detection of *R. salmoninarum*. The sampling and necropsy procedures used for this experiment were the same as described earlier.

#### PREPARATION OF INOCULUM

Feces were collected from domestic rainbow trout. Anaesthetized fish were gently massaged to release feces. The feces were autoclaved and aseptically homogenized (Polytron, Beckmann Instruments) to produce an easily injectable slurry.

Two-week old growth of *R. salmoninarum* (Pacific Biological Station, Nanaimo, #384) on SKDM-C was harvested and suspended in cold, sterile P-S. The bacterial suspension was aseptically homogenized to disrupt aggregates (microscopically confirmed), and absorbance readings at 420 nm used to estimate the concentration of *R. salmoninarum* (as described earlier). Appropriate volumes of the *R. salmoninarum* suspension were added to 2 tubes of sterile fecal slurry: one for injection and one for intubation. The concentration of *R. salmoninarum* in the fecal slurry to be used for injection was made 25 times more concentrated than the fecal slurry to be used for oral intubation. Different concentrations of *R. salmoninarum* were required because the volume of fecal slurry

administered by injection was 1/25th of the volume administered by intubation, and the same number of *R. salmoninarum* cells ( $10^5$ ) were to be administered to each fish. The larger volumes of the challenge material were orally intubated to compensate for losses due to anticipated regurgitation.

Serial dilutions (to  $10^{-8}$ ) of the *R. salmoninarum*/P-S suspension and the *R. salmoninarum*-fecal (Rs-fecal) material (actually used for the challenge), were drop-plated onto SKDM-C to determine the number of cfu's received by each fish.

#### CHALLENGE PROCEDURE

Coho were collected at random from the source netpen, anaesthetized (2-phenoxyethanol, 0.025% v/v), and challenged as outlined in Table 1.

**TABLE 1.** *R. salmoninarum* (Rs)-fecal challenge treatments received by coho salmon (mean weight 18 g).

<u>PEN</u>	<u>NO. COHO</u>	<u>CHALLENGE MATERIAL</u>	<u>CHALLENGE ROUTE</u>	<u>VOL.<sup>a</sup> (mL)</u>	<u>Rs<sup>b</sup> (cfu)</u>	<u>FIN CLIP</u>
Con. <sup>c</sup>	150	Feces	Oral <sup>e</sup>	0.25	0	No Clip
	150	Feces	Inj. <sup>f</sup>	0.10	0	Adipose
Exp. <sup>d</sup>	150	Rs+Feces	Oral <sup>e</sup>	0.25	$2 \times 10^5$	No Clip
	150	Rs+Feces	Inj. <sup>f</sup>	0.10	$5 \times 10^5$	Adipose

<sup>a</sup> Volume of challenge material administered to each coho.

<sup>b</sup> Actual number of Rs cells administered to each coho.

<sup>c</sup> Con. = Control pen.

<sup>d</sup> Exp. = Experimental pen.

<sup>e</sup> Oral = Oral intubation challenge route.

<sup>f</sup> Inj. = Intraperitoneal injection challenge route.

Intubation was performed using an Eppendorf Repeater Pipetteman 4700, set to deliver 0.25 mL aliquots via the attached Combitip syringe which contained the challenge material. The Combitip was inserted into the esophagus of the anaesthetized fish and 0.25 mL delivered. The ip injection (0.1 mL) was delivered from an area on the fish just anterior to the vent.

The fish were allowed to recover in oxygenated sea water (50 L) and were then placed in designated netpens. The netpens containing the experimental and control fish were located approximately 300 m apart. The netpens were covered with a predator net. The fish were fed half rations for the first week of the experiment and not at all thereafter.

#### SAMPLING AND DATA COLLECTION

Mortalities were collected periodically throughout the experiment. Daily feedings and mortality collections were planned but the salmon farm suffered financial problems, making this impossible after the first week of the experiment.

Weight and fork length measurements were taken from all dead fish. Kidney and fecal samples were aseptically removed from each dead specimen and examined for the presence of *R. salmoninarum* by the IFAT and culture



methods. Because of the emaciated state of the fish, sufficient amounts of kidney and feces were not available for the quantification of *R. salmoninarum* by the aforementioned drop-plate culture method. Kidney and fecal samples were therefore obtained with a sterile loop and inoculated onto SKDM-C plates (in triplicate). Plates were incubated as described previously. The samples were also smeared onto microscope slides (in duplicate). The prevalence of *R. salmoninarum* was expressed as the proportion of IFAT or culture-positive fish at each sampling.

The concentration of *R. salmoninarum* could not be determined by the culture method, therefore the relative concentrations of *R. salmoninarum* in the kidney and fecal samples were estimated from the IFAT results. The average number of *R. salmoninarum* cells per 100 fields was determined from four IFAT-stained smears of kidney and fecal material. An arbitrary grading scheme (described by Armstrong et al. 1989) was used to estimate the relative concentrations of *R. salmoninarum* present in the samples. The grading scheme used to evaluate the IFAT results was:

0 = No <i>R. salmoninarum</i> detected
+1 = 1-10 <i>R. salmoninarum</i> cells/100 fields
+2 = 11-25 <i>R. salmoninarum</i> cells/100 fields
+3 = 26-50 <i>R. salmoninarum</i> cells/100 fields
+4 = >50 <i>R. salmoninarum</i> cells/100 fields

The difference in prevalence between the experimental and control fish was analyzed using the Binomial Test (Zar 1974). This statistical test was also used to determine if the prevalence of *R. salmoninarum* in surviving fish was significantly different from that determined at the start of the experiment ( $T_0$ ).

## RESULTS

### **I. FARM SURVEY**

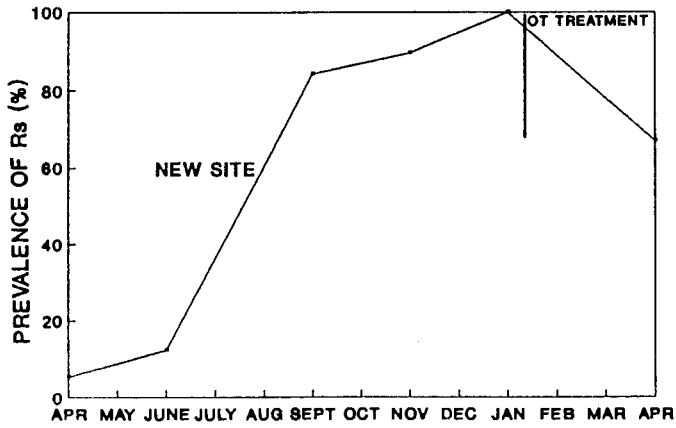
Results from the BKD survey indicate an overall increasing trend in the total prevalence of detectable *R. salmoninarum* with time (Figure 2). The percent positives at each sampling as detected by observation of clinical signs of disease, IFAT, and culture can be found in Appendix A (Company A), Appendix B (Company B), and Appendix C and D (Company C).

#### COMPANY A

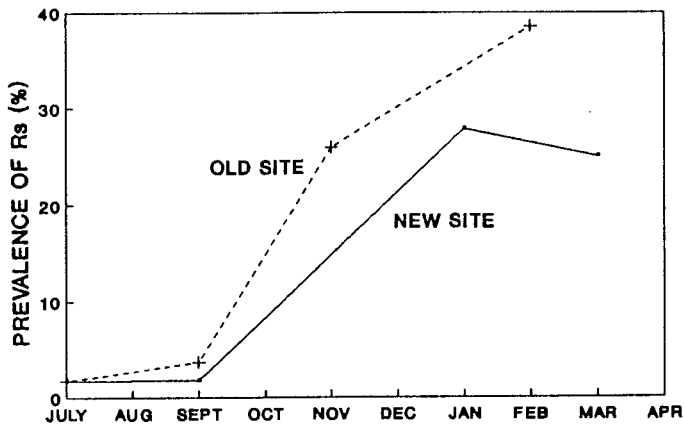
The Company A monitoring results reveal a large increase in prevalence of BKD infected chinook (Figure 2A). During the 10 month sampling period, the total prevalence of *R. salmoninarum* in the chinook population increased from 5% to 100%. This increase in prevalence with time was found to be statistically significant ( $p < 0.001$ ) (calculated from the start of the survey to the penultimate sample). The culture technique proved to be the most sensitive detection method, as more positives were detected by this method than by IFAT or by observation of clinical signs (see Appendix A). The number of *R. salmoninarum* cfu  $g^{-1}$  kidney material increased in the fish population over time (from

FIGURE 2. Results from the BKD survey conducted at Companies A, B and C. The total prevalence of *R. salmoninarum* (Rs) was calculated as the maximum number of fish positive per number tested using both IFAT and culture methods (expressed as a percent). (A) Prevalence of Rs within domestic and wild feminized chinook maintained at the new site belonging to Company A. Experimental fish had been in seawater 10 months when the first sample was taken (mean weight at first sample was 210 g). (B) Prevalence of Rs in domestic coho maintained at the new and old sites belonging to Company B. The first sample was taken at seawater entry (mean weight 9 g), subsequent sampling was performed at the separate sites. (C) Prevalence of Rs in 2 groups of chinook maintained by Company C. One group of wild feminized chinook was first sampled at the old site after approximately 5 months in sea water (mean weight at first sample was 221 g). The second group was a population of domestic chinook maintained 6 months at Company C's new site, then 6 months separated at the new and old sites. The first sample was taken at seawater entry (mean weight was 10 g).

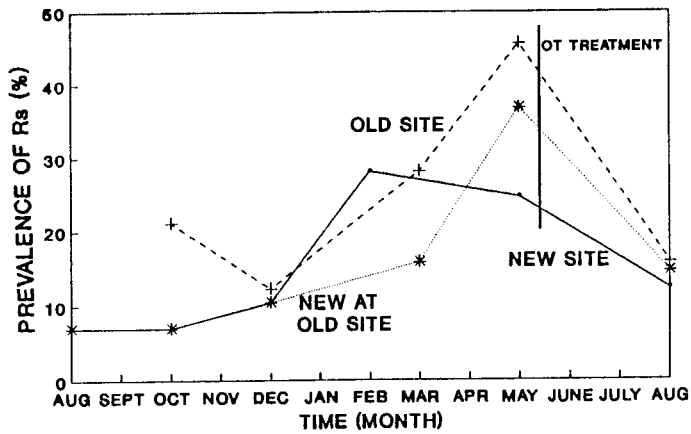
(A) COMPANY A



(B) COMPANY B



(C) COMPANY C



approximately  $10^4$  to  $10^{11}$  cfu  $g^{-1}$  kidney material) (refer to the Appendices).

#### COMPANY B

The survey conducted at the new and old sites belonging to Company B, revealed an increasing prevalence of *R. salmoninarum* over time (Figure 2B). However, the pathogen was detected only by IFAT (Appendix B). No clinical signs of BKD were noted at any time, and the culture results were always negative despite close to 40% prevalence of *R. salmoninarum* as detected by IFAT in the final sample at the old site. In both populations of salmon maintained at the old and the new sites, the prevalence of *R. salmoninarum* had increased significantly ( $p < 0.001$ ) over the sampling period. The difference between the prevalences of *R. salmoninarum* in the final samples taken from the old and new sites was not significantly different.

#### COMPANY C

The results from the BKD survey conducted at Company C's sites were similar to those found at the sites belonging to Companies' A and B (Figure 2C). The total prevalence of *R. salmoninarum* (as calculated from the first sample to the penultimate sample) increased significantly over time for fish held at the old site ( $p < 0.02$ ), the new

site ( $p < 0.05$ ), and for those fish moved from the new to the old site ( $p < 0.001$ ). The prevalence of *R. salmoninarum* as detected by clinical signs, IFAT, and culture can be seen in Appendix C and D.

There was no significant difference in the prevalence of *R. salmoninarum* between the chinook transferred from the new to the old site with either the old site or the new site chinook (calculated to the penultimate sample). There was however, a statistically significant difference ( $p < 0.05$ ) in prevalence between the chinook maintained continuously at the old and the new site.

A relationship between prevalence of *R. salmoninarum* and average percent monthly mortality could not be established because the causes of the mortalities were impossible to determine. Plankton blooms (harmful *Chaetocerus* spp.), vibriosis, premature sexual maturation, and BKD were all contributing factors to the observed mortalities.

The effect of seawater temperature on the prevalence of *R. salmoninarum* was inconclusive. Correlations between temperature and prevalence of *R. salmoninarum* were not significant. The chinook from Companies A and C showed increases in prevalence with increasing water temperature while the coho from Company B showed increasing prevalence with decreasing temperature. The prevalence of *R.*

*salmoninarum* increased significantly over time at all the survey sites, which suggests that horizontal transmission occurred whether the temperature was increasing or decreasing (refer to Appendices).

## II. WATER SAMPLING EXPERIMENT

Attempts to isolate and quantify *R. salmoninarum* from all the water depths sampled met with minimal success. The culture plates (SKDM-C) inoculated with fresh (i.e., not frozen or formalin preserved) sea water were overrun with faster growing seawater microflora. However, plates inoculated from the 1 m water sample did contain several small, white colonies that were confirmed as *R. salmoninarum* using the IFAT (see Table 2).

In an effort to determine the concentration of the viable pathogen in sea water, the remaining water samples (stored frozen) were thawed and cultured on SKDM-2 (rather than SKDM-C) agar plates. The type of culture medium used was changed from SKDM-C to SKDM-2 because the latter was believed to be more selective. *R. salmoninarum* was not isolated from the thawed water samples despite 8 weeks incubation.



**TABLE 2.** Prevalence of *R. salmoninarum* (Rs) within the chinook salmon and seawater samples collected from a netpen of chinook salmon experiencing a BKD epizootic. (Rs) was detected in the fecal and kidney samples using the IFAT and culture methods. The concentration of the pathogen was determined in the water samples by the MF-IFAT, and the presence of viable Rs cells was confirmed by culture (SKDM-C). Details of the detection methods are given in the Materials and Methods section.

<u>SAMPLE</u>	<u>PERCENT PREVALENCE OF Rs (NO. POS./TOTAL NO.)</u>			
	<u>CLINICAL BKD SIGNS</u>	<u>IFAT DETECTED Rs</u>	<u>CULTURE DETECTED Rs</u>	<u>TOTAL<sup>a</sup> PREVAL.</u>
<b>CHINOOK:</b>				
KIDNEY	87.7 (50/57)	94.7 (54/57)	100.0 (57/57)	100.0 (57/57)
FECES	--	80.7 (46/57)	86.0 (49/57)	94.7 (54/57)
<b>WATER:</b>				
1 m DEPTH	--	254.6 <sup>±</sup> 179.1 <sup>b</sup>	POS. <sup>c</sup>	--
2 m DEPTH	--	ND	ND	--
3 m DEPTH	--	ND	ND	--
4 m DEPTH	--	ND	ND	--

-- = Not Applicable, could not be determined.

ND = Not Detected.

<sup>a</sup> = Total prevalence calculated as the maximum number of fish in the sample determined as positive for the presence of Rs by the IFAT and culture detection methods.

<sup>b</sup> = Concentration of Rs  $\pm$  SE (Rs cells mL<sup>-1</sup>) determined by the MF-IFAT.

<sup>c</sup> = Presence of Rs determined by culture (SKDM-C) and confirmed by IFAT.

The MF-IFAT method was used to estimate the number of *R. salmoninarum* cells in the thawed sea water. An estimate of  $254.6 \pm 179.1$  (SE) *R. salmoninarum* cells mL<sup>-1</sup> was obtained from the 1m water sample. The pathogen could not be detected in any of the other water samples.

The sample of 57 chinook removed from the experimental pen was found to be severely affected with BKD. *R. salmoninarum* was detected by culture in 100% of the kidneys sampled (Table 2). The prevalence of *R. salmoninarum* in the fecal material was slightly lower than that found in the kidney. The culture method detected more positives in the fecal material than the IFAT ( 86.0% and 80.7%, respectively). The pathogen was detected by culture (SKDM-C) from the feces of 4 of the 7 fish that displayed no clinical signs of BKD.

The concentration of viable *R. salmoninarum* in the feces could not be accurately determined because faster growing bacteria present in the feces overwhelmed most of the plates. However, because *R. salmoninarum* was detected in the higher dilutions prepared from the fecal samples taken from heavily infected fish, it was estimated that feces could contain perhaps as many as  $1 \times 10^6$  cfu *R. salmoninarum* g feces<sup>-1</sup>.

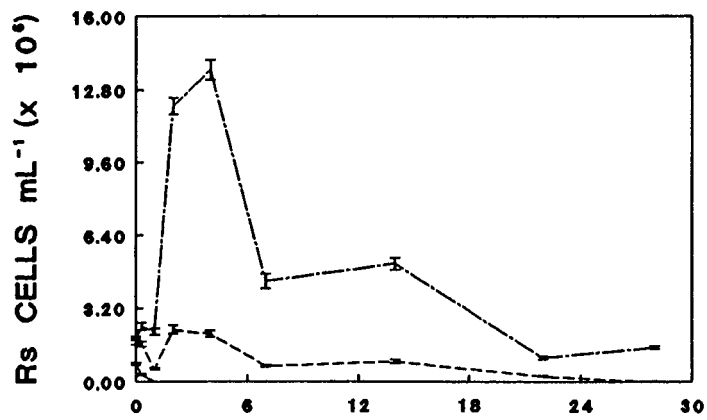
### III. R. salmoninarum SURVIVAL EXPERIMENT

The ability of *R. salmoninarum* to survive in the various test solutions can be seen in Figures 3 and 4. Direct counts were obtained using the DAPI staining technique described earlier, and confirmed as being *R. salmoninarum* using the MF-IFAT. The DAPI counts were preferred over the MF-IFAT derived counts for several reasons: DAPI counts were easier and quicker to perform, DAPI resulted in fewer unidentified fluorescing objects. This resulted in the DAPI-stained smears having a clearer and darker background, making enumeration easier and thus more accurate. In addition, the DAPI staining method was much less costly to perform; the fluorescence from the DAPI stain was more stable (i.e., faded much more slowly) than that used in the MF-IFAT and therefore more counts could be made from a single DAPI stained slide.

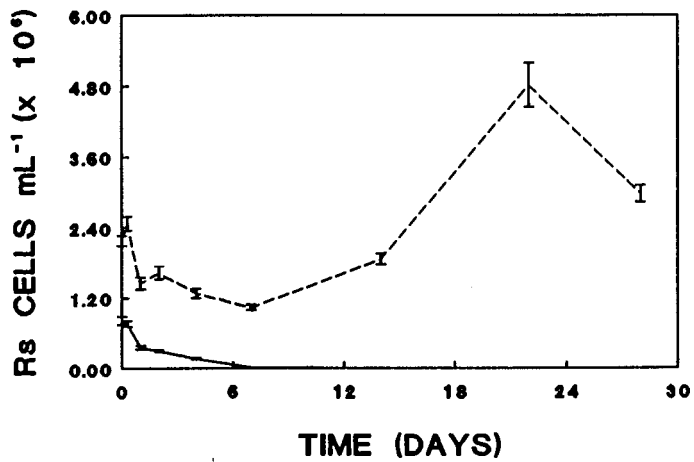
No significant difference ( $p < 0.001$ ) was found between the counts obtained by these two enumeration methods, except in the unfiltered seawater samples. The unfiltered seawater samples contained natural microflora, which were not detected using the MF-IFAT (but were detected by the DAPI staining method). The DAPI staining method revealed that there were significantly higher numbers of bacteria

**FIGURE 3.** Survival of *R. salmoninarum* (Rs) over a period of 28 days (at 10°C) in (A) natural seawater and (B) filter sterile seawater. The concentration of Rs was determined by culture onto SKDM-C — and MF-IFAT --. The concentration of other microbes was determined by direct microscopic enumeration --- (DAPI stain). Standard error bars indicate a 95% confidence interval. Details of the detection methods are given in the Materials and Methods section.

### (A) SEAWATER

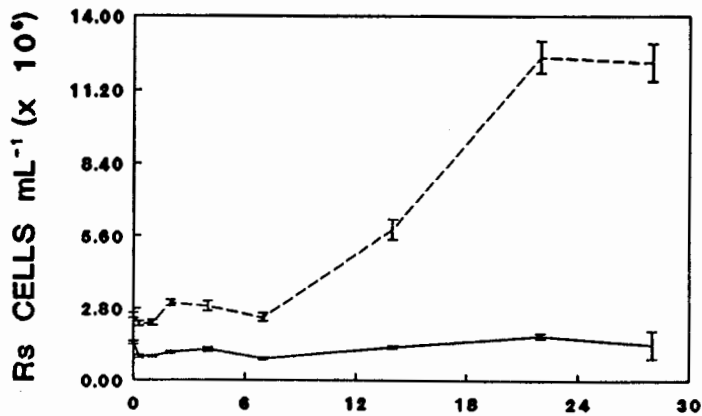


### (B) STERILIZED SEAWATER

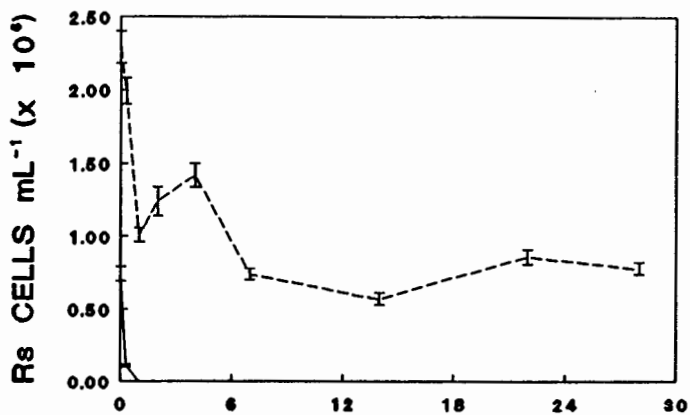


**FIGURE 4.** Survival of *R. salmoninarum* (Rs) over a period of 28 days (at 10°C) in (A) peptone saline, (B) saline, and (C) SKDM-2 broth. The initial concentration of Rs (i.e., at time 0) was approximately  $2 \times 10^6$  cells mL<sup>-1</sup>. The concentration of *R. salmoninarum* was determined by culture onto SKDM-C — and direct microscopic enumeration ---. Standard error bars indicate a 95% confidence interval. Details of the detection methods are given in the Materials and Methods section.

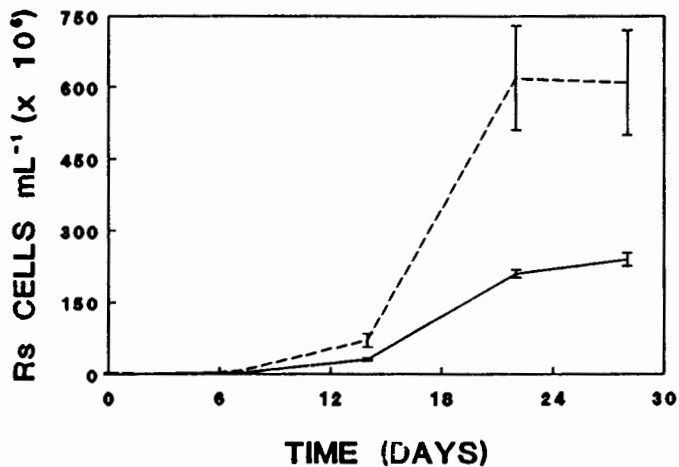
**(A) PEPTONE-SALINE**



**(B) SALINE**



**(C) SKDM-2 BROTH**



other than *R. salmoninarum* in all the sea water samples except for the  $T_0$  count. After 8 hours, the natural microflora present in the seawater had increased in numbers and remained significantly higher than the *R. salmoninarum* counts for the duration of the experiment (Figure 3).

There was no significant change in the microflora from the time the sea water was sampled from Burrard Inlet to the start of the experiment.

*R. salmoninarum* was detected by direct counts in each of the test solutions throughout the entire 28 d sample period. However, the number of *R. salmoninarum* cells was variable throughout this time period. In saline, the number of cells had decreased significantly ( $p < 0.001$ ) after the first sampling. The concentration of *R. salmoninarum* in the P-S and filtered seawater solutions was significantly different at 28 d (higher and lower, respectively) from those counts determined at  $T_0$  ( $p < 0.001$ ). In sea water both the DAPI and MF-IFAT counts differed significantly ( $p < 0.001$ ) from those at  $T_0$  (except for the day 4 MF-IFAT count). The concentration of *R. salmoninarum* in SKDM-2B at  $T_0$  differed from that detected at 4, 7 and 14 days ( $p < 0.05$ ).

The ability of *R. salmoninarum* to survive in the different test solutions was variable. In saline, *R. salmoninarum* remained culturable only 2 days (Figure 4B).



*R. salmoninarum* was cultured from the seawater solution up to day 7 but because of the rapid growth of seawater microflora on the agar plates, the concentration of *R. salmoninarum* could only be determined up to day 1. This corresponds to the increase in the number of rapidly growing non-*R. salmoninarum* cells (as detected by the DAPI staining method) after day 1 (Figure 3A). The ability to culture *R. salmoninarum* in the absence of the seawater microflora can be seen by the survival of *R. salmoninarum* in the FSW solution (Figure 3B). Culturable and quantifiable values were determined up to day 7. *R. salmoninarum* does not appear to survive longer than 7 days in sea water, regardless of the presence or absence of competing bacteria.

Viable *R. salmoninarum* concentrations in sea water, FSW, and saline all decreased significantly after the T<sub>0</sub> sample ( $p < 0.05$ ).

*R. salmoninarum* was cultured from the P-S and SKDM-2B solutions throughout the sample period (Figure 4). Throughout the 28 d sampling period the viable counts of *R. salmoninarum* were always significantly lower than direct counts based on microscopy.

#### IV. FECAL CHALLENGE EXPERIMENT

The pathogen could not be cultured from any of the kidney samples of the coho to be used in the experiment when sampling was done at the hatchery, at seawater entry, or just prior to the experiment ( $T_0$ ). The IFAT results revealed <10% prevalence of *R. salmoninarum* in the kidney samples (5.3% at  $T_0$ ). No *R. salmoninarum* was detected in the feces by the IFAT or culture methods at  $T_0$ .

The post-challenge mortality data can be seen in Table 3. The average weight of the surviving coho was slightly less than the starting average weight of 18 g. Under normal culture conditions these fish would have been approximately 60 g each.

Mortalities were collected regularly for 11 d post-challenge, but not for the following 40 days. At day 51, the pens were dived on to retrieve a mass of dead fish. The number of fish in this mass was estimated by subtracting the number of mortalities (at 11 d) and survivors from the starting number of fish. It can be seen from Table 3 that 90% and 68% of the fish in the experimental and control pens, respectively, died during that 40 day period. Dead fish were collected again 51 days post-challenge (3 days after the dive). The experiment was terminated 73 and 75 days post-challenge in the experimental and control pens,

**TABLE 3.** Number and average weight of coho mortalities collected from the (A) control and (B) experimental pens. Coho from the experimental and control pens were challenged (by either oral intubation or intraperitoneal (ip) injection) on Day 1 with *R. salmoninarum*/feces and feces, respectively. Average weight of all coho on Day 1 was 18g. One-hundred and fifty coho were challenged by each method. A total of 300 coho was placed in each pen.

<b>(A) CONTROL COHO PEN:</b>					
DAYS POST- CHALLENGE	NUMBER OF MORTALITIES		AVERAGE WEIGHT (g)		
	ORAL INTUBATION	IP INJECTION	ORAL INTUBATION	IP INJECTION	IP INJECTION
3	0	1	NA		16
5	0	4	NA		10
11	0	1	NA		7
NK	(102)	(103)	NK		NK
51	3	7	11		11
75a	45bc	34bc	15		17
TOTAL:		150			150
<b>(B) EXPERIMENTAL COHO PEN:</b>					
DAYS POST- CHALLENGE	NUMBER OF MORTALITIES		AVERAGE WEIGHT (g)		
	ORAL INTUBATION	IP INJECTION	ORAL INTUBATION	IP INJECTION	IP INJECTION
2	0	2	NA		7
4	0	2	NA		4
NK	(140)	(135)	NK		NK
50	7	7	12		11
73a	3bc	4bc	17		16
TOTAL:		150			150

Table 3. Continued on next page.

**Table 3. Continued.**

NK = Not Known. Mortalities that occurred between samplings at 11 and 51 d were estimated by subtracting the known number of mortalities plus survivors from starting number of coho.

NA = Not Available.

- a Experiment was terminated at this time. Therefore the numbers represent a count of the surviving coho.
- b Significant difference in mortalities between the control and experimental pen ( $p < 0.001$ ).
- c No significant difference in mortalities between the coho challenged by ip injection or oral intubation ( $p > 0.05$ ).

respectively. Survivors were sacrificed and kidney and fecal samples examined for the presence of *R. salmoninarum*.

There were significantly more ( $p < 0.001$ ) surviving fish in the control pen than in the experimental pen (Table 3). There was no significant difference between the number of survivors challenged by oral intubation and ip injection routes in both the control and experimental pens.

The prevalence of *R. salmoninarum* in the survivors and the intact dead fish was detected by IFAT only. No clinical signs of BKD were seen and no *R. salmoninarum* cells could be isolated by culture at any time. IFATs were performed on kidney and fecal smears from each surviving fish and the percent prevalence at each sample time calculated (Table 4). There was a significant difference in the prevalence of *R. salmoninarum* between the experimental and control fish ( $p < 0.05$ ) in almost all of the comparisons. The only exception was that there was no significant difference in the prevalence of *R. salmoninarum* in the feces between the experimental and control fish that were challenged by the ip injection route.

The surviving fish had IFAT detectable levels of *R. salmoninarum* in the feces and kidney. The prevalence of infection in the survivors was found to have increased significantly ( $p < 0.05$ ) in the feces and kidney samples from both the control and experimental fish (Table 4).

**TABLE 4.** A comparison of prevalence of *R.salmoninarum* (Rs) in the kidney and feces of coho before and after fecal challenge (75 and 73 d surviving post-challenge for the control and experimental coho respectively). Percent positives were detected by IFAT. Coho were challenged with feces containing Rs ( $10^5$  cfu each fish)(experimental test pen) or sterile feces (control pen) by oral intubation and intra-peritoneal (ip) injection methods.

CHALLENGE TREATMENT	PERCENT Rs POS. FISH (NO. POS/TOTAL NO.)		
	AT TIME 0	SURVIVORS <sup>a</sup> CONTROL PEN <sup>e</sup>	SURVIVORS <sup>a</sup> TEST PEN <sup>e</sup>
ORAL INTUBATION:			
KIDNEY <sup>c</sup>	5.3% (3/57)	24.4% (11/45)	100% (3/3)
FECES <sup>c</sup>	0 (0/57)	11.1% ( 5/45)	100% (3/3)
OVERALL <sup>bc</sup>	5.3% (3/57)	28.9% (13/45)	100% (3/3)
IP INJECTION:			
KIDNEY <sup>c</sup>	5.3% (3/57)	26.5% ( 9/34)	100% (4/4)
FECES <sup>d</sup>	0 (0/57)	29.4% (10/34)	75.0% (3/4)
OVERALL <sup>bc</sup>	5.3% (3/57)	47.1% (16/34)	100% (4/4)
COMBINED: <sup>f</sup>			
KIDNEY <sup>c</sup>	5.3% (3/57)	25.3% (20/79)	100% (7/7)
FECES <sup>c</sup>	0 (0/57)	19.0% (15/79)	85.7% (6/7)
OVERALL <sup>bc</sup>	5.3% (3/57)	36.7% (29/79)	100% (7/7)

- <sup>a</sup> Survivors were those coho alive when the experiment was terminated 75 and 73 d post-challenge for the control and experimental coho, respectively.
- <sup>b</sup> Overall refers to the maximum number of coho that were positive (i.e., contained Rs) using both feces and kidney samples.
- <sup>c</sup> Significant difference in the percent positives between the experimental and control pens ( $p < 0.05$ ).
- <sup>d</sup> No significant difference in the percent positives between the experimental and control pens ( $p = 0.076$ ).
- <sup>e</sup> Significant increase in the percent positives of survivors over the starting (Time 0) percent positives ( $p < 0.05$ ).
- <sup>f</sup> Combined refers to the results calculated using the total number of surviving coho in each pen (i.e., oral intubated plus ip injected).

*R. salmoninarum* was not isolated by culture from any of the fecal and kidney samples. The IFAT results were used to determine the relative concentrations of *R. salmoninarum* in the fecal and kidney samples (Figure 5). It can be seen that the kidney and fecal material from the experimental coho appeared to be more heavily infected with the pathogen than the fecal and kidney material from the control coho. The kidney material was found to be more heavily infected than the fecal material for the experimental and control coho.

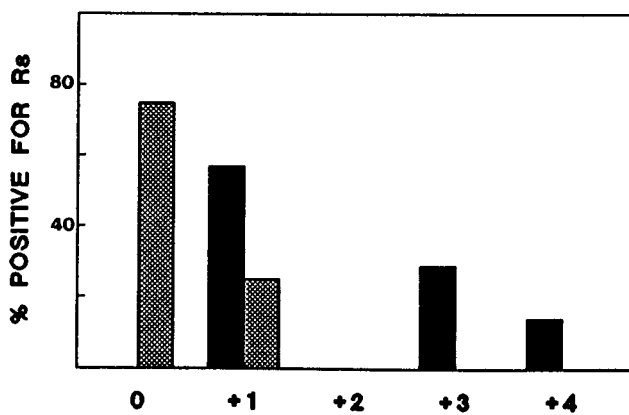
There was no significant difference in prevalence of infection (in survivors) between the intubated and the injected challenge groups in either pen (Table 4). There was also no significant difference between the prevalence of infection detected in the feces and kidney samples.

**FIGURE 5.** Relative concentrations of *R. salmoninarum* (Rs) in the kidney and fecal material collected from the experimental (n=7) and control (n=79) coho that survived 73 and 75 d post-challenge. Experimental coho were challenged by oral intubation and ip injection (150 coho by each method) with  $10^5$  cfu Rs and feces. Control coho were similarly challenged with sterile feces. The relative concentration of Rs was determined by the IFAT and expressed as the average number of Rs cells/100 field. The proportion of the sample of surviving coho in each of the following categories was then calculated for the (A) kidney and (B) fecal material:

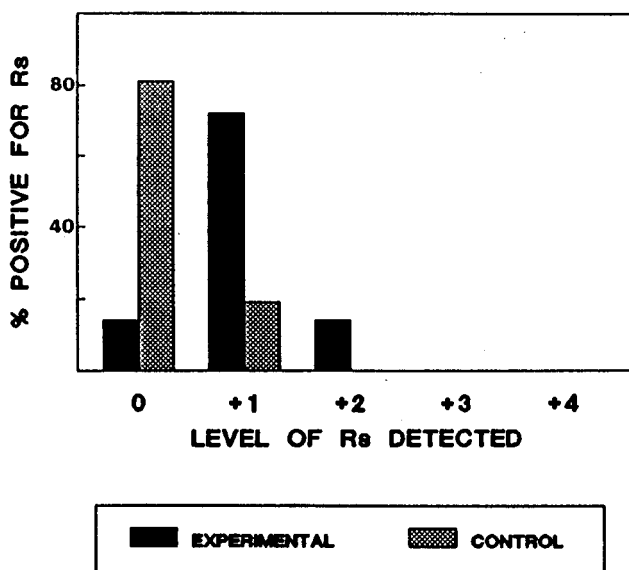
0 = 0 Rs cells/100 fields  
+1 = 1-10 Rs cells/100 fields  
+2 = 11-25 Rs cells/100 fields  
+3 = 26-50 Rs cells/100 fields  
+4 = >50 Rs cells/100 fields



### (A) KIDNEY



### (B) FECES



## DISCUSSION

The results from the research described herein support the hypothesis that horizontal transmission of *R. salmoninarum* occurs among salmonids reared in seawater netpens.

### **I. FECES AS A SOURCE OF R. salmoninarum**

Many researchers (Bullock et al. 1978; Bullock et al. 1980; Embley 1983; Austin and Rayment 1985) have detected *R. salmoninarum* in the fecal material of BKD affected salmonids. *R. salmoninarum* was also found in the fecal material of salmon examined during this research. Viable *R. salmoninarum* was detected in the feces of clinically and subclinically BKD affected chinook collected during the water sampling experiment (Table 2). *R. salmoninarum* was also detected in the fecal material of 5% of the coho sampled before the fecal challenge experiment was started (Table 4).

The fecal samples taken from the chinook collected during the water sampling experiment and cultured onto SKDM-C revealed that perhaps as many as  $1 \times 10^6$  cfu *R. salmoninarum* g feces<sup>-1</sup> may be present in fish heavily affected with BKD. Embley (1983) also found high numbers of *R. salmoninarum* ( $1 \times 10^7$  cfu g feces<sup>-1</sup>) in the fecal

material sampled from rainbow trout experimentally infected ( $10^7$  cfu ip injected per fish) with *R. salmoninarum*.

The high total prevalence of *R. salmoninarum* (95%) detected in the fecal material of the chinook collected during the water sampling experiment, indicates that it is likely that most, if not all of the infected chinook shed *R. salmoninarum* in their feces. The constant shedding of feces from BKD affected fish could, therefore, represent a significant source of *R. salmoninarum* to the sea water environment within netpens.

It is not known whether *R. salmoninarum* is a normal part of the microflora residing in the gastrointestinal tract of healthy salmonids. Studies examining the bacterial microflora of the gastrointestinal tract of healthy salmonids were directed towards the isolation of bacteria capable of growing on media that could not support the growth of *R. salmoninarum* (Strasidine and Dubetz, 1974; Colwell 1962; Trust et al. 1978; Austin and Al-Zahrani 1988).

The presence of *R. salmoninarum* in the gastrointestinal tract may be the result of ingesting *R. salmoninarum*-infected foodstuffs, or *R. salmoninarum* may enter the gastrointestinal tract during systemic infections. The manner in which *R. salmoninarum* contaminates fecal material in the gastrointestinal tract

is uncertain. *R. salmoninarum* may be released from the urinary tract and contaminate feces at the urogenital papillae. Another explanation is that *R. salmoninarum* may be released from the abdominal cavity to the feces via the abdominal pores that connect the intestine with the abdominal cavity (Goodrich 1958, as cited by Hoff 1989; Munro 1982).

Sera and Ishida (1972) have found that few bacteria are able to survive the adverse conditions in the stomach but that they can grow vigorously once in the intestine. The effect of the low pH and digestive enzymes present in the salmonid stomach on the viability of *R. salmoninarum* is not known. However, the ability of some researchers (Wood and Wallis 1955) to produce BKD infections in salmonids by feeding *R. salmoninarum* contaminated food indicates that some of these cells are able to withstand such conditions.

The results from the fecal challenge experiment conducted herein also support the hypothesis that some *R. salmoninarum* cells are able to survive the conditions within the stomach. Those coho that were orally intubated with *R. salmoninarum*-laden feces suffered significantly higher mortalities than the control fish (Table 3). The prevalence of *R. salmoninarum* in the kidney and fecal samples of the intubated fish was significantly higher than found for the control fish. These results indicate the

pathogen was able to survive the conditions of the stomach and result in a systemic infection of *R. salmoninarum* within those fish orally intubated with *R. salmoninarum*-laden feces.

## II. PRESENCE OF *R. salmoninarum* IN SEAWATER NETPENS

The water sampling experiment was successful in showing that viable *R. salmoninarum* cells occurred in the surface sea water taken from a netpen containing BKD-affected chinook. It was not possible to quantify the number of viable *R. salmoninarum* cells using the culture method because the culture plates were overwhelmed with faster growing seawater microflora. However, the MF-IFAT procedure used on the same water sample detected 255 ( $\pm$  179 SE) *R. salmoninarum* cells mL<sup>-1</sup> sea water (Table 2). This information suggests that sea water within netpens containing BKD-affected salmonids may contain "infectious doses" of *R. salmoninarum*. The source of the *R. salmoninarum* in the seawater sample was likely the infected fecal material shed from the chinook held in the experimental pen. *R. salmoninarum* was detected in fecal material from 95% of the chinook sampled from within the experimental pen (Table 2).

The hydrophobic characteristic (Bruno 1988) of *R. salmoninarum* may serve to prolong its survival in sea water. Gurijala and Alexander (1990) have demonstrated that hydrophobic bacteria are able to escape predation by protozoans because the bacteria tend to adsorb to surface microlayers and particulate materials too large to be ingested by the protozoans. These findings also suggest that most of the *R. salmoninarum* in the feces shed from an infected fish, will likely remain adsorbed to the organic matter in the feces rather than disperse throughout the water column.

Gowen and Bradbury (1987) estimate that approximately 26% of the food eaten by a salmon is eventually released as feces. At some commercial salmon farms (especially those farms that have BKD-affected fish on site) in B.C. this may have implications with respect to the transmission of pathogens such as *R. salmoninarum*.

Water currents play an important role in the distribution of fecal material and thereby probably affect the potential range for horizontal transmission. Based on the settling velocity of fecal material ( $0.017-0.06 \text{ m s}^{-1}$ ) (Warrer-Hansen 1979, cited by Gowen and Bradbury 1987), it has been calculated that on a typical salmon farm in B.C., feces may be distributed as far as 200 m from the source (Gowen and Bradbury 1987). This calculation considers the

water depth and current as well as the settling velocity of fecal pellets.

### III. SURVIVAL OF *R. salmoninarum* IN SEA WATER

Results from the survival experiments indicate that *R. salmoninarum* is capable of remaining viable in sea water for up to one week at 10°C (Figure 3). *R. salmoninarum* was detected by direct microscopic counts for up to 28 d, thereby suggesting that the pathogen may be able to survive in sea water for longer than the one week detected by culture. Evelyn (1988) reported that at 15°C, *R. salmoninarum* remained viable for 12 d in filter-sterilized sea water. Paclibare (1989) showed that *R. salmoninarum* was viable for less than 4 d in autoclaved sea water held at 17.6°C. Clearly, it can be seen that the survivability of *R. salmoninarum* in sea water is variable and depends on many factors such as temperature, nutrient availability and the initial (time zero) concentration of *R. salmoninarum*. Regardless, the survival of *R. salmoninarum* in sea water is limited. Consequently, the constant shedding of *R. salmoninarum* (via the feces of infected fish) into the sea water probably accounts for horizontal transmission of the pathogen between neighbouring fish.

The survival of *R. salmoninarum* in sterilized saline was similar to that found in sterilized sea water, except

that *R. salmoninarum* was viable for 2 d in saline and 7 d in the latter. This suggests that, unlike the organic nutrient depleted saline, sea water may have contained some nutrients that enabled *R. salmoninarum* to maintain its viability for a longer period of time. Viability in sea water may be enhanced because it contains components (i.e., inorganic salts and minerals) vital for such processes such as osmoregulation (Munro et al. 1989). Non-viable cells were detected by the MF-IFAT over the 28 d sampling period in both saline and filtered sea water.

The addition of peptone to the saline appeared to provide *R. salmoninarum* with an enriched environment because *R. salmoninarum* was found to be viable in P-S over the entire 28 d period (versus 2 d in saline alone).

The survival of *R. salmoninarum* in SKDM-2 broth demonstrates what occurs under close to ideal conditions. Growth of *R. salmoninarum* during the 28 d period may have been optimized further if nutrients such as L-cysteine were added at intervals to replenish the original levels (assumed to be depleted by growing cells).

*R. salmoninarum* cells were detected in the sterilized and unsterilized seawater solutions, (using the MF-IFAT) over the entire 28 day sampling period. The average number of *R. salmoninarum* cells detected on d 28 in the unsterilized sea water was significantly lower than that



detected in the sterilized sea water. It is possible that the flagellates and ciliates present in the unsterilized seawater may have grazed on the *R. salmoninarum* cells, thereby accounting for the lower number of cells detected.

The MF-IFAT detected significantly higher numbers of *R. salmoninarum* than detected by culture in the saline, P-S, filtered sea water, and seawater solutions. An explanation for the discrepancy between the numbers of *R. salmoninarum* cells detected by the two methods is that the MF-IFAT can detect both dead and living *R. salmoninarum* cells whereas the culture method can detect only living cells.

The disagreement in the results obtained with the two detection methods may also be related to problems associated with the culture of an organism as fastidious as *R. salmoninarum* (discussed later). Roszak and Colwell (1987) have found that bacterial populations can become sublethally stressed when maintained under conditions where nutrients may be limited. Highly variable culture data often result with stressed cells even with the use of selective growth media. Marine bacteria have developed survival mechanisms to survive in nutrient limited (stressed) environments, and these mechanisms have been used to explain the disagreement between culture and direct count data (Roszak and Colwell 1987). One survival strategy

is for stressed bacteria to enter a state of dormancy, akin to a state of metabolic arrest brought on by a lack of available energy. This would occur under conditions of nutrient starvation (Morita 1988). The problem of enumerating these dormant cells (i.e., the live but non-culturable cells) may be solved by using methods that measure such parameters as cellular respiration, enzyme activity and energy charge. The ability to 'resuscitate' dormant cells by the addition of nutrients has been demonstrated (Amy and Morita 1983; Roszak et al. 1984). There was no evidence found here to indicate that *R. salmoninarum* uses survival strategies to survive for extended periods of time in sea water. Therefore, the ability of *R. salmoninarum* to become resuscitated (or indeed whether resuscitation was necessary) by the addition of nutrients was not assessed.

Novisky and Morita (1978) have shown that some marine bacteria divide in response to limited nutrient conditions such as low organic carbon and nitrogen. The result is an increase in population number due to the formation of daughter cells. The daughter cells are proportionally reduced in size but have an increased surface area to volume ratio. This survival strategy may explain the observed increase in *R. salmoninarum* cells detected by the MF-IFAT in the P-S, SKDM-2 broth and FSW solutions. The MF-

IFAT stained *R. salmoninarum* cells were not sized during the survival experiment to provide information to support this hypothesis, but future work should include measurements of cell size in order to establish whether the reduction division survival strategy is used by nutrient stressed *R. salmoninarum* cells.

In general, slow growing bacteria with low metabolic rates have been shown to be better suited for survival in nutrient depleted environments (Moyer and Morita 1989; Roszak and Colwell 1987). *R. salmoninarum* is a slow growing bacterium closely related to the Coryneform group of bacteria - a group that has been shown to be well suited for survival during periods of nutrient deprivation (Boylen and Mulks 1978). This suggests the possibility that the survival of *R. salmoninarum* may be similarly suited for survival during periods of nutrient deprivation (i.e., as would occur when shed in the feces into sea water) by using a strategy like those described above.

#### **IV. EXPRESSION OF BKD IN SALMON IN CHALLENGED WITH R. salmoninarum-LADEN FECES.**

In the fecal-challenge experiment, the prevalence of *R. salmoninarum* detected in the experimentally infected coho was found to be significantly higher than detected in the control coho (Table 4). The numbers of mortalities in the experimentally infected group of coho were also

significantly higher (Table 3). In addition, Figure 5 shows that the number of *R. salmoninarum* cells in the surviving experimentally infected coho were much higher than the numbers detected in the surviving control coho. Together, these results clearly indicate that the experimentally treated fish were experiencing an active *R. salmoninarum* infection.

Wolf and Dunbar (1959) were not successful in producing BKD infections in brook trout (*Salvelinus fontinalis*) fed *R. salmoninarum*-laden feed probably because the fish were not observed long enough for the expression of BKD. These researchers considered BKD to have been transmitted if *R. salmoninarum* could be detected in Gram-stained kidney or liver smears. If the fish were observed for a longer period of time then the infection would have progressed further. This would have enabled the detection of *R. salmoninarum* by the Gram stain method (as is not a very sensitive method and higher numbers of the pathogen are required for its detection in infected tissue). Therefore the transmission of BKD (by their definition) would have been observed to have occurred. The chronic nature of BKD and the dose of *R. salmoninarum* fed to experimental fish are also key factors in the ability to produce disease by any challenge method. The pathogen level during initial

exposure to the pathogen is a major contributing factor to mortality rate (Wood 1974).

In general, the variability in mortality results from orally challenged fish may be due to the lack of detailed experimental information as to how *R. salmoninarum* is taken up from the gastro-intestinal tract to produce a systemic infection. Klontz (1983) suggests that macrophages enter the lumen, engulf *R. salmoninarum* and then return (with the pathogen) to the lamina propria. Another suggested mechanism is that *R. salmoninarum* gains access to the fish when intestinal mucosa are sloughed off (Klontz 1983).

#### **V. PREVALENCE OF BKD IN FARMED SALMON**

The inability of some researchers (Bjornn and Moffitt 1988) to demonstrate horizontal transmission may be attributed to the characteristic slow pathogenesis of BKD infections. The slow chronic progression of BKD is such that systemic lethal infections seldom show up in fish until they are six to twelve months of age (Evelyn 1988). Bjornn and Moffitt (1988) found no difference in the mortality between chinook salmon reared in groups and those reared individually, perhaps because the experiment was not carried out long enough for expression of BKD. Because these researchers did not measure changes in the prevalence of *R. salmoninarum* infection, they were only able to

conclude that if horizontal transmission of *R. salmoninarum* was occurring, it did not result in increased mortality.

Similarly, if in the present research the sampling had been continued for a longer period of time, site history may have had a more pronounced effect on the prevalence of BKD found at new and old sites. Perhaps if the salmon surveyed at Companies B and C were sampled several more times, a significant difference in the prevalence of *R. salmoninarum* would have been detected between the new and old site populations.

Water temperature has been found to play an important role in the pathogenesis of BKD in salmonids. Belding and Merrill (1935) were the first to report on the relationship between changing water temperature and mortality.

Sanders et al. (1978) reported that the shortest time to death occurs between 15 and 20.5 °C, which is the temperature range for the optimal growth of *R. salmoninarum* in vitro. A relationship between mortality and temperature could not be determined from the data collected during the BKD survey experiment because the cause of any mortality could not be attributed exclusively to BKD (refer to the Appendices). However, the prevalence of *R. salmoninarum* did appear to increase with increasing water temperature as seen for the sites belonging to Companies A and C. This was not observed in the coho sampled at Company B's sites where

temperatures were actually decreasing as the prevalence increased. Perhaps a clearer relationship could have been established if sampling at Company B's sites had continued through the summer months when water temperature would have presumably increased.

The chronic nature of BKD combined with low water temperatures may have contributed to the absence of disease signs in the coho that were challenged with the *R. salmoninarum*-laden feces. If the fecal challenge experiment had been continued for a longer period of time, then perhaps the experimental coho would have shown a greater prevalence of clinical BKD than the control coho.

The *R. salmoninarum*-treated coho of the fecal-challenge experiment did not at any time display clinical signs of BKD, nor was it possible to culture *R. salmoninarum* from any of the fish. Although the culture method has been found to be one of the most sensitive methods available for detecting *R. salmoninarum* (Paclibare 1989) it has some shortcomings (discussed below). Such drawbacks may be the reasons why the less sensitive IFAT was able to detect *R. salmoninarum* in this experiment while the culture method could not.

Despite the incorporation of antibiotics into the media of KDM-2 (Evelyn 1977) and KDM-C (Daly and Stevenson 1985), contamination of the agar plates was frequently a

problem when trying to isolate *R. salmoninarum* from fecal or seawater samples. It was found during this study that the presence of contaminants even inhibited the growth of the 'nurse culture' (Evelyn et al. 1989) used to accelerate *R. salmoninarum* growth.

Although not evident in this research, a newly discovered problem with the culture technique is the effect that different peptone sources have on the ability to culture *R. salmoninarum* (Evelyn and Prosperi-Porta 1989). The peptone source was changed during the course of this research but the effects of this change are not known. The inhibitory effects of salmonid tissue constituents on the growth of *R. salmoninarum* (as described by Daly and Stevenson 1988) is not likely to have reduced the sensitivity of the culture method, because the tissues were washed with P-S prior to culture.

Despite the limitations associated with the culture technique it is still a valuable detection method because it provides a measure of the viability of *R. salmoninarum*. In contrast, the IFAT does not distinguish between live and dead cells. It is because of this discrepancy between the two detection methods that the total prevalence was calculated and used in this research.



## SUMMARY AND CONCLUSIONS

The results from the experiments described herein suggest that high concentrations of *R. salmoninarum* are found in the feces of BKD-affected salmon. *R. salmoninarum* can be found in sea water of net pens that contain BKD-affected salmonids, and it survived in sea water for at least one week - long enough for horizontal transmission to occur among neighbouring fish. The prevalence of *R. salmoninarum* increased in young salmon orally challenged with *R. salmoninarum*-treated feces and survival of the challenged fish was significantly lower than that of the unchallenged controls.

The foregoing data are consistent with the hypothesis that horizontal transmission of *R. salmoninarum* in sea water occurs and that infections by this route account for the increases in prevalence of *R. salmoninarum* infections that were documented as occurring in the penned salmon with time. The data also suggest that horizontal infections may occur as a result of ingestion of *R. salmoninarum*-contaminated feces.

APPENDICES

APPENDIX A. Prevalence of *Renibacterium salmoninarum* (Rs) among wild and domestic chinook monitored at Company A. Sample size (N) was 57 at each sample date.

SAMPLE DATE	MEAN FISH WEIGHT	% PREVALENCE AS DETERMINED BY: <sup>a</sup>					TOTAL <sup>b</sup> PREVAL.	AVE. RS CFU g <sup>-1</sup> KIDNEY <sup>c</sup>	AVERAGE MONTHLY SW TEMP <sup>d</sup>	AVERAGE MONTHLY MORTAL <sup>d</sup>
		CLINICAL BKD SIGNS	KIDNEY IFAT	CULTURE SKDM-C	RS	TEMP				
04/87	208g	1.8 <sup>f</sup>	3.5 <sup>f</sup>	5.3 <sup>f</sup>	5.3 <sup>f</sup>	5.3 <sup>f</sup>	confl.	8.1 <sup>o</sup> C	0.2%	
06/87	329	0	5.3	7.0	12.3	12.3	2x10 <sup>3</sup>	11.4	1.0	
09/87	548	42.1	42.1	84.2	84.2	84.2	6x10 <sup>8</sup>	10.8	11.9	
11/87	557	15.8	49.1	89.5	89.5	89.5	9x10 <sup>9</sup>	8.9	12.2	
01/88	918	47.4 <sup>f</sup>	61.4 <sup>f</sup>	100.0 <sup>f</sup>	100.0 <sup>f</sup>	100.0 <sup>f</sup>	9x10 <sup>9</sup>	8.3	10.7	
04/88 <sup>e</sup>	1355	17.5	36.8	59.7	66.7	66.7	5x10 <sup>11</sup>	12.3	6.0	

- a Details of the detection methods are given in the Materials and Methods section.
  - b Total prevalence calculated as the total number of fish in that sample determined as being positive for the presence of Rs by either the IFAT or culture method.
  - c Results expressed as average colony forming units (cfu) of Rs g<sup>-1</sup> kidney material.
  - d Data obtained from records kept by personnel at Company A.
  - e Chinook received a 2 week oxytetracycline treatment two months prior to this sample.
  - f Statistically significant difference (p<0.001) between results from 04/87 and 01/88 sample dates.
- confl. = confluent growth of Rs.

**APPENDIX B.** Prevalence of *Renibacterium salmoninarum* (Rs) among normal domestic coho monitored at Company B. No mortality data was collected.

OLD SITE:

SAMPLE DATE	SAMPLE SIZE	MEAN FISH WEIGHT	% PREVALENCE AS DETERMINED BY: <sup>a</sup>				TOTAL <sup>b</sup> % PREVAL.	AVE. RS CFU g <sup>-1</sup> KIDNEYC	AVERAGE MONTHLY SW TEMPd
			CLINICAL BKD SIGNS	KIDNEY IFAT	CULTURE SKDM-C				
07/87	60	9.3g	0	1.7e	0	1.7e	0	14.30C	
09/87	54	45.6	0	3.7	0	3.7	0	14.9	
11/87	54	92.7	0	25.9	0	25.9	0	11.5	
02/88	39	90.1	0	38.5e	0	38.5e	0	8.0	

NEW SITE:

SAMPLE DATE	SAMPLE SIZE	MEAN FISH WEIGHT	% PREVALENCE AS DETERMINED BY: <sup>a</sup>				TOTAL <sup>b</sup> % PREVAL.	AVE. RS CFU g <sup>-1</sup> KIDNEYC	AVERAGE MONTHLY SW TEMPd
			CLINICAL BKD SIGNS	KIDNEY IFAT	CULTURE SKDM-C				
07/87	60	9.3g	0	1.7e	0	1.7e	0	14.30C	
09/87	54	49.0	0	1.8	0	1.8	0	14.9	
01/88	54	259.0	0	27.8	0	27.8	0	8.2	
03/88	60	344.4	0	25.0e	0	25.0e	0	8.7	

Appendix B. Continued on next page.

**Appendix B. Continued.**

- a Details of the detection methods are given in the Materials and Methods section.
- b Total prevalence calculated as the total number of fish in that sample determined as being positive for the presence of Rs by either the IFAT or culture method.
- c Results expressed as average colony forming units (cfu) of Rs g<sup>-1</sup> kidney material.
- d Data obtained from records kept by personnel at Company B.
- e Statistically significant difference ( $p < 0.001$ ) between results from the first sample (07/87) and the last sample (03/88 & 02/88).

**APPENDIX C.** Prevalence of *Renibacterium salmoninarum* (Rs) among wild feminized chinook monitored at Company C. Sample size (N) was 57 at each sample date.

OLD SITE:		% PREVALENCE AS DETECTED BY: <sup>a</sup>										AVERAGE MONTHLY MORTALITY	
SAMPLE DATE	FISH WEIGHT	CLINICAL BKD SIGNS	KIDNEY IFAT	CULTURE SKDM-C	TOTAL <sup>b</sup> % PREVAL.	AVE. RS CFU g <sup>-1</sup> KIDNEYC	AVERAGE MONTHLY SW TEMP <sup>d</sup>	AVERAGE MONTHLY MORTALITY		AVERAGE MONTHLY MORTALITY			
10/87	216g	0h	21.1h	0f	21.1g	0	11.0°C	0.5%		0.5%			
12/87	271	0	12.3	0	12.3	0	8.5	1.2		1.2			
03/88	422	3.5	15.8	24.6	28.1	3x10 <sup>9</sup>	8.5	0.3		0.3			
05/88	565	8.8h	22.8h	33.3f	45.6g	1x10 <sup>9</sup>	11.9	3.4		3.4			
08/88e	1222	0	15.8	0	15.8	0	14.8	na		na			

- a Details of the detection methods are given in the Materials and Methods section.
- b Total prevalence calculated as the total number of fish in that sample determined as being positive for the presence of Rs by either the IFAT or culture method.
- c Results expressed as average colony forming units (cfu) of Rs g<sup>-1</sup> kidney material.
- d Data obtained from records kept by personnel at Company C.
- e Chinook received a 2 week oxytetracycline treatment two months prior to this sample.
- f Statistically significant difference (p<0.001) between results from 10/87 and 05/88 sample dates.
- g Statistically significant difference (p<0.01) between results from 10/87 and 05/88 sample dates.
- h Statistically significant difference (p<0.05) between results from 10/87 and 05/88 sample dates.
- na = Information not available

**APPENDIX D.** Prevalence of *Renibacterium salmoninarum* (Rs) among domestic chinook monitored at Company C. Sample size (N) was 57 at each sample date.

<u>NEW SITE:</u>												
SAMPLE DATE	MEAN FISH WEIGHT	% PREVALENCE AS DETECTED BY: <sup>a</sup>			TOTAL <sup>b</sup> % PREVAL.	AVE. RS CFU g <sup>-1</sup> KIDNEYC	AVERAGE MONTHLY SW TEMP <sup>d</sup>	AVERAGE MONTHLY MORTAL <sup>d</sup>				
		CLINICAL BKD SIGNS	KIDNEY IFAT	CULTURE SKDM-C					CONFL.	14.8°C	11.0	1.0%
08/87	7g	3.5h	7.0hi	1.89h	7.0fi	confl.	14.8°C	na				
10/87	74	0	7.0	0	7.0	0	11.0	1.0%				
12/87	81	0	10.5	0	10.5	0	8.5	na				
**CHINOOK GRADED 01/88. SMALL GRADES (<1kg) REMAINED @ NEW SITE & LARGE GRADES (>1kg) MOVED TO OLD SITE. MONITORING OF THE CHINOOK CONTINUED IN THE TWO SEPARATED POPULATIONS. RESULTS BELOW:												
<u>NEW @ NEW SITE:</u>												
02/88	58	0	28.1	0	28.1	0	8.0°C	1.1%				
05/88	185	7.0h	21.1h	8.8h	24.6i	1x10 <sup>4</sup>	11.9	1.1				
08/88 <sup>e</sup>	694	0	12.3	0	12.3	0	14.8	na				
<u>NEW @ OLD SITE:</u>												
03/87	224g	0	15.8	0	15.8	0	8.5°C	0.6%				
05/88	355	3.5h	22.2i	21.19	36.8f	5x10 <sup>9</sup>	11.9	3.3				
08/88	760	3.6	14.6	1.8	14.6	2x10 <sup>9</sup>	14.8	na				

Appendix D. Continued on next page.

**Appendix D. Continued.**

- a Details of the detection methods are given in the Materials and Methods section.
  - b Total prevalence calculated as the total number of fish in that sample determined as being positive for the presence of Rs by either IFAT or culture method.
  - c Results expressed as average colony forming units (cfu) of Rs g<sup>-1</sup> kidney material.
  - d Data obtained from records kept by personnel at Company C.
  - e Chinook received a 2 week oxytetracycline treatment two months prior to this sample.
  - f Statistically significant difference ( $p < 0.001$ ) between results from 8/87 and 05/88 sample dates.
  - g Statistically significant difference ( $p < 0.01$ ) between results from 8/87 and 5/88 sample dates.
  - h Statistically significant difference ( $p < 0.05$ ) between results from 8/87 and 5/88 sample dates.
  - i No statistically significant difference ( $p > 0.05$ ) between results from 8/87 and 5/88 sample dates.
- na = Information not available  
confl = confluent growth of Rs.

## REFERENCES

- Amy, P.S. and R.Y. Morita. (1983). Starvation-survival patterns of sixteen freshly isolated open-ocean bacteria. *Appl. Environ. Microbiol.* 45: 1109-1115.
- Armstrong, R.D., S.W. Martin, T.P.T. Evelyn, B. Hicks, W.J. Dorward and H.W. Ferguson. (1989). A field evaluation of an indirect fluorescent antibody-based broodstock screening test used to control the vertical transmission of *Renibacterium salmoninarum* in chinook salmon (*Oncorhynchus tshawytscha*). *Can. J. Vet. Res.* 53: 384-389.
- Austin, B. and A.M. Al-Zahrani. (1988). The effect of antimicrobial compounds on the gastrointestinal microflora of rainbow trout, *Salmo gairdneri* Richardson. *J. Fish. Biol.* 33: 1-14.
- Austin, B. and J.N. Rayment. (1985). Epizootiology of *Renibacterium salmoninarum*, the causal agent of bacterial kidney disease in salmonid fish. *J. Fish Dis.* 8: 505-509.
- Austin, B., T.M. Embley and M. Goodfellow. (1983). Selective isolation of *Renibacterium salmoninarum*. *FEMS Microbiol. Lett.* 17: 111-114.
- Belding, D.L. and B. Merrill. (1935). A preliminary report upon a hatchery disease of the Salmonidae. *Trans. Am. Fish. Soc.* 65: 76-84.
- Bell, G.R., D.A. Higgs and G.S. Traxler. (1984). The effect of dietary ascorbate, zinc, and manganese on the development of experimentally induced bacterial kidney disease in sockeye salmon (*Oncorhynchus nerka*). *Aquacult.* 36: 293-311.
- Bjornn, T.C. and C.M. Moffitt. (1988). Mortality from bacterial kidney disease in chinook salmon reared individually or in groups in seawater. In: Abstracts, American Fisheries Society Fish Health Section, International Fish Health Conference Handbook, Vancouver, B.C., Canada. July 19-21, 1988. p.30.
- Boylen, C.W. and M.H. Mulks. (1978). The survival of Coryneform bacteria during periods of prolonged nutrient starvation. *J. Gen. Microbiol.* 105: 323-334.



- Bruno, D.W. (1986). Histopathology of bacterial kidney disease in laboratory infected rainbow trout, *Salmo gairdneri*, Richardson, and Atlantic salmon, *Salmo salar* L., with reference to naturally infected fish. *J. Fish Dis.* 9: 523-537.
- Bruno, D.W. (1988). The relationship between auto-agglutination, cell surface hydrophobicity and virulence of the fish pathogen *Renibacterium salmoninarum*. *FEMS Microbiol. Lett.* 51: 135-140.
- Bullock, G.L. (1980). Bacterial kidney disease of salmonid fishes caused by *Renibacterium salmoninarum*. U.S. Fish. Wildl. Serv. Fish Dis. Leaflet 60. 10 pp.
- Bullock, G.L., H.M. Stuckey and D. Mulcahy. (1978). Corynebacterial kidney disease: egg transmission following iodophore disinfection. *Fish Health News* 7: 51-52.
- Bullock, G.L., B.R. Griffin and H.M. Stuckey. (1980). Detection of *Corynebacterium salmoninus* by direct fluorescent antibody test. *Can. J. Fish. Aquat. Sci.* 37: 719-721.
- Colwell, R.R. (1962). The bacterial flora of Puget Sound fish. *J. Appl. Bacteriol.* 25: 147-158.
- Daly, J.G. and R.M.W. Stevenson. (1985). Charcoal agar, a new growth medium for the fish disease bacterium *Renibacterium salmoninarum*. *Appl. Environ. Microbiol.* 50: 868-871.
- Daly, J.G. and R.M.W. Stevenson. (1988). Inhibitory effects of salmonid tissue on the growth of *Renibacterium salmoninarum*. *Dis. Aquat. Org.* 4: 169-171.
- Elliott, D.G., R.J. Pascho and G.L. Bullock. (1989). Developments in the control of bacterial kidney disease of salmonid fishes. *Dis. Aquat. Org.* 6: 201-215.
- Embley, T.M. (1983). Aspects of the biology of *Renibacterium salmoninarum*. PhD Thesis, University of Newcastle-upon-Tyne. 317 pp.
- Evelyn, T.P.T. (1977). An improved growth medium for the kidney disease bacterium and some notes on using the medium. *Bull. Off. int. Epiz.* 87: 511-513.

- Evelyn, T.P.T. (1988). Bacterial kidney disease in British Columbia, Canada: comments on its epizootiology and on methods for its control on fish farms. In: AQUA NOR 87 Trondheim International Conference, Norske Fiskeoppdretteres Forening-Fiskeoppdretternes Salgslag A/L, Trondheim, Norway. p. 51-57.
- Evelyn, T.P.T., G.R. Bell, L. Prosperi-Porta and J.E. Ketcheson. (1989). A simple technique for accelerating the growth of the kidney disease bacterium *Renibacterium salmoninarum* on a commonly used culture medium (SKM2). Dis. Aquat. Org. 7: 231-234.
- Evelyn, T.P.T., J.E. Ketcheson and L. Prosperi-Porta. (1981). The clinical significance of immunofluorescence-based diagnoses of the bacterial kidney disease carrier. Fish Pathol. 15: 293-300.
- Evelyn, T.P.T., J.E. Ketcheson and L. Prosperi-Porta. (1984). Further evidence for the presence of *Renibacterium salmoninarum* in salmonid eggs and for the failure of povidone-iodine to reduce the intra-ovum infection rate in water-hardened eggs. J. Fish Dis. 7: 173-182.
- Evelyn, T.P.T., and L. Prosperi-Porta. (1989). Inconsistent performance of KDM2, a culture medium for the kidney disease bacterium *Renibacterium salmoninarum*, due to variation in the composition of its peptone ingredient. Dis. Aquat. Org. 7: 227-229.
- Fryer, J.L. and J.E. Sanders. (1981). Bacterial kidney disease of salmonid fish. Ann. Rev. Microbiol. 35: 273-298.
- Gowen, R.J. and N.B. Bradbury. (1987). The ecological impact of salmonid farming in coastal waters: a review. Oceanogr. Mar. Biol Ann. Rev. 25: 563-575.
- Gurijala, K.R. and M. Alexander. (1990). Effect of growth rate and hydrophobicity on bacteria surviving protozoan grazing. Appl. Environ. Microbiol. 56: 1631-1635.
- Hoff, K.A. (1989). Occurrence of the fish pathogen *Vibrio salmonicida* in faeces from Atlantic salmon, *Salmo salar* L., after experimental infection. J. Fish Dis. 12: 595-597.

- Klontz, G.W. (1983). Bacterial kidney disease in salmonids: an overview. In: D.P. Anderson, M. Dorson, and P.H. Dubourget (eds.) *Antigens of Fish Pathogens*. Collection Foundation Marcel Marioux. p. 177-199.
- Lee, E.G.-H. (1989). Technique for enumeration of *Renibacterium salmoninarum* in fish kidney tissues. *J. Aquat. Anim. Health* 1: 25-28.
- Mitchum, D.L. and L.E. Sherman. (1981). Transmission of bacterial kidney disease from wild to stocked hatchery trout. *Can. J. Fish. Aquat. Sci.* 38: 547-551.
- Morita, R.Y. (1988). Bioavailability of energy and its relationship to growth and starvation survival in nature. *Can. J. Microbiol.* 34: 436-441.
- Moyer, C.L. and R.Y. Morita. (1989). Effect of growth rate and starvation-survival on cellular DNA, RNA, and protein of a psychrophilic marine bacterium. *Appl. Environ. Microbiol.* 55: 2710-2716.
- Munro, A.L. (1982). The pathogenesis of bacterial diseases in fishes. In: R.J. Roberts (ed.) *Microbial Diseases of Fish*. Academic Press, New York. p. 130-150.
- Munro, P.M., M.J. Gauthier, V.A. Breittmayer, and J. Bongiovanni. (1989). Influence of osmoregulation processes on starvation survival of *Escherichia coli* in seawater. *Appl. Environ. Microbiol.* 55: 2017-2024.
- Novitsky, J.A. and R.Y. Morita. (1978). Possible strategy for the survival of marine bacteria under starvation conditions. *Mar. Biol.* 48: 289-295.
- Ossiander, F.J. and G. Wedemeyer. (1973). Computer program for sample sizes required to determine disease incidence in fish populations. *J. Fish. Res. Board Can.* 30: 1383-1384.
- Paclibare, J.O. (1989). Aspects of the epizootiology of bacterial kidney disease in salmon farms in British Columbia. MSc. Thesis, Simon Fraser University, Burnaby, B.C. 49 p.
- Roszak, D.B. and R.R. Colwell. (1987). Survival strategies of bacteria in the natural environment. *Microbiol. Rev.* 51: 365-379.

- Roszak, D.B., D.J. Grimes and R.R. Colwell. (1984). Viable but nonrecoverable stage of *Salmonella enteritidis* in aquatic systems. *Can. J. Microbiol.* 30: 334-338.
- Sanders, J.E. and J.L. Fryer. (1980). *Renibacterium salmoninarum* gen. nov., sp. nov., the causative agent of bacterial kidney disease in salmonid fishes. *Intl. J. Syst. Bacteriol.* 30: 496-502.
- Sanders, J.E., K.S. Pilcher and J.L. Fryer. (1978). Relation of water temperature to bacterial kidney disease in coho salmon (*Oncorhynchus kisutch*), sockeye salmon (*O. nerka*), and steelhead trout (*Salmo gairdneri*). *J. Fish. Res. Board Can.* 35: 8-11.
- Sera, H. and Y. Ishida. (1972). Bacterial flora in the digestive tracts of marine fish-II. Changes of bacterial flora with time lapse after ingestion of diet. *Bull. Jap. Soc. Sci. Fish.* 38: 633-637.
- Smith, I.W. (1964). The occurrence and pathology of Dee disease. Dept. Agric. Fish. Scot. Freshwater and Salmon Fisheries Research No. 34, Her Majesty's Stationery Office, Edinburgh. 12 pp.
- Strasdine, G.A. and L. Dubetz. (1974). Coliform flora of migrating sockeye salmon (*Oncorhynchus nerka*). *J. Fish. Res. Board Can.* 31: 1559-1560.
- Trust, T.J., J.I. MacInnes and K.H. Bartlett. (1978). Variations in the intestinal microflora of salmonid fishes. In: M.W. Loutit and J.A.R. Miles (eds.) *Microbial Ecology*, Springer-Verlag, Berlin. pp.250-254.
- Velji, M.I. and L.J. Albright. (1986). Microscopic enumeration of attached marine bacteria of sea water, marine sediment, fecal matter and kelp blade samples following pyrophosphate and ultrasound treatments. *Can. J. Microbiol.* 32: 121-126.
- Wolf, K. and C.E. Dunbar. (1959). Methods of infecting trout with kidney disease and some effects of temperature on experimental infections. U.S. Dept. Inter. Fish. Wildl. Serv. Spec. Sci. Rep. Fish. No. 286. 8 pp.
- Wood, J.W. (1974). Diseases of Pacific salmon, their prevention and treatment. Dept. Fish., Olympia, Wash. 82 pp.

- Wood, J.W. and J. Wallis. (1955). Kidney disease in adult chinook salmon and its transmission by feeding to young chinook salmon. Res. Briefs, Fish. Comm. of Oregon. 6: 32-40.
- Yoshimizu, M. and T. Kimura. (1976). Study on the intestinal microflora of salmonids. Fish Pathol. 10: 243-259.
- Young, C.L. and G.B. Chapman. (1978). Ultrastructural aspects of the causative agent and renal histopathology of bacterial kidney disease in brook trout (*Salvelinus fontinalis*). J. Fish. Res. Board Can. 35: 1234-1248.
- Zar, J.H. (1974). Biostatistical Analysis. Prentice-Hall, Englewood Cliffs, N.J. 620 p.