ASPECTS OF THE EPIZOOTIOLOGY OF BACTERIAL KIDNEY DISEASE IN SALMON FARMS IN BRITISH COLUMBIA

by

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ABSTRACT

Bacterial kidney disease (BKD), caused by *Renibacterium salmoninarum*, is one of the most important diseases of farmed salmonids in British Columbia and, although the disease has been recognized for many years, knowledge of its epizootiology in the marine environment is incomplete. In particular, little is known of the reservoirs of infection in sea water and of the mechanisms of transmission to farmed salmonids.

The objectives of this investigation were: 1) to determine whether *R. salmoninarum* occurs in non-salmonid fish species and in the blue mussel *Mytilus edulis* (commonly found in and around salmon farm pens) from selected salmon farm sites in British Columbia , 2) to study the clearing of *R. salmoninarum* from sea water by *M. edulis,* and 3) to determine, experimentally, the relative susceptibility to *R. salmoninarum* of a non-salmonid fish species and a salmonid (i.e., *Cymatogaster aggregata* (shiner perch) and *Oncorhynchus tshawytscha* (chinook salmon), respectively).

A comparative evaluation of various techniques for the detection of *R. salmoninarum* was also conducted to determine the most appropriate technique for assaying for the pathogen. Techniques evaluated were the culture method, the indirect fluorescent antibody technique (IFAT), the immunoenzyme assay method (conducted on nitrocellulose membranes) (IEA), and the counterimmunoelectrophoresis method (CIE). Based on this evaluation, the culture and IFAT methods were chosen for routine assays of *R. salmoninarum*.

A total of 288 non-salmonid fishes was collected from inside and around pens of salmon farms, in which BKD was known to occur. These were *Cymatogaster aggregata*, *Gasterosteus aculeatus*, *Clupea harengus pallasi*, *Sebastes caurinus*, *Hydrolagus colliei*, and *Ophiodon elongatus*. In addition, 146 *M. edulis* were removed from the net pens at 3 *R. salmoninarum*-infected farms. The pathogen was not detected in any of the non-salmonid finfishes or in the mussels. During the survey, high agglutinating titres against *R. salmoninarum* were found in the plasma of *R. salmoninarum*-free *Sebastes caurinus* caught under salmon farm nets but similar titres were also found in samples of this species collected well away from salmon farms.

M. edulis concentrated *R. salmoninarum* cells from sea water (although inefficiently when the cells occured singly) and, based on *in vitro* tests, was capable of rapidly inactivating the ingested *R. salmoninarum* cells, apparently by digestion.

The chinook salmon proved to be far more susceptible to *R. salmoninarum* infection than the shiner perch. Based on these findings, non-salmonid fishes and mussels do not appear to be reservoirs for BKD infection of farmed salmonids.

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INTRODUCTION

Bacterial kidney disease (BKD) is a chronic systemic infection of salmonids characterized by the presence of nodular lesions (granulomata) in the kidneys, spleen, and liver; pinpoint ulcerations in the epidermis; bilateral exophthalmia; and a pale empty gastrointestinal tract (Klontz, 1982). Since its first report in Scotland in 1930, under the name Dee disease in Atlantic salmon, *Salmo salar*, it has become widely recognized as one of the most important infectious diseases of hatchery-reared salmonids (Sanders and Fryer, 1980). It has been found to occur in almost all species of hatchery-reared salmonids throughout North America, Europe, and Japan. BKD is the most serious disease of pen-reared salmonids in British Columbia (T.P.T. Evelyn, pers. comm.).

The causative agent of BKD is *Renibacterium salmoninarum*. It is a small (0.3 - 0.5 µm by 1.0 - 1.5 µm) Gram-positive diplobacillus. It is nonmotile, noncapsulated, non-acid fast, and nonsporulating (Sanders and Fryer, 1980). Culture of *R. salmoninarum* on kidney disease medium 2 (KDM 2) produces creamy (non-pigmented), smooth, round, raised, entire, 2-mm diameter colonies after incubation at 15 ^{o}C for 20 days (Austin and Austin, 1988). It is aerobic and requires cysteine for growth (Fryer and Sanders, 1981).

R. salmoninarum has been isolated from populations of 13 finfish species in the genera- *Oncorhynchus*, *Salmo*, *Salvelinus*, and *Thymallus* - members of the family Salmonidae (Fryer and Sanders, 1981; Kettler *et al.*, 1986; Souter *et al.*, 1987). It has been presumed that *R. salmoninarum* is an obligate pathogen of salmonid fish, as there has been no report of this organism occurring in non-salmonid fishes (Evelyn, 1987).

R. salmoninarum is transmitted horizontally (i.e., among individuals) in fresh water (Mitchum and Sherman, 1981; Bell *et al.*, 1984) and in sea water (Evelyn, 1987) , and vertically via eggs (Evelyn *et al.*, 1984). The bacterium appears to be an obligate fish pathogen because it does not survive indefinitely outside its host. However, Austin and Rayment (1985) demonstrated that *R. salmoninarum* could survive for up to 28 days in sterilized river water. They were, however, unable to detect *R. salmoninarum* in water or sediments from freshwater fish farms that they surveyed.

The epizootiology of this disease in sea water is only imperfectly understood. One aspect that needs elucidation is the reservoir of infection in sea water. There is good circumstantial evidence to suggest that horizontal transmission among farmed salmonids occurs in sea water (Evelyn, 1987). However, whether non-salmonid fishes and mussels, living in association with farmed salmonids, also serve as reservoirs of *R. salmoninarum* is not known.

To date, certain non-salmonid fish species i.e., *Anoplopoma fimbria* (sablefish) and *Clupea harengus pallasi* (Pacific herring) have been shown to be capable of harbouring the bacterium under experimental conditions (Bell *et al.*, 1988; Traxler and Bell, 1988). However, another non-salmonid species, *Lampetra tridentata* (Pacific lamprey), was shown to be refractory to infection (Bell and Traxler, 1986).

To address the question- Do non-salmonid fishes and mussels serve as reservoirs of infection? - studies were conducted with the following objectives:

1) To determine whether *R. salmoninarum* occurs in non-salmonid fish species and in the blue mussel *Mytilus edulis* commonly found in and around salmon farm pens,

2) To study the clearing of *R. salmoninarum* from sea water by *M. edulis* , and

3) To determine, experimentally, the relative susceptibility to *R. salmoninarum* of a non-salmonid fish species and a salmonid (i.e., *Cymatogaster aggregata* (shiner perch) and *Oncorhynchus tshawytscha* (chinook salmon), respectively).

A comparative evaluation of various techniques for the detection of *R. salmoninarum* was also conducted to determine the most appropriate technique for assaying for the pathogen. Techniques evaluated were the culture method, the indirect fluorescent antibody technique (IFAT), the immunoenzyme assay method (conducted on nitrocellulose membranes) (IEA), and the counterimmunoelectrophoresis method (CIE).

MATERIALS AND METHODS

COMPARATIVE EVALUATION OF VARIOUS METHODS FOR DETECTING *R. salmoninarum* IN SALMONIDS

R. salmoninarum cells mixed with kidney homogenates

A suspension of *R. salmoninarum* (Pacific Biological Station, Nanaimo, B.C. strain 384) in 0.1% peptone + 0.85% saline (P-S) was prepared using cells obtained from a culture grown on kidney disease medium 2 (KDM 2) (Evelyn, 1977). The suspension was divided into four equal portions and each was serially diluted in ten-fold steps using P-S. Kidney tissue (2 g) from a healthy rainbow trout, *Salmo gairdneri*, was homogenized in ice cold P-S (10% w/v) using a Polytron homogenizer for 1 min and equal volumes of the kidney homogenate were added to each of the dilutions of the cell suspension. The first and second series of cell suspension : kidney homogenate mixtures were used as test materials for culture and indirect fluorescent antibody technique (IFAT) assays, respectively. The remaining two series were each heat-treated (100 ^{o}C for 30 min) and centrifuged at 500 g for 10 min. The supernatant fluids were used as test materials for the immunoenzyme (IEA) and counterimmunoelectrophoresis (CIE) assays .

Culture method

Twenty-five ul aliquots of the homogenate dilutions were drop-inoculated onto plates of selective kidney disease medium (SKDM) (Austin *et al.*, 1983). The plates were incubated, inverted, in plastic bags at 15 °C and colonies were counted on the 40th day of incubation.

Indirect fluorescent antibody technique

The IFAT was carried out using the method of Bullock and Stuckey (1975) with several modifications. Smears were prepared by uniformly spreading 10 µl aliquots of the homogenate dilutions within 8 mm diameter circles (drawn by a Manostat Tech Pen, N.Y.) on a microscope slide. Cells were detected using rabbit anti- *R. salmoninarum* serum (Microtek Ltd., Sidney, B.C.) and goat anti- rabbit globulin conjugated to fluorescein isothiocyanate (Sigma, St. Louis, MO.).

Immunoenzyme assay

The IEA was carried out using the procedure of Bio-Rad (undated). A 3 µl aliquot of test material was blotted onto a nitrocellulose membrane. Antigen was detected using rabbit anti- *R. salmoninarum* serum (Microtek Ltd.) and goat anti-rabbit globulin - horseradish peroxidase conjugate (Bio-Rad, Richmond, CA.). The compound 4-chloro-1-naphthol served as the colour development reagent. The presence of antigen was indicated by a purple-violet spot against a white background.

Counterimmunoelectrophoresis

The CIE assay was performed using the method of Cipriano *et al.*(1985), with some modifications. Electrophoresis was run using a 1% agarose gel with opposing rows of 6 mm diameter wells. The anodic and cathodic wells were filled with 50 µl of anti- *R. salmoninarum* serum (Microtek Ltd.) and with 50 µl of test material, respectively. A positive reaction was indicated by the formation of a visible precipitin band between the antibody- and antigen-containing wells.

Kidney homogenates from *R. salmoninarum*-infected chinook salmon

The foregoing assays were also carried out on kidney homogenates derived from chinook salmon, *Oncorhynchus tshawytscha*, showing overt BKD.

Five 20% w/v kidney homogenates in P-S were prepared from five chinook salmon as described previously. Each homogenate was divided into four equal parts, each of which was serially diluted in ten-fold steps in P-S. As before, one series of unheated dilutions was examined by culture and the other by IFAT; the other two series of dilutions were heat-treated, one of them being tested by the IEA and the other by CIE.

TEST FOR THE PRESENCE OF *R. salmoninarum* IN NON-SALMONID FINFISHES AND *Mytilus edulis* SAMPLED FROM SELECTED *R. salmoninarum*-INFECTED FARMS IN BRITISH COLUMBIA

Survey

A survey was conducted to determine whether *R. salmoninarum* occurred in non-salmonid finfish species and in the blue mussel from selected farm sites in British Columbia i.e., at Port Alberni, Egmont, Tofino, Quadra Island, and Nanaimo (Fig. 1). The farms all contained *R. salmoninarum*-infected stocks of salmon. A total of 288 non-salmonid finfishes was collected by angling from inside and within 5 m of farm pens. The non-salmonid finfishes were *Cymatogaster aggregata* (shiner perch), *Gasterosteus aculeatus* (three-spine stickleback), *Clupea harengus pallasi* (Pacific herring), *Sebastes caurinus* (copper rockfish), *Hydrolagus colliei* (ratfish), and *Ophiodon elongatus* (lingcod). In addition, 146 *M. edulis* (blue mussels) were removed from the net pens at 3 *R. salmoninarum*-infected farms (Table 1). Samples were taken from the outermost and innermost portions of the mussel aggregates on the nets.

Each fish and mussel was examined for the presence of *R. salmoninarum* by culture and by microscopy (IFAT). In addition, plasma samples from the finfish specimens were tested for agglutinating activity versus *R. salmoninarum* and other fish pathogens (see below) using the standard microtitre agglutinating technique (Cooke Engineering Co., Alexandria,Va.).

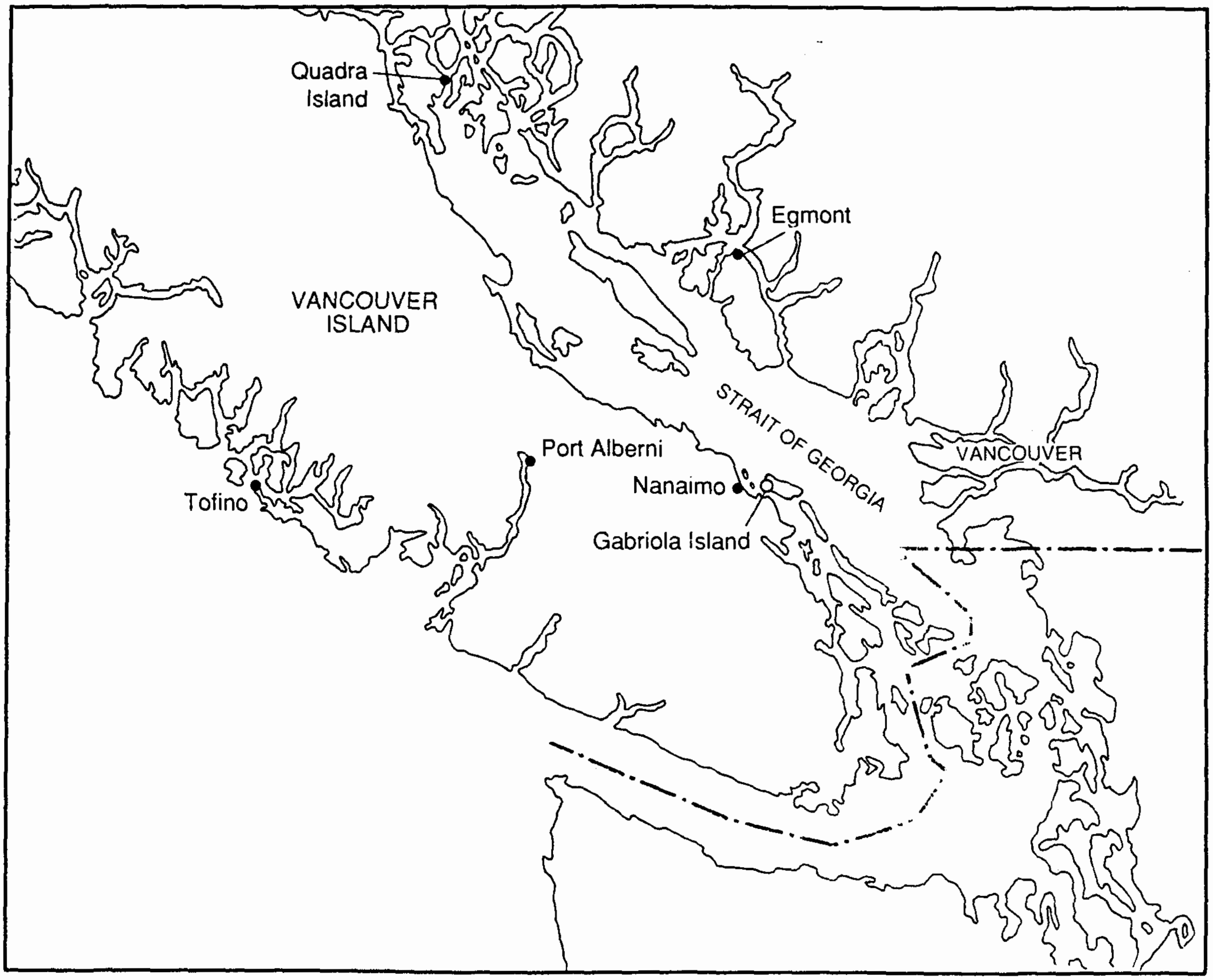

Fig. 1. Salmon farm sites surveyed in British Columbia, Canada.

Table 1. List of fish and shellfish species collected from salmon farm sites surveyed in British Columbia, showing sample size per site and mean weight of each sample lot.

Species	Site	Sample size	Mean weight (g)
Cymatogaster aggregata	Port Alberni	51	19.27
	Egmont	50	42.16
	Nanaimo	25	9.6
Gasterosteus aculeatus	Port Alberni	25	0.74
	Tofino	14	0.82
Clupea harengus pallasi	Quadra Island	48	7.88
	Nanaimo	60	109.02
Sebastes caurinus	Egmont	2	332.54
	Nanaimo	4	346.08
Hydrolagus colliei	Nanaimo	8	498.32
Ophiodon elongatus	Nanaimo	1	235.20
Mytilus edulis	Port Alberni	70	8.24
	Quadra Island	26	7.86
	Egmont	50	4.28

Samples were weighed and dissected aseptically, taking note of any gross pathology. Kidney materials from fish and intestinal materials from the mussels were streaked onto plates of SKDM. Positive controls consisting of plates inoculated with a known strain of *R. salmoninarum* were also included with each batch of cultured samples to ensure that the medium supported the growth of the pathogen. Culture plates were incubated at 15 ^{o}C in plastic bags for approximately 6 weeks. Plates were examined for the presence of slow growing, circular, smooth edged, creamy-white colonies typical of the pathogen. Gram and IFAT stains were done on smears from representative colonies to determine whether the colonies were indeed *R. salmoninarum*.

Duplicate smears of kidney tissue from the finfish samples and gut material from the mussels were prepared and stained by IFAT. Positive control smears prepared from a known strain of the pathogen were also made and IFAT-stained to ensure that the stain performed as expected. The IFAT-stained smears were examined using an epifluorescence microscope. A total of 100 microscopic fields per sample was examined.

Plasma agglutinating titres against killed cells of *R. salmoninarum* (PBS 384) and of other fish pathogens, namely *Aeromonas salmonicida* (PBS 76-30), *Vibrio anguillarum* (PBS C1), and *Yersinia ruckeri* (PBS 75-199) were determined using 25 µl of plasma in two-fold dilutions in microtitre "U" plates with saline as diluent. Equal volumes of killed cells adjusted to an optical density of 2.5 at 540 nm absorbance were added to each well and mixed. Cells for the assays were formalin-killed (0.3 % v/v) and, except for *V. anguillarum*, were heated at 70 °C for 30 min. The lowest dilution of plasma tested was 1:2. The plates were incubated overnight at 15 °C and then examined for agglutination. The highest plasma dilution showing agglutination was recorded as the titre. Tests were done in duplicate. Mussel samples were not assayed by this technique.

Comparison of *S. caurinus* plasma agglutinating titres

During the course of the survey, high agglutinating titres against *R. salmoninarum* were found in the plasma of *Sebastes caurinus* (rockfish) caught under salmon farm nets (Table 4). An investigation was therefore carried out to compare the plasma agglutinating titres from samples collected from a salmon farm with BKD infected stocks and from samples collected at a site well away from a salmon farm. Samples of an additional 15 and 10 *S. caurinus* were collected from the salmon farm of Pacific Biological Station, Nanaimo and from a site near Gabriola Island, well away from any salmon farm, respectively. The presence of *R. salmoninarum* was examined by culture of and IFAT staining on kidney materials. Plasma agglutinating titres against *R. salmoninarum* were determined by the standard procedure already described. The titres against the other bacterial pathogens i.e., *A. salmonicida*, *V. anguillarum*, and *Y. ruckeri* were also determined.

EXPERIMENTAL CLEARING OF *R. salmoninarum* FROM SEA WATER BY *M. edulis*

Uptake and retention of *R. salmoninarum* by *M. edulis*

Two hundred and forty *M. edulis* with mean weight of 3.7 g were collected from the intertidal region of Departure Bay, Nanaimo, B.C.. The specimens were immediately placed in a tank containing 10 L of 25 ppt. salinity sea water (at 15 ^{o}C) to which *R. salmoninarum* had been added to a concentration of 5 x 10^6 cells ml^{-1}. The mussels remained in this water for 20 hr. Following this exposure to *R. salmoninarum*, each mussel was removed and briefly rinsed in flowing sea water for several minutes. Twenty mussels were then sacrified (time 0 sample) and the remaining 220 mussels were placed in a net which was hung in the sea water at Departure Bay, B.C..

Samples of 20 mussels each were removed from the net after 1, 3, 5, 7, 9, 11, 24, 48, 72, 96, and 120 h. Smears and histological sections of mantle, gill and gut of each animal were then examined for the presence of *R. salmoninarum* using the Gram stain technique. (In this early experiment, IFAT examination of the smears was not conducted because of inexperience with the technique on the part of the investigator.)

Inactivation of *R. salmoninarum* by *M. edulis* gut extract

The gut materials from 30 *M. edulis* were collected using 2.5 ml of sterile physiological saline (0.85% NaCl) (S); the resulting suspension was 61% (w/v) gut material. Mantle tissue was also collected in a similar fashion; the resulting suspension was 65% (w/v) mantle tissue. After collection, each suspension was immediately homogenized and centrifuged at 15,000 rpm for 20 min. The supernatant fluids were then collected and an equal volume of *R. salmoninarum* cell suspension was added to each.

The *R. salmoninarum* suspension was prepared by growing the bacterium on KDM 2 for 10 days and harvesting the cells using physiological saline. The absorbance of the suspension was adjusted to 4.0 at 540 nm.

A positive control consisting of a 1:1 suspension of the original *R. salmoninarum* cell suspension and physiological saline was also prepared. The initial concentrations of *R. salmoninarum* in each suspension were determined by the culture method.

The pH of the suspensions containing gut extract, mantle extract, and physiological saline were 6, 6, and 7, respectively, as taken by indicator pH paper.

After incubating all three suspensions at 10 ^{o}C for 24 and 48 h, samples were removed and examined for *R. salmoninarum* by culture, Gram-staining, and IFAT. At each sampling, the three suspensions were vortex-mixed, aliquots were removed and each serially diluted in ten-fold steps prior to drop-inoculation onto SKDM. Smears of each preparation were also stained by the IFAT and Gram technique.

The SKDM plates were incubated at 15 ^{o}C and colony counts were noted at day 73. Smears from representative colonies were stained by the Gram and IFAT methods to ensure that the colonies were indeed *R. salmoninarum*.

Clearing of *R. salmoninarum* from sea water by *M. edulis*

R. salmoninarum cells were harvested from an 11 day culture on SKDM plates, using 9.5 ml P-S . The suspension with an absorbance of 0.35 (at a 1:100 dilution in P-S) at 540 nm, was added to autoclaved sea water to make 4 L of final suspension. The concentration of viable *R. salmoninarum* in this suspension was determined by plating 25 µl aliquots on SKDM and counting the resulting *R. salmoninarum* colonies following 40 days of incubation at 15 ^{o}C.

The *R. salmoninarum* suspension was divided into two parts and each was placed in a 2 L beaker equipped with aeration. Beaker A contained 14 *M. edulis* animals (total weight of 100.2 g); Beaker B contained no *M. edulis* and served as the control. The animals used were free from *R. salmoninarum* since an additional sample of 5 mussels was negative for the bacterium by culture and IFAT of their gut, gill, and mantle materials.

Aliquots (25 μl) from each suspension were removed after 24, 48, 72, 96, 120, 144, 168, 192, and 216 hr and cultured on SKDM for *R. salmoninarum*. Culture plates were incubated at 15 ^{o}C for 38 d.

After 216 hr, mussels from A were removed and smears from mantle, gills, and gut were prepared and examined for *R. salmoninarum* by culture and IFAT.

The mean temperature over the 216 hr period was 17.6 ^{o}C.

RELATIVE SUSCEPTIBILITY TO *R. salmoninarum* OF A NON-SALMONID FISH SPECIES AND A SALMONID

The selected test organisms were *Cymatogaster aggregata* (shiner perch) and *Oncorhynchus tshawytscha* (chinook salmon), with mean weights of 10 and 27 g, respectively. The shiner perch used were collected by netting in Departure Bay. The chinook salmon were taken from the Rosewall Hatchery, which uses eggs certified free from *R. salmoninarum,* and filter- and ultraviolet- sterilized water. Until used in the susceptibility experiments, the test species were held separately in flowing seawater (8 °C) at low densities (2 shiner perch /li; 1 chinook salmon /7 li).

Fishes experimentally challenged with *R. salmoninarum* , either by intraperitoneal injection or by immersion, were allowed to cohabit with the unchallenged fishes in a tank of sea water (described below).

The experimental design permitted observations on whether or not infections with *R. salmoninarum* could be established in each of the test fish species by injection or by the waterborne route.

The experimental design was as follows :

Tank A: Fishes were challenged by intraperitoneal injection.

Species name	Dose of *R. salmoninarum* (cells/fish)	No. of indiv./ group	Fin clip
Cymatogaster aggregata	3.15×10^5	10	left pelvic
	3.15×10^3	10	right pelvic
	0	50	none
Oncorhynchus tshawytscha	3.15×10^7	10	left pelvic
	3.15×10^5	10	right pelvic
	3.15×10^3	10	adipose
	0	20	none

Tank B: Fishes were challenged by immersion.

Species name	Concentration of *R. salmoninarum* in challenge suspension (cells ml^{-1})	No. of indiv./ group	Fin clip
C. aggregata	4.70×10^6	50	none
	0	50	left and right pelvic
O. tshawytscha	4.70×10^6	20	none
	0	20	adipose

The *R. salmoninarum* cells used were harvested in P-S from a 10 day-old culture on KDM 2 plates. The suspension was vortexed and passed four times through a syringe fitted with a 26G needle. Ten-fold serial dilutions in P-S were prepared for plate count and to obtain the desired bacterial concentration for subsequent use. The 1:100 dilution of the suspension had an absorbance of 0.6 at 540 nm.

The challenged groups received *R. salmoninarum* either by intraperitoneal injection (0.1 ml bacterial suspension) or by immersion for 30 minutes in 10 L of bacterial suspension contained in a plastic bucket.

The experimentally challenged groups were held together with the unchallenged groups in their respective tanks. The various groups were tagged by fin clipping to denote the treatment received (see preceeding tables). Tanks (650 L volume) were supplied with filtered and UV-sterilized sea water, flowing at a rate of 140 ml sec^{-1}. The water temperature ranged from 8 - 10.8 ^{o}C.

At the start of the experiment, both the chinook salmon and the shiner perch were examined by the IFAT and culture methods to determine whether they were infected with *R. salmoninarum*. Kidney homogenates (5% w/v in P-S) from 10 chinook salmon and 25 shiner perch were prepared using a Polytron homogenizer and a syringe fitted with a 26G needle, respectively. The homogenates were assayed for *R. salmoninarum* by IFAT and by culture on SKDM 2. Plasma samples from these fishes were also examined for their titres of anti-*R. salmoninarum* agglutinating antibodies using the standard procedure described previously.

On day 80, when most of the challenged fishes in Tank A and when the first chinook salmon in Tank B had died of BKD, half of each of the remaining treatment groups were sampled and examined for the presence of *R. salmoninarum* using IFAT. Kidney homogenates (50 % w/v in P-S) from a total of 105 chinook salmon and shiner perch from Tanks A and B were prepared. All kidney homogenate smears for IFAT examination from the chinook and shiner perch in this experiment were prepared using the blood smear technique; 10 µl samples of homogenate (5% w/v or 50% w/v) were used per microscope slide.

The remaining half of the populations were kept in their respective tanks for further observation and will be reported on in a later paper.

RESULTS

COMPARATIVE EVALUATION OF VARIOUS METHODS FOR DETECTING *R. salmoninarum* IN SALMONIDS

R. salmoninarum cells mixed with kidney homogenates

When *R. salmoninarum* cells were mixed with kidney homogenates, the culture method proved the most sensitive, the limit of detection being 3 x 10^2 *R. salmoninarum* cells ml^{-1} (Table 2). The IFAT was the next most sensitive method but it could only detect *R. salmoninarum* at concentrations of 3 x 10^7 or more cells ml^{-1}. Both IEA and CIE could only detect *R. salmoninarum* in preparations derived from samples containing 3 x 10^8 or more cells ml^{-1}.

Kidney homogenates from *R. salmoninarum*-infected chinook salmon

The detection methods tested showed the same relative sensitivities as in the preceeding experiment when kidney homogenates from *R. salmoninarum*-infected chinook salmon were examined (Table 3). In 3 of the 5 kidney homogenate samples, IFAT was observed to be as sensitive as the culture method while in the remaining 2 samples it proved 10-fold less sensitive.

Table 2. Limits of detection of various assay methods for *R. salmoninarum* (R.s.) using serial dilutions of bacterial cell suspension mixed with kidney homogenates.

No. of R.s. cells/ml	Assay Methods			
	Culture	IFAT	IEA	CIE
3×10^{10}	+	+	+	+
3×10^{9}	+	+	+	+
3×10^{8}	+	+	+	+
3×10^{7}	+	+	-	-
3×10^{6}	+	-	-	-
3×10^{5}	+	-	-	-
3×10^{4}	+	-	-	-
3×10^{3}	+	-	-	-
3×10^{2}	+	-	-	-
3×10^{1}	-	-	-	-
3×10^{0}	-	-	-	-

+ = *R. salmoninarum* detected; - = *R. salmoninarum* not detected

Table 3. Limits of detection of various assay methods for *R. salmoninarum* (R.s.) in kidney homogenates prepared from chinook salmon with overt bacterial kidney disease (n=5).

Dilutions of homogenate	Assay Methods			
	Culture	IFAT	IEA	CIE
1×10^{0}	+	+	+	+
1×10^{1}	+	+	+	+
1×10^{2}	+	+	+	+
1×10^{3}	+	+	+	+4/-1
1×10^{4}	+	+	+	-
1×10^{5}	+	+	+3/-2	-
1×10^{6}	+	+	-	-
1×10^{7}	+	+	-	-
1×10^{8}	+	+3/-2*	-	-
1×10^{9}	-	-	-	-
1×10^{10}	-	-	-	-

* In 3 out of 5 samples, *R. salmoninarum* could be detected

ABSENCE OF *R. salmoninarum* IN NON-SALMONID FINFISHES AND *M. edulis* SAMPLED FROM SELECTED *R. salmoninarum*-INFECTED FARMS IN BRITISH COLUMBIA

Survey

None of the non-salmonid finfishes and *M. edulis* examined showed any signs of gross pathology; rather, they all appeared to be healthy. Further, *R. salmoninarum* was not detected in any of the samples examined by culture or IFAT (Table 4). Plasma agglutinating titres against *R. salmoninarum* were generally low (mean titres ranged from 0.14 to 4.0), except for the *Sebastes caurinus* (rockfish) samples, the titres of which were much higher (120 and 240). The agglutinating titres against the three other bacterial pathogens *A. salmonicida*, *V. anguillarum*, and *Y. ruckeri* were very low for all finfish species tested.

Comparison of *S. caurinus* plasma agglutinating titres

Table 5 shows the results of the comparison of plasma agglutinating titres against *R. salmoninarum*, *A. salmonicida*, *V. anguillarum*, and *Y. ruckeri* of rockfish samples collected from the farm at the Pacific Biological Station, Departure Bay and from a site near Gabriola Island. These results did not confirm the high agglutinating titres previously detected in rockfish samples during the course of the survey.

Table 4. Prevalence of *R. salmoninarum* (R.s.) in various non-salmonid fishes and mussels from selected R. s.-infected salmon farms in British Columbia and mean plasma agglutinating titres against R.s., *A. salmonicida* (A.s.), *V. anguillarum* (V.a.), and *Y. ruckeri* (Y.r.) in these non-salmonid fishes.

Species	Sample size/ site	Prevalence of R.s.*	Mean Agglutinating Titres			
			R.s.	A.s.	V.a.	Y.r.
C. aggregata	51	0	1.75	0.18	0.00	0.00
	50	0	2.44	0.00	ND	ND
	25	0	4.00	ND	ND	ND
G. aculeatus	25	0	ND	ND	ND	ND
	14	0	ND	ND	ND	ND
C. harengus	48	0	0.33	0.00	ND	ND
pallasi	60	0	0.14	2.33	0.16	0.00
S. caurinus	2	0	120.00[1]	3.50	2.00	0.00
	4	0	240.00[2]	3.00	8.00	0.00
H. colliei	8	0	4.00	3.12	5.71	2.00
M. edulis	70	0	ND	ND	ND	ND
	26	0	ND	ND	ND	ND
	50	0	ND	ND	ND	ND

* 0% prevalence= *R. salmoninarum* was not detected in samples
ND = not determined
[1] Range : 48-192 Standard deviation : 102
[2] Range : 96-384 Standard deviation : 204

Table 5. Comparison of plasma agglutinating titres against *R. salmoninarum* (R.s.), *A. salmonicida* (A.s.), *V. anguillarum* (V.a.), and *Y. ruckeri* (Y.r.) of rockfish from an R.s.-infected salmon farm (PBS farm) and site well away from a salmon farm (Gabriola Island site).

Site	Sample Size	Mean Plasma Agglutinating Titres			
		R.s.	A.s.	V.a.	Y.r.
PBS farm	15	28.27^a	2.38^b	1.85^c	0.00^d
Gabriola Island	10	35.20^a	2.64^b	1.33^c	0.00^d

Values marked by same superscript are not significantly different ($P > 0.01$).

EXPERIMENTAL CLEARING OF *R. salmoninarum* FROM SEA WATER BY *M. edulis*

Uptake and retention of *R. salmoninarum* by *M. edulis*

At time 0, the gills of the *M. edulis* tested positive for *R. salmoninarum*. From time 0 to 7 h both the gills and gut of these animals had bacteria which were Gram-positive with a diplobacillus morphology typical of *R. salmoninarum* (Table 6).

Inactivation of R.salmoninarum by mussel gut extract

Viable cells of *R. salmoninarum* remained in the mantle extract and saline suspensions after 48 h of incubation; however, viable *R. salmoninarum* cells could not be recovered from the gut extract suspension after 24 and 48 h of incubation (Table 7). However, all three suspensions tested positive for *R. salmoninarum* at 24 and 48 h when assayed using the Gram stain and IFAT (Table 8).

Clearing of *R. salmoninarum* from sea water by *M. edulis*

Viable counts of *R. salmoninarum* decreased with time in sea water in the absence of mussels. However, in the presence of mussels the decline in the concentrations of

Table 6. Presence of *R. salmoninarum* cells as detected by Gram stain from both smears and histological sections of mantle, gills, and gut of *M. edulis* over a 120 hr period post exposure to the bacterium.

Time (hr)	Mantle	Gills	Gut
T_0	-	+	+
T_1	-	+	+
T_3	-	+	+
T_5	-	+	+
T_7	-	+	+
T_9	-	-	-
T_{11}	-	-	-
T_{24}	-	-	-
...			
T_{120}	-	-	-

Table 7. Viable counts on SKDM of *R. salmoninarum* (cells ml^{-1}) from suspensions containing *M. edulis* gut extract, mantle extract, or physiological saline after 0, 24, 48 hr periods of incubation of the suspensions at 10 °C.

Gut Extract			Mantle Extract			Saline		
T0	T24	T48	T0	T24	T48	T0	T24	T48
$1x10^{11}$	ND	ND	$1x10^{11}$	$6x10^8$	$2x10^8$	$1x10^{11}$	$4x10^7$	$2x10^7$

ND = not detected

Table 8. Presence of *R. salmoninarum* as detected by Gram stain and IFAT in suspensions containing *M. edulis* gut extract, mantle extract, or physiological saline after 0, 24, 48 hr incubation of the suspensions at 10 °C.

Time (hr)	Suspensions		
	Gut Extract	Mantle Extract	Saline
T_0	+	+	+
T_{24}	+	+	+
T_{48}	+	+	+

viable cells was much greater, such that by 96 h no viable cells remained (Table 9). Table 10 shows the percentage reduction in concentration of *R. salmoninarum* in sea water with or without *M. edulis*. The difference in values (A-B) indicates the estimated reduction attributable to the presence of *M. edulis*.

The gill, mantle, and gut samples from mussels at T_{216} were negative for *R. salmoninarum* by IFAT and culture.

Table 9. Viable counts on SKDM of *R. salmoninarum* (cells ml^{-1}) from sea water with (A) or without (B) *M. edulis* over a 216 hr period.

Time	A	B
T_0	2.28×10^8	2.28×10^8
T_2	1.02×10^7	2.52×10^7
T_{24}	2.27×10^5	4.88×10^6
T_{48}	1.04×10^5	9.24×10^5
T_{72}	2.24×10^3	5.00×10^4
T_{96}	ND	3.64×10^3
T_{120}	ND	4.40×10^2
T_{144}	ND	3.20×10^2
T_{168}	ND	1.20×10^2
T_{192}	ND	8.00×10^1
T_{216}	ND	ND

ND = not detected

Table 10. Percentage reduction in concentrations of *R. salmoninarum* in sea water with or without *M. edulis* (M.e.).

Time	With M.e. (A)	Without M.e. (B)	A-B
T_0	0	0	0
T_2	95.53	88.95	6.58
T_{24}	99.90	97.86	2.04
T_{48}	99.95	99.59	0.36
T_{72}	99.99	99.97	0.02
T_{96}	100.00	99.99	0.01

RELATIVE SUSCEPTIBILITY TO *R. salmoninarum* OF A NON-SALMONID FISH SPECIES AND A SALMONID

The initial samples of *C. aggregata* (shiner perch) and *O. tshawytscha* (chinook salmon) , taken prior to the experiment, were found to be negative for *R. salmoninarum* by IFAT and culture on SKDM. The plasma agglutinating titres against the bacterium were low; shiner perch and chinook salmon had mean titres of 4 and 5.6, respectively.

The fishes challenged by injection were killed by the bacterium much earlier than the ones challenged by immersion. Table 11 shows the mean time to death (days) of fishes challenged by injection. *R. salmoninarum* was confirmed as the cause of mortalities by IFAT and culture on SKDM.

Table 12 shows the prevalence values for *R.salmoninarum* among fishes sampled on day 80 as determined by IFAT on kidney homogenates (50 % w/v in P-S). Samples from both Tanks A and B , which contained fishes challenged by injection and by immersion, respectively, were examined.

Table 11. Mean time to death (days) of fishes challenged by intraperitoneal injection of *R. salmoninarum* (R.s.).

Species name	Dose of R.s./ fish	Mean time to death (days)
C. aggregata	3.15×10^5	64*
	3.15×10^3	62**
O. tshawytscha	3.15×10^7	32
	3.15×10^5	57
	3.15×10^3	67

* Mean time to death of 80% of the population as of day 80
** Mean time to death of 60% of the population as of day 80

Table 12. Prevalence of *R. salmoninarum* among fishes sampled on day 80 as determined by IFAT on kidney homogenates. Tanks A and B contained fishes challenged by injection and by immersion, respectively.

		Sample size	Prevalence (%)
Tank A :			
C. aggregata	Challenged groups[1]		
	Unchallenged group	25	0*
O. tshawytscha	Challenged groups[2]		
	Unchallenged group	10	10
Tank B :			
C. aggregata	Challenged group	25	20[3]
	Unchallenged group	25	0*
O. tshawytscha	Challenged group[4]	10	60
	Unchallenged group	10	20

* = *R. salmoninarum* was not detected in any of the samples

[1] 70% of the population have died of BKD as of day 80

[2] 100% of the population have died of BKD as of day 80

[3] Positive slides had 1-5 *R. salmoninarum* cells per 100 fields

[4] 30% of the sample had kidney lesions characteristic of BKD

DISCUSSION

COMPARATIVE EVALUATION OF VARIOUS METHODS FOR DETECTING *R. salmoninarum* IN SALMONIDS

Diagnosis of BKD involves the examination of fish for clinical signs of the disease; it also involves examining fish tissues (eg., kidney) for the presence of *R. salmoninarum* using one or more of the following techniques: the Gram stain, the culture method (Evelyn, 1977), fluorescent antibody techniques (Bullock and Stuckey, 1975; Bullock *et al.*, 1980), the staphylococcal coagglutination method (Kimura and Yoshimizu, 1981), immunodiffusion (Chen *et al.*, 1974; Kimura *et al.*, 1978),counterimmunoelectrophoresis (Cipriano *et al.*, 1985), and immunoenzyme assays like the peroxidase-antiperoxidase procedure (PAP) (Sakai *et al.*, 1987a), and the enzyme-linked immunosorbent assay (ELISA) (Manfredi, 1986; Dixon, 1987; Pascho *et al.*, 1987).

Previous reports on the comparative sensitivities of various detection methods indicate disagreements in results among workers. Table 13 shows the ranking in sensitivities of various methods in the present study in relation to that of previous investigations.

The results of the the present study are in agreement with that of Evelyn *et al.*, 1978, Evelyn, 1981, and Shortt *et al.*, 1988. Evelyn and co-workers reported that culture is

Table 13. Comparative sensitivities of various detection methods as reported. Numbers indicate rankings in sensitivity (1 = most sensitive; 5 = least sensitive).

Methods		Sources : a	b	c	d	e	f	g	h	i	j	k	l
Gram stain		3	2	3	2	5		4.5					
Culture		1		1		3		4.5		3		1	1
FAT :	IFAT	2		2			2	2.5	4			2	2
	DFAT		1			4				2	3.5		
IEA's :	ELISA						1			1	1		
	PAP							1	2.5				
	DIEA								3.5				
	IIEA								2.5				
	ABC								1				
	Bio-Rad												3
CIE						1	3		3.5	4			4
ID		4			3		4			5			
CoA					1	2		2.5			2		
LA								3			3.5		

Notes:

[a]Evelyn *et al.*, 1978
[b]Bullock *et al.*, 1980
[c]Evelyn *et al.*, 1981
[d]Kimura & Yoshimizu, 1981
[e]Cipriano *et al.*, 1985
[f]Manfredi, 1986
[g]Sakai *et al.*, 1987a
[h]Sakai *et al.*, 1987b
[i]Pascho *et al.*, 1987
[j]Dixon, 1987
[k]Shortt *et al.*, 1988
[l]Present study

FAT = Flourescent antibody techniques (Indirect or Direct)
IEA's = Immunoenzyme assays
ELISA= Enzyme-linked immunosorbent assay
PAP = Peroxidase-antiperoxidase procedure
DIEA = Direct immunoenzyme assay
IIEA = Indirect immunoenzyme assay
ABC = Avidin-biotin complex
Bio-Rad = IEA on nitrocellulose membrane, present study
CIE = Counterimmunoelectrophoresis
ID = Immunodiffusion
CoA = Staphylococcal coagglutination
LA = Latex agglutination

approximately 10^2-fold more sensitive than IFAT. This was also found by Shortt *et al.*, 1988 who reported that the former is approximately 10^3 times more sensitive. Based on their findings, the culture method could detect *R. salmoninarum* at concentrations as low as 10^3 colony forming unit (CFU) g^{-1} tissue while IFAT could only detect the bacterium at concentrations of 10^6 CFU g^{-1} tissue or more.

The increase in sensitivity of IFAT in the experiment where kidney homogenates from BKD infected chinook salmon were assayed over that of the first experiment (in which the concentration of the homogenate was held constant) is undoubtedly due to the differences in the preparation of the test materials, i.e., in this particular experiment, the "masking effect" of the kidney homogenate on *R. salmoninarum* was reduced by serially diluting the sample with P-S instead of kidney homogenate. Under practical assay conditions, however, where dilution of the kidney sample would be minimal, IFAT would likely prove somewhat less sensitive than found here.

The IEA and CIE were less sensitive than the culture and IFAT assays (Tables 2 and 3). The higher sensitivities reported by Sakai *et al.*, (1987) and Cipriano *et al.*, (1985) for a similar IEA and CIE, respectively, were not supported by the present findings. However, the present findings with respect to CIE is in aggreement with that of Pascho *et al.*, (1987) who also found it to be relatively insensitive (Table 13).

The culture method and IFAT were both chosen for most of the routine assays for *R. salmoninarum* in this study because they were found to be most sensitive. The exception to this is mentioned in the experiment with clearance of *R. salmoninarum* from mussel tissues (p. 15).

ABSENCE OF *R. salmoninarum* IN NON-SALMONID FINFISHES AND *M. edulis* FROM SELECTED *R. salmoninarum*-INFECTED FARMS IN BRITISH COLUMBIA

R. salmoninarum was not found to occur in any of the non-salmonid finfishes and *M. edulis* in the present study even though the samples were removed from salmon farms in which the pathogen was known to occur. These results suggest that these species of fishes are not likely to be sources of *R. salmoninarum* infections on salmon farms, possibly because, as discussed later, they did not appear to be susceptible to the waterborne pathogen. However, the pathogen may have been present in some of the samples at undetectable levels but the detection methods used i.e. culture and IFAT methods may not have been sensitive enough.

Despite the foregoing, it is relevant to indicate that, while a non-salmonid fish like the Pacific lamprey proved refractory to infection with *R. salmoninarum* (Bell and Traxler, 1986), Pacific herring can be infected by injection with *R. salmoninarum*. In fact, the infected herring died of BKD (Traxler and Bell, 1988). In addition, sablefish also proved susceptible to the injected pathogen and, in one case, carried the pathogen for up to 165 days (Bell *et al.*, 1988). It is conceivable, therefore, that non-salmonid species could, under certain circumstances, serve as reservoirs of infection for salmonids. However, judging from the results of the present study, these conditions do not prevail on salmon farms.

The high anti-*R. salmoninarum* titres detected in plasma of rockfish during the initial survey (Table 4) may have been due to naturally occurring agglutinins which occur in certain fishes (Ingram, 1980), rather than an indication of exposure to *R. salmoninarum*. It is also possible that the titres observed are normal for rockfish and that they are a response to a harboured commensal organism sharing antigens in common with *R. salmoninarum*.

The plasma agglutinating titres against *R. salmoninarum* of the rockfish sampled from the PBS farm and Gabriola Island were lower than those found in the initial survey samples (cf. Tables 4 and 5) but this difference may have been more apparent than real and probably reflected the fact that different *R. salmoninarum* antigen suspensions were used in the two assays. Nevertheless, rockfish anti-*R. salmoninarum* agglutininins tended to be high relative to agglutinins against the other pathogens. However, because they were "high" at both the farm and non-farm sites, it is unlikely that their presence was related to an exposure to *R. salmoninarum*. Whether or not the agglutinins were immunoglobulins was not determined.

EXPERIMENTAL CLEARING OF *R. salmoninarum* FROM SEA WATER BY *M. edulis*

There has been a concern among sectors of the salmon farming industry that mussels, which are commonly attached on farm pen nets, may be harbouring *R. salmoninarum* and serving as reservoirs of infection for BKD. The three experiments reported on herein showed that this concern is likely unjustified.

First of all, the initial experiment showed that the mussel rapidly becomes free of the pathogen following exposure to it. Secondly, the ingested pathogen is very likely rapidly inactivated by the digestive enzymes of the mussel gut because extracts of gut material proved lethal to the pathogen. This may be attributed to the bacteriolytic activity of lysozymes abundantly present in the digestive gland and style of *M. edulis* (McHenery *et al.*, 1979; Birkbeck and McHenery, 1982). However, digestive enzymes other than lysozyme may be the ones responsible since *R. salmoninarum* was reported to be lysozyme resistant (Fryer and Sanders, 1981). Finally, the mussel may actually serve in clearing the pathogen from sea water (Table 9). *M. edulis* was reported to be capable of clearing several bacteria at a rapid rate- C_{90} = 1.93 h (time required to reduce the bacterial concentration by 90%) (Birkbeck and McHenery, 1982). Although in the present experiment it proved relatively inefficient in this function; under natural conditions, where clumps of the pathogen (as in fecal material) rather than single *R*. *salmoninarum* cells would be encountered, one would expect a higher clearance efficacy. However, an experiment involving the clearance of the pathogen as it exists in clumps was not conducted because of technical constraints.

The rapid decline in concentration of *R. salmoninarum*, which is however relatively lower, in mantle extract or saline may be attributed to the natural death of the bacterium in the presence of saline. Evelyn, 1987 stated that the bacterium has limited survival in saline. *R. salmoninarum* cells with initial viable count of 1.71 x 105 ml^{-1} had survival rate of 0.01% in filter-sterilized saline after 2 days of incubation at 15 ^{o}C. The present findings are in agreement with those of Evelyn. In the present study, *R. salmoninarum* cells suspended in sterile saline (initial viable count of 1 x 10^{11} ml^{-1}) and incubated at 10 ^{o}C, had survival rates of 0.04% and 0.02% after 1 and 2 days, respectively (Table 7). Another factor which may have contributed to the seemingly lower subsequent viable counts was the autoaggregation of the *R. salmoninarum* cells (*R. salmoninarum* cells undergo autoaggregation when held in heavy suspensions). Nevertheless, this result does not in any way discount the fact that the gut extract indeed destroyed the viability of the cells, since after 24 hr incubation the bacterial concentration was reduced from 10^{11} to an undetectable level. In contrast mantle extract and saline still contained 10^8 and 10^7 viable *R. salmoninarum* cells ml^{-1} at 48 hr, respectively.

RELATIVE SUSCEPTIBILITY TO *R. salmoninarum* OF A NON-SALMONID FISH SPECIES AND A SALMONID

The shiner perch, as well as the chinook salmon, was found to be susceptible to *R. salmoninarum* challenge by intraperitoneal injection (Table 11). However, chinook salmon proved to be more susceptible. While 100% of the challenged chinook salmon were killed by BKD as of day 80, only 70% of the challenged shiner perch had died by this stage.

Chinook salmon, again, is far more susceptible to *R. salmoninarum* infection through the waterborne route than the shiner perch. The first mortality in the immersion-challenged chinook salmon was observed on day 80. The killed animal and 30% of the samples taken on day 80 showed gross clinical signs of BKD i.e., granulomatous lesions in the kidney. Further, 60% of the samples were positive to *R. salmoninarum* by IFAT. Shiner perch, on the other hand, were only subclinically infected and only 20% of the challenged group were positive for *R. salmoninarum*.

It appears, based on the results, that horizontal transmission of *R. salmoninarum* from challenged fishes to the unchallenged chinook salmon actually occurred. At the start of the experiment, none of the chinook salmon contained detectable *R. salmoninarum*. However, after 80 days of cohabitation with experimentally infected groups, 10 and 20 % of the chinook salmon in tanks A and B, respectively, contained detectable *R. salmoninarum* (Table 12). Horizontal transmission among salmonids in sea water was previously confirmed by Evelyn, 1987 who found that 66% to 78% of initially uninfected sockeye salmon (*O. nerka*) died of BKD within 12 months of exposure to *R. salmoninarum*-infected salmon in adjacent netpens.

Horizontal transmission of the bacterium to the shiner perch was not demonstrated. There was no increase in the prevalence of *R. salmoninarum* in the unchallenged groups of shiner perch in any of the tanks. Even after cohabitation with infected groups for 80 days, none of the shiner perch contained detectable *R. salmoninarum* (Table 12).

In view of the foregoing, it is not surprising that non-salmonid finfishes surveyed on fish farms proved *R. salmoninarum*-free.

CONCLUSIONS

The culture method proved to be the most sensitive technique in the detection of *R. salmoninarum* and is at least 10-fold more sensitive than the indirect fluorescent antibody technique (IFAT). The immunoenzyme assay on nitrocellulose membrane (IEA) and counterimmunoelectrophoresis (CIE) were the least sensitive.

Based on the survey results, on the laboratory challenges of shiner perch with *R. salmoninarum,* and on the observations on the clearing of *R. salmoninarum* from sea water by *M. edulis* , non-salmonid fishes and mussels do not appear to be reservoirs of *R. salmoninarum.*

The most likely source of *R. salmoninarum* infection for farmed salmon is another stock of *R. salmoninarum*-infected salmon.

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