THE USE OF ANTIBIOTICS FOR THE CONTROL OF VERTICAL TRANSMISSION OF BACTERIAL KIDNEY DISEASE IN COHO SALMON (*ONCORHYNCHUS KISUTCH*)

by

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B.Sc., McGill University, 1982

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THE USE OF ANTIBIOTICS FOR THE CONTROL OF VERTICAL TRANSMISSION OF BACTERIAL KIDNEY DISEASE IN COHO SALMON (ONCORHYNCHUS KISUTCH)

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The Use of Antibiotics for the Control of Vertical Transmission of Bacterial Kidney Disease in Coho Salmon (*Oncorhynchus kisutch*)

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ABSTRACT

This study examines the efficacy of prophylactic injection of selected antibiotics into maturing, pre-spawning female salmon as a means of reducing intra-ovum infections with *Renibacterium salmoninarum* the causative agent of bacterial kidney disease (BKD).

Saline solutions of 5 antibiotics (erythromycin phosphate, penicillin G, oxytetracycline, cephradine, and rifampicin) were injected into 5 groups of maturing female coho salmon (*Oncorhynchus kisutch*) - one antibiotic per group. Fish in a sixth group, the controls, were injected with sterile saline only. Eggs from all fish were experimentally infected with *R.salmoninarum*, using a microinjection technique, The eggs were then fertilized and incubated. The prevalence of infection with *R.salmoninarum* was determined within the eyed eggs, and within alevins at hatch. Cephradine and rifampicin brought about a significant reduction in the prevalence of infection at the eyed stage. All of the antibiotics were effective in significantly reducing the prevalence of infection due to *R.salmoninarum* within the alevins. Histological examination revealed the pathogen within the yolk sac of alevins from control fish, i.e., spawners that had not received an injection of antibiotic.

Concentrations of the antibiotics were measured within eggs at spawning, and within the alevins at hatch. All five of the antibiotics were detected in the eggs at spawning. Only erythromycin and rifampicin were present in measurable amounts within the newly hatched alevins.

It was demonstrated in *in vitro* tests that cells of *R.salmoninarum* were killed by all of the antibiotics. The concentrations tested approximated those detected within the eggs in the previously described experiment. The minimum lethal concentration and the minimum exposure time required to kill the bacterium varied with each antibiotic.

This study indicates that prophylactic injection of antibiotics into maturing female salmon is effective in reducing the prevalence of intra-ovum infections due to *R.salmoninarum*. The procedure should therefore prove useful in controlling vertical transmission of BKD.

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INTRODUCTION

Bacterial kidney disease (BKD) is a chronic, systemic, bacterial infection in salmonids. It was first described in the 1930's as "Dee Disease", following its finding in an Atlantic salmon (*Salmo salar*) population in Aberdeenshire, Dee, Scotland (Smith 1964).

Since that time, BKD has been reported to occur throughout the world, where salmonids are cultured or occur naturally (Bell 1961; Evelyn et.al. 1973; de Kinkelin 1974; Ellis 1978; Bylund 1987). The exceptions to this are Australia, New Zealand, and the Soviet Union. (Evelyn 1988).

To date, natural occurrences of BKD have been observed only in salmonids (Fryer & Sanders 1981; Evelyn 1988), and outbreaks of BKD have been documented in both cultured and feral salmonids (Rucker et.al. 1954; Mitchum et.al. 1979; Banner et.al. 1983; Banner et.al 1986). Experimental infections in non-salmonids such as sablefish (G.R. Bell, pers.comm.) have been achieved, but natural infections in non-salmonids have not been reported.

The disease is a systemic infection with a normally slow progress (Fryer & Sanders 1981; Klontz, 1983; Evelyn 1988). Overt pathological signs of an epizootic are not usually observed before the fish are 6 to 12 months old (Evelyn 1988, Post 1987). High incidences of pre-spawning mortalities have been reported in returning salmon at government and privately owned hatcheries in Canada and the United States, with the

resulting loss of progeny (DeCew 1972; Fryer & Sanders 1981, Klontz 1983, Evelyn 1988).

The pathology of the disease is variable (Fryer & Sanders 1981; Austin & Rayment 1985; Post 1987; Evelyn 1988). Among the signs that may be exhibited externally by fish suffering from the disease are exophthalmia, distended abdomens, skin blisters (this is particularly common within maturing, pre-spawning adult fish that are suffering from BKD), and haemorrhaging around the base of the fins and vent (Fryer & Sanders 1981; Post 1987; Evelyn 1988). Internal signs may include haemorrhage of the organs and internal body walls. Grey, granulomatous lesions in the kidney, and sometimes in the spleen and liver, are characteristic (but not inevitable) signs of BKD (Evelyn 1988, Post 1987).

The causative agent of BKD is a small, Gram-positive diplo-bacillus identified as *Renibacterium salmoninarum* (Sanders & Fryer 1980). The bacterium is non-acid-fast, non-sporogenic, non-encapsulated and non-motile, extremely slow-growing, and fastidious in its growth media requirements (Evelyn 1977; Sanders & Fryer 1980; Fryer & Sanders 1981). The difficulty involved in culturing *R.salmoninarum*, and the length of time required for growth is a factor in the slow progress in studies on the control of this disease (Evelyn 1988; Elliott et.al. 1989).

Additional properties of *R.salmoninarum* contribute to its success as a pathogenic organism. It appears that the bacterium is able to survive and multiply within the phagocytic cells of salmonids (Young & Chapman 1978; Bruno 1986). This ability implies that antibiotic therapy may be ineffective against the bacterium, because the drugs

would be unable to penetrate the macrophages. Thus, outbreaks of the disease usually re-occur after antibiotic treatment is stopped (Young & Chapman 1978; Getchell et.al. 1985; Bruno 1986; Elliott et.al. 1989). Similarly, any antibody response mounted by the host system would be unsuccessful in combatting a pathogen that is "protected" by the phagocytic cells (Baudin Laurencin et.al. 1977; Paterson et.al. 1981). To date, attempts to develop a vaccine against *R.salmoninarum* have been unsuccessful (Baudin Laurencin et.al. 1977; Evelyn et.al. 1984c; Kaattari et.al. 1986; Elliott et.al. 1989).

Vertical transmission is an important route for the persistence of BKD within salmonid populations. Cells of *R.salmoninarum* have been isolated from the coelomic fluid of spawning salmon (Bullock et.al. 1978; Evelyn et.al. 1984a,b; 1986a,b,c) and from within the eggs of salmon and trout (Evelyn et.al. 1984a,b; 1986a,b,c). The bacterium is thought to enter the egg via the micropyle, after ovulation. The source of infection is the contaminated coelomic fluid that surrounds the eggs within the body cavity of the female (Evelyn et.al. 1984a,b; 1986a,b,c). A possible second source of infection is ovarian tissue, the eggs being infected during oogenesis; the bacterial cell may enter the developing oocyte early in oogenesis (Bruno & Munro 1986b; Lee & Gordon 1987; Lee & Evelyn 1989).

Evelyn et.al. have demonstrated that the male plays little or no role in vertical transmission of *R.salmoninarum* (Evelyn et.al. 1984a,b; 1986b,c).

The egg and developing embryo can harbour cells of *R.salmoninarum* without overt pathological changes. High numbers of *R.salmoninarum*

cells have been isolated from salmonids at all stages in their life cycle, with no apparent signs of the disease (Evelyn 1988). This ability of the bacterium to be transmitted from parent to progeny via the egg and to be maintained within the growing salmonid without killing its vehicle is one of the factors in the success of *R.salmoninarum* as a pathogenic organism (Evelyn et.al. 1984a,b; 1986a,b,c). One explanation for this ability is that the cells of *R.salmoninarum* do not appear to produce any acutely lethal toxins (Evelyn 1986b). In contrast, *Aeromonas salmonicida*, the causative agent of furunculosis in salmonids (Post 1987), does produce lethal toxins (Munro 1984). Although *A.salmonicida* has been isolated from the coelomic fluid of maturing, pre-spawning salmon (Evelyn et.al. 1984a,b; Bullock & Stuckey 1987), it is generally thought to be transmitted horizontally rather than vertically (McCarthy & Roberts 1980; Bullock & Stuckey 1987). High numbers of *A.salmonicida* cells result in the death of the fish, probably due to the presence of the toxin (Munro 1984).

Several techniques are used in hatcheries to prevent vertical transmission of *R.salmoninarum* (Evelyn 1988; Elliott et.al. 1989). These include broodstock screening, disinfection of eggs with povidone-iodine solutions, water-hardening the eggs in solutions of antibiotics, and injections of antibiotics into pre-spawning female salmon. The use of some of these techniques has proved effective in controlling the disease within hatchery populations (Wood 1979; Klontz 1983; Elliott et.al. 1989). However, attempts to break the cycle of infection from parent to progeny via the egg using the above-mentioned methods have not been successful (Evelyn 1988; Elliott et.al. 1989).

Screening of broodstock is carried out at public and private hatcheries in Canada and the United States in order to identify those fish that are infected with *R.salmoninarum*. Eggs from heavily infected fish are discarded (Evelyn 1988). Samples of coelomic fluid or kidney are usually used for this screening procedure - the pathogen within infected fluid or tissue is identified by fluorescent antibody tests (Bullock & Stuckey 1975) and/or culture (Evelyn 1977; Austin et.al. 1983; Daly & Stevenson 1985). Application of this procedure has not proved successful in controlling vertical transmission of *R.salmoninarum* (Evelyn 1988). This lack of success may be due to the low sensitivity of the detection methods used (Cipriano et.al. 1985; Paclibare et.al. 1988; Sakai et.al. 1989). Also, the bacterial cells are not evenly dispersed throughout the coelomic fluid. Clumps of bacterial cells occur (Lee & Evelyn 1989), and thus a sample may be falsely identified as negative for the presence of *R.salmoninarum*. Finally, ovarian tissue rather than ovarian fluid might be the best sample to take in broodstock screening if reports that the eggs can acquire infections due to *R.salmoninarum* from ovarian tissue (Lee & Gordon 1987; Lee & Evelyn 1989) are confirmed.

Povidone-iodine solutions are effective in disinfecting the surface of water-hardened salmonid eggs (Evelyn et.al.1984a). However, these treatments are ineffective in eliminating intra-ovum infections of *R.salmoninarum* (Evelyn et.al. 1984a,b; 1986c).

Other methods for controlling vertical transmission of BKD include the use of antibiotics. To determine the most effective antibiotic against *R.salmoninarum*, investigators surveyed a number of antimicrobials, using *in vitro* and *in vivo* methods. Wolf and Dunbar (1959), and Austin

(1985) examined numerous antimicrobial compounds for their efficacy as chemotherapeutants against *R.salmoninarum*. Wolf and Dunbar (1959) reported success with erythromycin. Austin (1985) found that eleven antibiotics were useful as prophylactic compounds, when added to the feed of juvenile rainbow trout (*Oncorhynchus mykiss*). Among these eleven antibiotics were erythromycin phosphate, penicillin G, cephradine, and rifampicin. That same study showed oxytetracycline to be ineffective as a prophylactic agent.

One method for reducing the prevalence of *R.salmoninarum* within eggs that involves the use of antibiotics is water-hardening the eggs in a solution of erythromycin. Klontz (1983) reported that this technique was effective in preventing vertical transmission of *R.salmoninarum* in chinook salmon (*O.tshawytscha*). However, other investigators were unable to duplicate these findings (Groman 1983, Evelyn et.al. 1986a,b,c). Evelyn et.al. (1986a,b,c) have demonstrated that erythromycin is taken up by coho (*O.kisutch*) and chinook salmon (*O.tshawytscha*) eggs when they are hardened in aqueous solutions of the antibiotic. The drug, however, apparently accumulates in the perivitelline space rather than in the yolk, where the pathogen is reported to be located (Evelyn et.al. 1984c). Further, the antibiotic leaches quickly from the egg, before any bactericidal action would occur (Evelyn et.al. 1986a).

Injections of erythromycin into maturing salmon before spawning is a recommended procedure for reducing pre-spawning mortalities due to BKD (DeCew 1972,; Groman & Klontz 1983; Klontz 1983; Evelyn 1988). Success has been reported with this treatment; pre-spawning

mortalities were significantly reduced after the fish received antibiotic injections (DeCew 1972, Fryer & Sanders 1981; Klontz 1983; Elliott et.al. 1989). Reports have indicated that the progeny of fish receiving such injections suffered fewer mortalities due to BKD (Klontz 1983), or that the onset of epizootics of the disease was significantly delayed (Armstrong et.al. 1989).

Investigators have also shown that when maturing female salmon are injected with erythromycin phosphate before spawning, the drug is accumulated in detectable concentrations within the egg (Bullock & Leek 1986; Evelyn et.al. 1986b; Armstrong et.al. 1989). It was reported that the antibiotic persists within the egg, even after water-hardening. This led to the conclusion that the antibiotic was in the yolk material of the egg and not in the perivitelline space (Bullock & Leek 1986; Evelyn et.al. 1986b; Armstrong et.al. 1989).

The timing of injection is important for the deposition of the maximum amount of antibiotic within the egg. Evelyn et.al. (1986b) reported that injections done more than 70 days before spawning did not result in detectable drug levels within the eggs. Armstrong et.al. (1989) detected significant concentrations of erythromycin phosphate in eggs from chinook salmon (*O.tshawytscha*) that had been injected 9 days before spawning. These authors postulated that the drug entered the egg during vitellogenesis.

The reports described above indicate that injection of antibiotics into female broodstock salmon is promising as a means of reducing intra-ovum infections due to *R.salmoninarum*. The studies described in this thesis were designed to evaluate the efficacy of that technique.

Five antibiotics were selected: erythromycin phosphate, penicillin G, oxytetracycline, cephradine, and rifampicin. All were shown effective against *R.salmoninarum in vitro* and all, with the exception of oxytetracycline, were effective as prophylactic agents in juvenile rainbow trout (Austin 1985). It was decided to include oxytetracycline, however, as other investigators have indicated that injection of oxytetracycline into broodstock salmon was effective in reducing pre-spawning mortalities due to BKD (Fryer & Sanders 1981). Moreover, oxytetracycline is at this time the only chemotherapeutant available that is approved for use against Gram - positive bacteria in food fish in the United States (Meyer 1989). The five selected antibiotics represent a diversity of types, classified according to their chemical structures and modes of action (Lancini & Parenti 1982) (Table 1). Erythromycin phosphate acts by binding to the 50S subunit of the bacterial ribosome and thus prevents elongation of protein by blocking the addition of amino acids by RNA. Penicillin G and cephradine inhibit the cross-linking of peptidoglycan molecules within a bacterial cell wall. In a growing bacterial cell the cell wall is weakened and cell lysis occurs. Oxytetracycline binds to the 30S subuni of the bacterial ribosome and prevents initiation of the translation of the messenger RNA codons to the appropriate amino acids. Rifampicin acts by binding to RNA polymerase, thus blocking transcription of bacterial genomic DNA to RNA.

The objectives of the studies described herein are as follows:

First, to evaluate the efficacy of injection of each antibiotic into pre-spawning female coho salmon (*O.kisutch*) as a means of eliminating infections of *R.salmoninarum* within the eggs and alevins from these

fish; second, to determine the concentration of each antibiotic deposited within the egg after injection of the drug. Also, to examine the persistence of the antibiotics within the developing eggs and emerging alevins; and third, to quantify the susceptibility of *R.salmoninarum* to each of the five antibiotics.

Table 1. Types and modes of action of antibiotics selected for study.

Antibiotic	Type (according to chemical structure)	Mode of action
Erythromycin phosphate	macrolide	Inhibits protein elongation
Penicillin G	β - lactam	Inhibits cell wall synthesis
Oxytetracycline	tetracycline	Inhibits initiation of translation
Cephradine	β - lactam	Inhibits cell wall synthesis
Rifampicin	ansamycin	Inhibits initiation of transcription

MATERIALS AND METHODS

Experimental Design:

The experiment designed to meet the first two objectives is shown schematically in Figure 1. This experiment involved several steps, each of which is described in detail later in this section. An outline of the experiment (Fig.1) is given below.

Thirty-six mature female coho salmon (*Oncorhynchus kisutch*) were held at the Capilano Hatchery (Department of Fisheries and Oceans), in North Vancouver, British Columbia. Six groups of six fish each were given antibiotic injections. Drug dosages were based on fish weight (Table 2).

Evelyn et.al. (1986b) reported that, even when eggs are bathed in coelomic fluid contaminated with high numbers of *R.salmoninarum* cells, a maximum of 15% of those eggs will become infected with the bacterial cells. Because of this report it was decided to experimentally infect each egg with *R.salmoninarum*, rather than rely on the natural prevalences of infection. This would ensure that most of the eggs contained viable *R.salmoninarum* just after spawning, and that bacterial cell concentrations within each egg would be similar. Also, this would reduce the need for the large sample sizes normally required for statistically valid results, when using eggs from a natural infection situation.

Eggs were taken at spawning and 50 eggs from each fish were experimentally infected with *R.salmoninarum* using a microinjection technique (Brown et.al. 1989).

Figure 1. Schematic flow-diagram of the experiment designed to evaluate the efficacy of antibiotic injection into female broodstock salmon as a means of controlling vertical transmission of *Renibacterium salmoninarum*, and to determine the concentration of antibiotics deposited within the eggs.

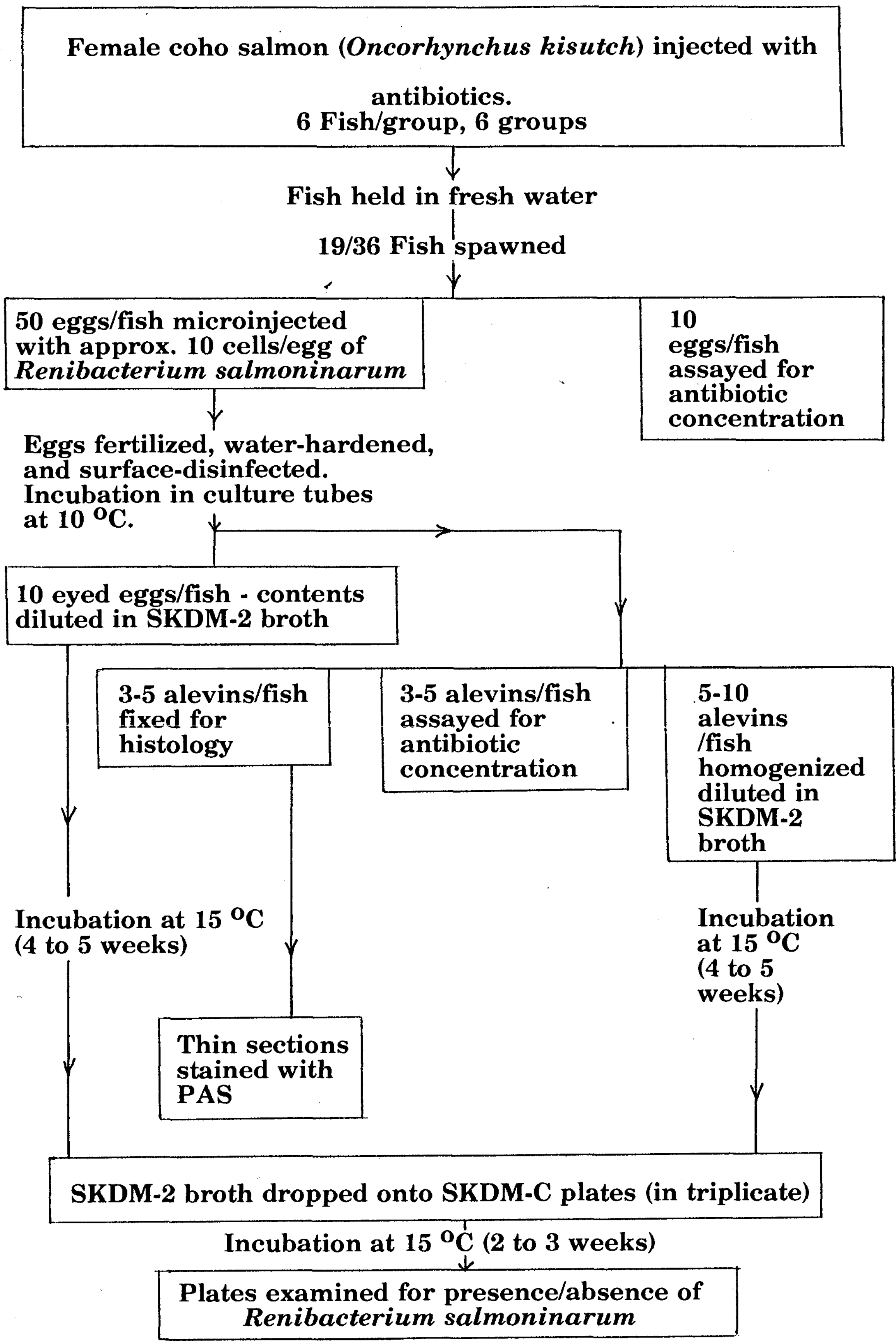

Female coho salmon (Oncorhynchus kisutch) injected with antibiotics.
6 Fish/group, 6 groups
Fish held in fresh water
19/36 Fish spawned
50 eggs/fish microinjected with approx. 10 cells/egg of Renibacterium salmoninarum
10 eggs/fish assayed for antibiotic concentration
Eggs fertilized, water-hardened, and surface-disinfected. Incubation in culture tubes at 10 °C.
10 eyed eggs/fish - contents diluted in SKDM-2 broth
3-5 alevins/fish fixed for histology
3-5 alevins/fish assayed for antibiotic concentration
5-10 alevins /fish homogenized diluted in SKDM-2 broth
Incubation at 15 °C (4 to 5 weeks)
Incubation at 15 °C (4 to 5 weeks)
Thin sections stained with PAS
SKDM-2 broth dropped onto SKDM-C plates (in triplicate)
Incubation at 15 °C (2 to 3 weeks)
Plates examined for presence/absence of Renibacterium salmoninarum

Table 2. Concentration of antibiotics injected into pre-spawning female coho salmon (*Oncorhynchus kisutch*).

Antibiotic	Concentration of antibiotic injected [a] (mg kg^{-1} fish wt.)	Volume injected (mL kg^{-1} fish wt.)	# Fish injected[b]	# Fish spawned
Erythromycin phosphate	20	1.00	6	4
Penicillin G	50	0.30	6	4
Oxytetracycline	10	0.50	6	3
Cephradine	25	0.60	6	2
Rifampicin	5	0.36	6	3
Control [c]		1.00	6	3

a All antibiotics injected were dissolved in 0.85% (w/v) NaCl

b Injections were into the dorsal sinus, after fish were anaesthetized with 2 - phenoxyethanol.

c Control fish were injected with 0.85% (w/v) NaCl only.

The eggs were then fertilized, water-hardened, and following surface-disinfection with iodine, were incubated in sterile culture tubes. At the eyed stage, samples of eggs were assayed for prevalence of infection due to *R.salmoninarum* Mean % survival at the eyed stage was 45%. At hatch, samples of alevins were also assayed for the prevalence of infection due to *R.salmoninarum.* Mean % survival of the remaining embryos at hatch was 47%. Samples of the alevins were also taken at hatch and fixed for histological examination, to determine the location of the pathogen within the developing embryo and/or yolk sac.

In addition, 10 eggs and 3 to 5 injected alevins from each fish were taken at spawning and at hatch, respectively, and were assayed for the concentrations of the antibiotic that had been injected into the source broodfish.

Fish:

The fish used in this study were maturing, pre-spawning coho salmon (*O.kisutch*) held at the Capilano Hatchery. Thirty-six females were selected at random and divided into six groups of six fish each. The fish were anaesthetized with 2-phenoxyethanol (0.025% v/v) and weighed. They were treated as shown in Table 2. The salmon were given opercular clips to distinguish the treatment groups, and held in one pen. Two to three weeks after injection the fish were spawned. The eggs were taken, transported to the laboratory at Simon Fraser University in sterile, iced, plastic containers, to which oxygen had been added to ensure viability, and held overnight at 10 ^{O}C. Each time that a group of the female salmon was spawned, milt was taken from two or three

males, pooled, and transported to the laboratory in the same manner as the eggs.

All salmon used were examined internally and externally. No clinical signs of BKD were observed. Culture and indirect fluorescent antibody tests (IFAT) (Bullock & Stuckey 1975; Daly & Stevenson 1985) done on samples of coelomic fluid and milt failed to detect any *R.salmoninarum* cells.

Antibiotics:

Antibiotics selected were erythromycin phosphate, penicillin G, oxytetracycline, cephradine, and rifampicin. All antibiotics were purchased from Sigma Chemical Company (St.Louis, Missouri). Solutions of the drugs were made in sterile saline (0.85% w/v NaCl). As stated in the Introduction, drug dosages were determined from previous work done on erythromycin injections (20 mg kg^{-1}) (Evelyn et.al.1986a,b,c; Armstrong et.al. 1989), and from reports on *in vitro* studies for the other four antibiotics (Wolf & Dunbar 1959; Austin 1985). Dosages were based on fish weight (Table 2). Control fish were injected with sterile saline only.

Bacterial Isolate:

Isolate #384 of *R.salmoninarum* was used for all experiments (Evelyn et.al. 1984a,b). The isolate was grown for 21 days at 15 ^{o}C on charcoal medium that had been made selective (SKDM-C) (Daly & Stevenson 1985) for the pathogen by the addition of antibiotics described by Austin et.al. (1983). Growth on the plates was aseptically scraped off

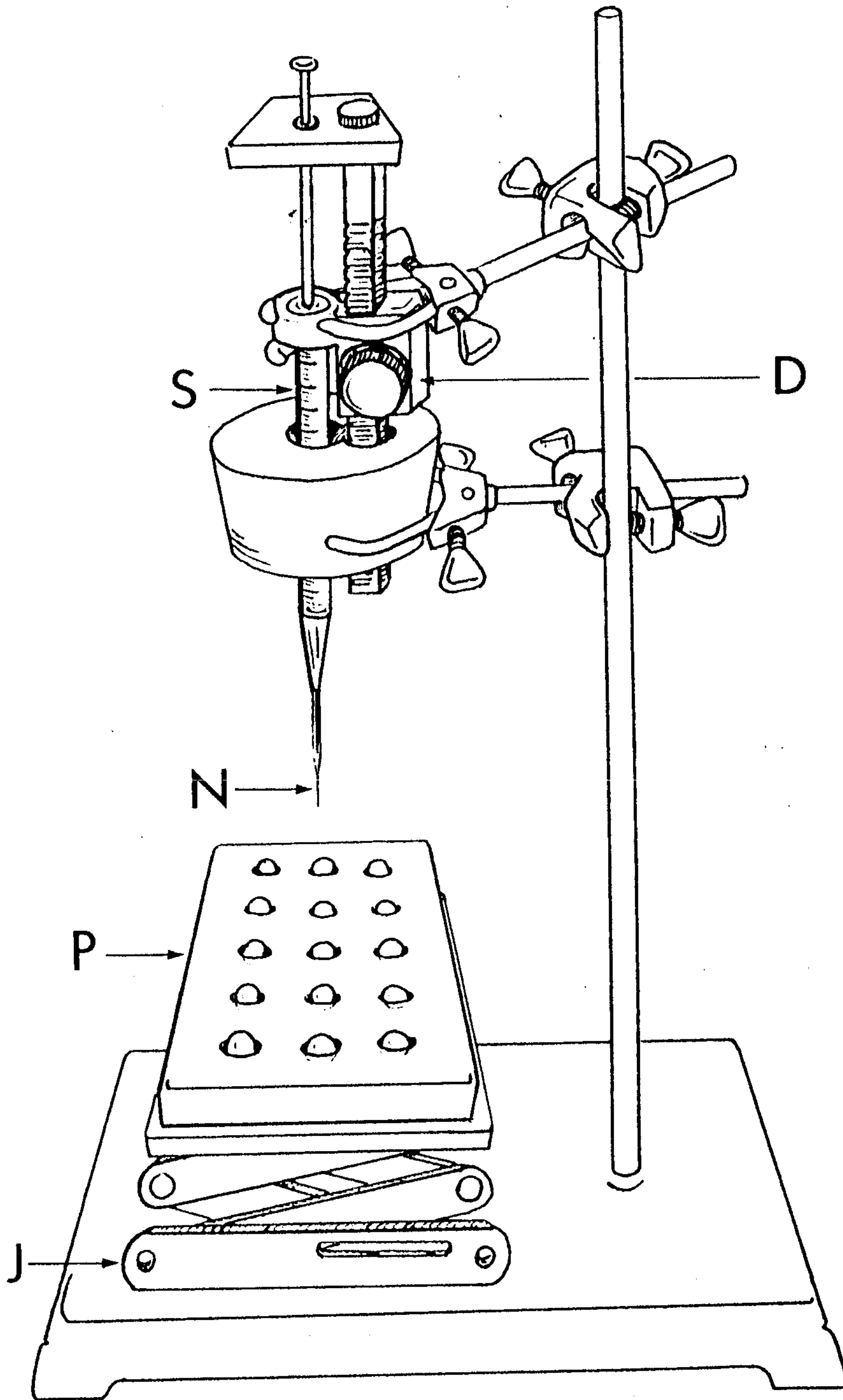
and suspended in sterile peptone-saline (P-S) (0.1%-0.85%). The suspension was adjusted to an absorbance of 2.0 at 420 nm. This had been previously determined to be approximately 1 x 10^8 cells of *R.salmoninarum* mL^{-1} by the "drop-plate" technique (Evelyn 1977). Each time that an experiment was performed, a fresh suspension of actively growing bacterial cells at an absorbance of 2.0 (420 nm) was prepared and diluted to the required concentration. Twenty-five μL samples of the diluted suspension were then dropped onto SKDM-C plates, incubated at 15 ^{o}C, and the resultant colonies counted to determine the actual concentration of viable *R.salmoninarum* cells used for each experiment.

Microinjection Procedure:

Eggs were experimentally infected with viable *R.salmoninarum*, using a microinjection technique (Brown et.al. 1989) (Figure 2). Glass needles of approx. 10 μm internal bore size were used. One μL of a suspension of *R.salmoninarum* (approximately 1 x 10^4 cells mL^{-1}), was injected into each unfertilized, non-water-hardened egg for a total injection dose of approximately 10 *R.salmoninarum* cells per egg. Fifty eggs from each fish were injected in this manner.

Figure 2. Diagram of the microinjection apparatus used to experimentally infect eggs from injected coho salmon (*Oncorhynchus kisutch*), with viable cells of *Renibacterium salmoninarum*. The 50 µL syringe (S) is attached to an automatic dispenser (D), and fitted with a glass needle (N). Eggs held in wells in a plexiglass block (P) were raised to the needle via a labjack (J).

(from Brown et.al. (1989). Reproduced with permission of the publishers.)


S
D
N
P
J

Incubation Procedure:

Eggs were fertilized after injection by placing them in a clean, dry beaker, on ice, and adding the pooled milt (approximately 0.5 mL milt per 50 eggs). After 1 minute the eggs were rinsed with sterile, chilled (10 ^{0}C) water, to free them of excess coelomic fluid and milt, and then water-hardened for 2 hours in at least 10 egg volumes of sterile, chilled, distilled water. The eggs were then surface-disinfected in a solution of 400 ppm Ovadine (Syndel Laboratories, Vancouver, B.C.) for 10 minutes, after which they were thoroughly rinsed 3-times in at least 10 volumes of sterile, chilled, distilled water. Each egg was then carefully transferred to a separate sterile polystyrene culture tube (17 x 100 mm, Falcon) containing 5 ml of sterile, chilled distilled water. The tubes were incubated at 10 ^{0}C, in the dark. The average times to the eyed and hatch stages were 41 days, and 86 days, respectively.

Surface Disinfection of Eggs:

In order to be assured that the povidone-iodine treatment was effective in disinfecting the surface of the eggs the following test was made, using the procedure of Evelyn et.al. (1984b).

Seven microinjected, fertilized, water-hardened, and surface-disinfected eggs in individual culture tubes were randomly chosen. The water in the tubes was poured off. Five mL of non-selective (KDM-2) broth (Evelyn et.al. 1977) was added to each culture tube. The tubes were incubated at 15 ^{0}C for 3 months, during which time the broth in the tubes was checked for evidence of bacterial growth. After 3 months, triplicate samples of 25 µL from each tube were streaked onto SKDM-C

plates. The plates were then incubated at 15 ^{0}C for 3 to 4 weeks. Any growth in the culture tubes or on the plates was tested by IFAT to see if it was that of *R.salmoninarum.*

To confirm that the KDM-2 broth used in the above test was capable of supporting growth of *R.salmoninarum*, and therefore that a lack of growth in the culture tubes described above reflected a complete disinfection of the surface of the eggs, the following test was done:

Three additional microinjected, fertilized, water-hardened and surface-disinfected eggs were chosen at random, and treated as was described above. After 5 mL of KDM-2 broth was added to each tube, it was inoculated with approximately 1 x 10^3 viable cells of *R.salmoninarum.* The tubes were incubated as described above. The broth from each tube was streaked onto SKDM-C plates, as above. The plates were incubated at 15 ^{0}C for 3 to 4 weeks. Any growth observed in the KDM-2 broth or on the SKDM-C plates was confirmed as that of *R.salmoninarum* by IFAT.

Determination of Prevalence of Infection due to *Renibacterium salmoninarum*:

At the eyed stage, and at hatch, 10 injected eggs and 5 to 10 alevins from each fish were taken and assayed for the prevalence of infection. Eggs or alevins were removed from the culture tubes, blotted dry on sterile filter paper, and transferred to clean, dry, sterile culture tubes. The eggs were then pierced with a sterile needle, and the contents were expressed. The "egg shell" (chorion) was removed. Alevins were killed by crushing the skulls with a sterile glass rod. The alevins were then homogenized whole with a Polytron homogenizer (Brinkmann Instruments). Egg contents and whole homogenized alevins were diluted in 5 ml of selective KDM-2 broth (SKDM-2) (Evelyn 1977; Austin et.al.1983), and then incubated at 15 ^{o}C for 5 to 6 weeks. The volume of SKDM-2 added ensured that any antibiotic remaining within the eggs or alevins was diluted to below the concentration that would inhibit the growth of any cells of *R.salmoninarum* that were also present. The tubes were then centrifuged at 3000 x g for 30 minutes to pellet the bacteria. The supernatant was decanted, leaving enough to resuspend the pellet. Twenty-five µL of the resuspended pellet was dropped peripherally and in triplicate onto SKDM-C plates, which were then incubated at 15 ^{o}C for 2 to 3 weeks. Growth was enhanced by the "nurse culture" technique which involved the addition of a 25 µL drop of a heavy suspension (1 x 10^8 cell mL^{-1}) of viable *R.salmoninarum* cells to the centre of the plate (Evelyn et.al. 1989). After incubation, the plates were examined for the presence or absence of growth. Any growth was confirmed as that of *R.salmoninarum* by the IFAT technique. Results

are expressed as the percentage of eggs from fish treated with an antibiotic that were found to contain viable *R.salmoninarum*.

Statistical Analysis:

Comparison of the prevalence of infection between the experimental groups and the control group was done using a Binomial Proportions Test (Zar 1974). The probability that the observed differences were due to chance is expressed as $p > 0.05$.

IFAT Technique:

The indirect fluorescent antibody technique (IFAT) (Bullock & Stuckey 1975) was used to identify growth on SKDM-C plates as that of *R.salmoninarum*. Growth was aseptically removed from experimental plates with a sterile loop, and smeared onto a microscope slide in a drop of sterile saline. The smear was then air-dried and heat-fixed.

Antisera used were rabbit immunoglobulins (IgG) against *R.salmoninarum*, diluted 1:50 in sterile phosphate-buffered-saline (PBS) (0.85% NaCl, 0.12% K_2HPO_4, 0.34% KH_2PO_4, pH 7.2), and goat IgG against rabbit IgG, conjugated to the fluorescent dye, fluorescein isothiocyanate. The counterstain, (rhodamine), (BBL Laboratories, Cockeysville, Maryland) was added to the second antiserum before use. Final dilution of the second antiserum was 1:100 in sterile PBS. Each antiserum was incubated on the slides for 30 minutes in the dark. After staining, slides were mounted and examined with an epifluorescent microscope, under oil immersion at 1000x magnification. Positive control

slides of known *R.salmoninarum* cells were prepared each time an IFAT test was done.

Assay for Antibiotic Concentration Within Eggs and Alevins:

Eggs were assayed at spawning (10 eggs from each fish) for the intra-ovum concentration of the antibiotic that had been injected into the fish serving as the source of the eggs. Also, 3 to 5 whole alevins were taken from each incubating egg lot (from the 50 injected eggs from each fish) and assayed for antibiotic concentration. Egg contents and whole alevins were prepared as described above. Triplicate samples of 10 uL each from each egg or homogenized alevin were dropped onto sterile filter paper disks, for use in a modified cylinder plate bioassay method (Difco 1968; Bell et.al. 1969; Bullock & Leek 1986; Evelyn et.al. 1986a,c). These disks were placed on Difco Antibiotic Medium #1 (Difco) plates.

Plates were prepared by adding heavy suspensions, in sterile P-S, of a sensitive bacterial species (approximately 10^{10} cells mL^{-1}) to melted agar. For determination of erythromycin phosphate concentrations, a sensitive strain of *Micrococcus luteus* was used. A sensitive strain of *Staphylococcus aureus* was used for the four other antibiotics. The seeded agar was poured into Petri plates, and allowed to solidify before assay disks were placed on them.

The plates were incubated at room temperature overnight, and the zones of inhibition measured. To determine the amount of antibiotic present, the zones were compared to standard curves. These were determined by preparing plates in the same manner, and dropping

known concentrations of antibiotics onto the sterile filter disks. Standard curves were done in triplicate, each time that an antibiotic assay was performed. To ensure that the bacterial cells on each plate were sensitive to that antibiotic, one disk containing a known amount of antibiotic was placed on each test plate along with the disks saturated in the samples of egg contents or homogenized alevins.

Histology:

To attempt to locate the pathogen within the developing embryo, 3 to 5 alevins from each lot of the original 50 injected eggs from each fish were taken at hatch for histological examination. The alevins were killed with 2-phenoxyethanol, and immediately placed in Stockard's fixative (J. Bagshaw, pers. comm.). This fixative was replaced with a fresh batch after 24 hours. Another 24 hours later, this was replaced with a buffered formalin saline solution (BFS) (Drury & Wallington 1967). The BFS was then changed three times every 24 hours. The fixed tissues were imbedded in paraffin, and sagittal sections of the alevin tissues, including the yolk sac, were taken. The sections were deparaffinized, and then stained with Periodic Acid-Schiff stain (PAS) (Lillie 1969; Bruno & Munro 1986a; Hoffmann et.al. 1989).

In vitro Assay for the Susceptibility of *Renibacterium salmoninarum* to Antibiotics:

This experiment was designed to meet the third objective of this study. Conditions such as concentrations of *R.salmoninarum* cells and incubation temperature were chosen to most closely resemble those likely to be encountered by the developing egg in the first experiment.

Culture tubes containing 5 mL of SKDM-2 broth were inoculated with approximately 2×10^3 cells of *R.salmoninarum*. The tubes each contained one of three concentrations of one of the five antibiotics. The three concentrations were: 0.1 $\mu g\ ml^{-1}$, 0.5 $\mu g\ ml^{-1}$, and 1.0 $\mu g\ ml^{-1}$. Control tubes contained no added experimental antibiotics. There were three replicates of all experimental tubes, as well as of the control tubes, for a total of 48 tubes. The tubes were incubated at 10 ^{o}C for 22 days. At selected intervals (0, 5, 16, and 22 days), 1 mL was aseptically removed from each tube, and transferred to sterile polypropylene tubes. These were then centrifuged at approximately 12,000 x g. for 15 minutes. The supernatant was then decanted and the pellet was resuspended in 1 mL sterile P-S. The procedure was repeated, in order to wash all antibiotics from the bacterial cells. The resuspended pellet was dropped peripherally and in triplicate onto SKDM-C plates, and the plates were then incubated at 15 ^{o}C for 3 to 4 weeks. A "nurse-culture" suspension was dropped onto the centre of each plate to facilitate growth of the antibiotic-exposed bacteria. Samples on the plates were checked for the presence or absence of growth, and the growth was confirmed as that of *R.salmoninarum* by IFAT.

RESULTS

Surface Disinfection of Eggs:

After incubation for 3 months at 15 ^{0}C, the KDM-2 broth in the first 7 culture tubes remained clear - no bacterial growth was observed. No bacterial growth was observed on the SKDM-C plates that had been streaked with triplicate samples of the broth from those 7 culture tubes.

After incubation for 3 months at 15 ^{0}C, the KDM-2 broth in the 3 culture tubes that had each received an inoculation of *R.salmoninarum* cells, was cloudy - evidence of bacterial growth. Bacterial colonies were noted on the SKDM-C plates that had been streaked with triplicate samples of the cloudy KDM-2 broth. The growth in the broth and on the plates was identified as that of *R.salmoninarum* by IFAT, indicating that the KDM-2 used was capable of supporting growth of the bacterium.

These observations indicate that the procedure used for disinfecting the surface of the coho salmon eggs was effective.

Antibiotic Concentrations Within Eggs and Alevins:

All five of the antibiotics were detected within the eggs that were taken at spawning (Table 3). Two of the antibiotics, erythromycin phosphate and rifampicin, were present within homogenized alevins in detectable concentrations (Table 3). There was little variation observed in the antibiotic concentrations detected within eggs and alevins from an individual fish. However, there was considerable variation between the average antibiotic concentration of eggs or alevins from one fish and the average antibiotic concentration within the progeny of another (Appendices A & B).

Histology:

Bacterial cells with morphological and staining characteristics typical of *R.salmoninarum* (i.e., they were short (0.5 µm - 1.0 µm), PAS-positive rods) were observed within the yolk sacs of 5 of the alevins from 2 of the 3 control fish (those that were not injected with antibiotics) (from a total of 9 examined from control fish) (Fig. 3).

No bacterial cells were observed within the sections of alevins from the experimental fish.

No bacterial cells were observed within the tissues of any of the embryos, nor was there evidence of any inflammatory response in the alevin tissues. No phagocytic or hyperplastic cells were observed. Examination of the alevin tissues did not reveal any gross abnormalities in cell morphology.

Table 3. Average antibiotic concentrations detected within the coho salmon (*Oncorhynchus kisutch*) eggs at spawning, and within whole alevins at hatch.

Antibiotic	Antibiotic concentration [a] (µg/ml)	
	Eggs	Alevins
Erythromycin phosphate	1.10 + 0.57[b]	0.30 + 0.33
Penicillin G	0.25 + 0.01	n.d.[c]
Oxytetracycline	1.20 + 0.00	n.d.
Cephradine	0.50 + 0.00	n.d.
Rifampicin	2.67 + 0.66	0.25 + 0.00

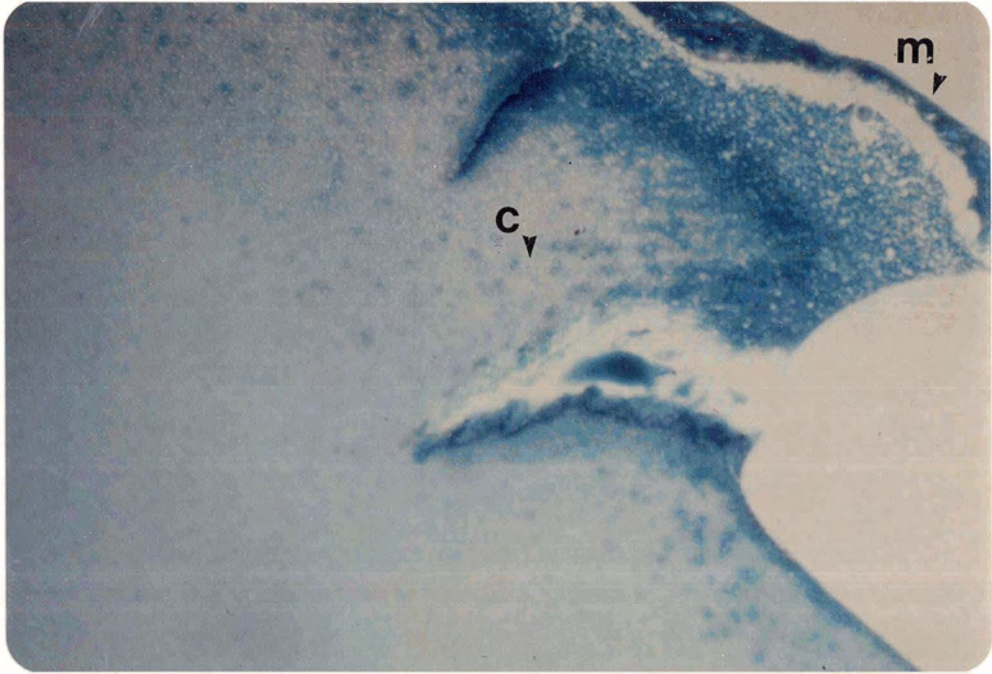
a Determined by a modified Cylinder Plate Method, using sensitive strains of *Micrococcus luteus* or *Staphylococcus aureus*.

b Standard error

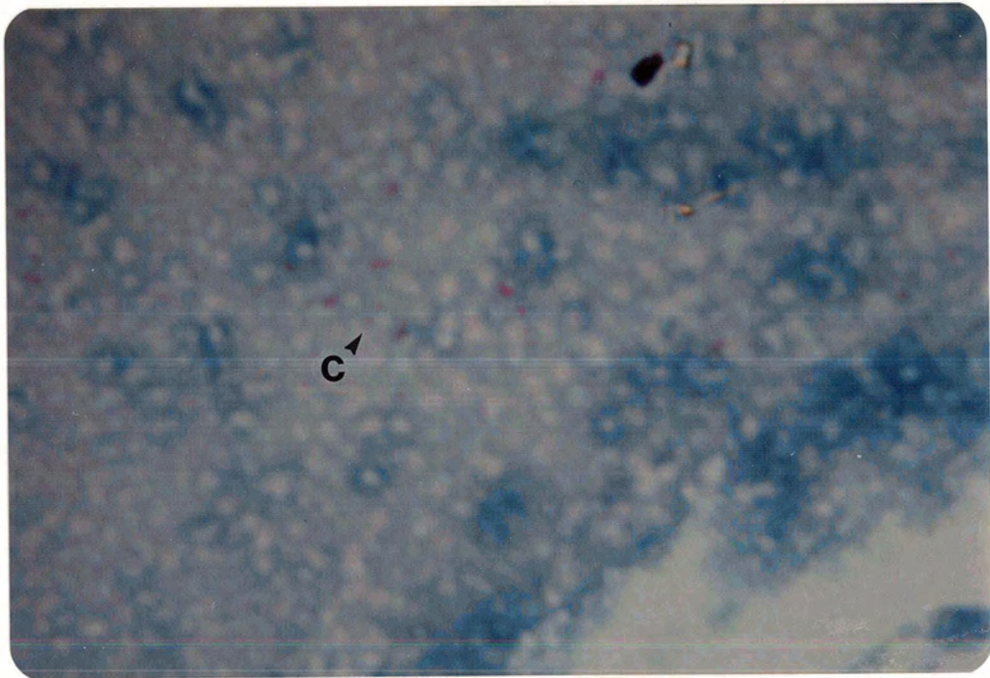
c n.d. = not detected.

Figure 3. Section through an alevin from a control fish (one that received no antibiotic injection), showing cells with morphological and staining characteristics typical of *Renibacterium salmoninarum*. Sections were stained with Periodic-Acid-Schiff stain (PAS). Bacterial cells were observed exclusively within the yolk sac of the alevins. (A) Low magnification (x100) - showing location of bacterial cells (c). The margin of the yolk sac is also shown (m). (B) High magnification (x1000) - same section as (A). The location of the bacterial cells (c) is shown.

A



B



Prevalence of Infection due to *Renibacterium salmoninarum*:

The prevalence of infections due to *R.salmoninarum* within the eyed eggs and alevins from each fish is shown in Figure 4. There was considerable variation observed between fish (Figure 4). The mean prevalence of infection due to *R.salmoninarum* for each experimental group was calculated and the results are shown in Table 4. A comparison of experimental groups with the control group was also done and the levels of significance are shown in Table 4.

The proportions of eyed eggs from salmon injected with cephradine or rifampicin that contained viable *R.salmoninarum* were significantly smaller than the proportion from the control fish ($p < 0.01$) (Table 4).

At hatch, viable cells of *R.salmoninarum* were isolated from a significantly smaller percentage of alevins from each of the five experimental groups than from the control group ($p < 0.01$) (Table 4). There was no significant difference in the prevalence of infection among the experimental groups of alevins.

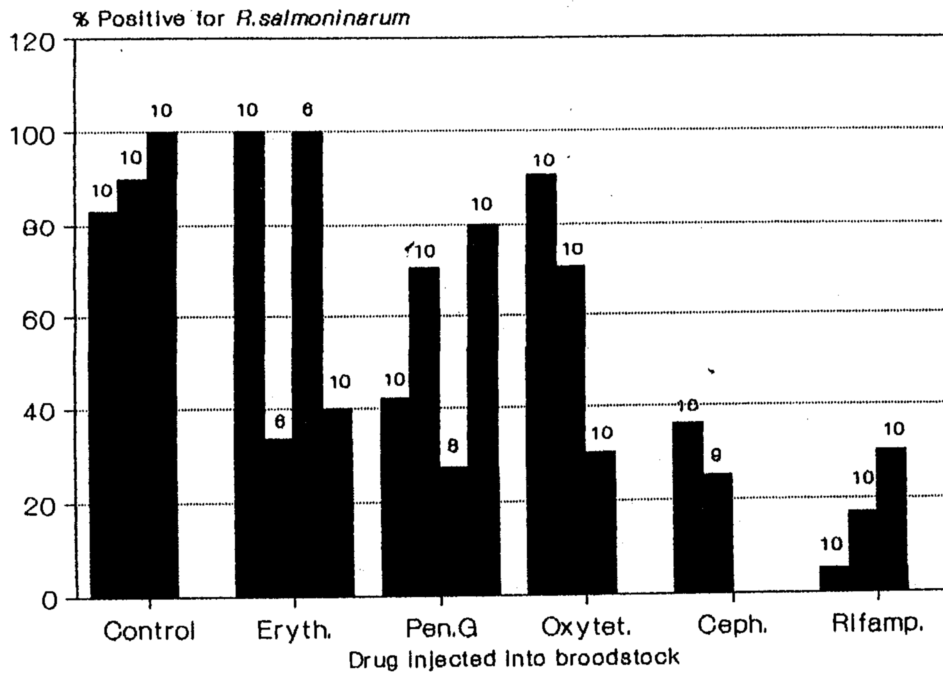
In none of the experimental groups was the prevalence of infection due to *R.salmoninarum*, at either the eyed or the hatch stage, reduced to zero.

Figure 4. **A.** Prevalence of infection due to *Renibacterium salmoninarum* within eyed eggs from each fish (each bar represents the proportion of eggs from a fish, which were found to be positive for *R.salmoninarum*). Numbers above each bar indicate the sample size from each fish.

B. Prevalence of infection due to *Renibacterium salmoninarum* within alevins at hatch, from each fish (each bar represents the proportion of alevins from a fish, which were found to be positive for *R.salmoninarum*). Numbers above each bar indicate the sample size from each fish.

Eryth. = Erythromycin phosphate
Pen.G = Penicillin G
Oxytet. = Oxytetracycline
Ceph. = Cephradine
Rifamp. = Rifampicin

(A)



(B)

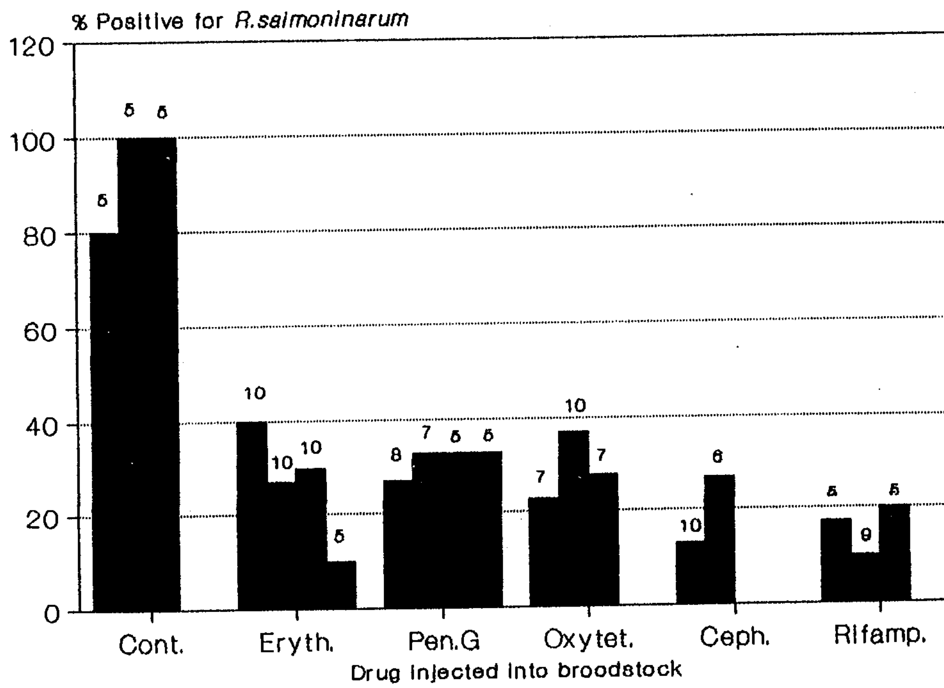


Table 4. Mean of the prevalences of infections due to *Renibacterium salmoninarum* (Rs) within eyed eggs and alevins at hatch.

Antibiotic	Mean proportion of eyed eggs or alevins found to contain viable Rs.					
	Eyed Eggs			Alevins		
	n	% +ve for Rs	p[a]	n	% +ve for Rs	p
Control[b]	30	91 ± 8.5		15	93 ± 11.5	
Eryth.	32	68 ± 36.8	>0.05	35	27 ± 12.5	<0.01
Pen.G	38	55 ± 24.5	>0.05	25	32 ± 3.0	<0.01
Oxytet.	30	63 ± 30.5	>0.05	24	29 ± 7.1	<0.01
Ceph.	19	30 ± 7.8	<0.01	16	20 ± 9.9	<0.01
Rifamp.	30	17 ± 12.5	<0.01	19	16 ± 5.1	<0.01

[a] p indicates the level of significance (confidence interval). Comparisons of the mean prevalence of infection in each experimental group with the mean of the control group was done using a Binomial Proportions Test. $p>0.05$ indicates no statistical significance, $p<0.01$ indicates statistical significance.

[b] Control fish were injected with saline (0.85% w/v) only.

Eryth. = Erythromycin phosphate
Pen.G = Penicillin G
Oxytet. = Oxytetracycline
Ceph. = Cephradine
Rifamp. = Rifampicin

In vitro Assay for the Susceptibility of *Renibacterium salmoninarum* to Antibiotics:

At 10 ^{o}C, the minimum lethal concentration (MLC) and minimum time of exposure required for a lethal effect on cells of *R.salmoninarum* (MLT), varied with each antibiotic (Table 5).

The cells of *R.salmoninarum* were killed when they were exposed to concentrations of erythromycin phosphate greater than 0.1 $\mu g\ mL^{-1}$ for at least 16 days. The MLC of penicillin G was 0.5 $\mu g\ mL^{-1}$ with an MLT of at least 16 days. The MLC of oxytetracycline under these conditions was also observed to be 0.5 $\mu g\ mL^{-1}$. However, the MLT for this antibiotic, 22 days, was the longest observed for all five drugs. The bacterial cells were killed when exposed to cephradine or rifampicin at 0.1 $\mu g\ mL^{-1}$ concentration, for 16 or 5 days, respectively.

Table 5. Recovery of viable *Renibacterium salmoninarum* (R.s.) cells from SKDM-2 medium with added antibiotics at selected concentrations and times of exposure.

Antibiotic	Concentration [a] (µg ml^{-1})	Number of drops yielding growth of R.s. [b] — Time of exposure (days) 0	5	16	22
Erythromycin phosphate	0.1	9 [c]	9	3	3
	0.5	9	3	0	0
	1.0	9	0	0	0
Penicillin G	0.1	9	9	9	9
	0.5	9	3	3	0
	1.0	9	0	0	0
Oxytetracycline	0.1	9	9	9	9
	0.5	9	9	3	0
	1.0	9	3	0	0
Cephradine	0.1	9	3	0	0
	0.5	9	0	0	0
	1.0	9	0	0	0
Rifampicin	0.1	9	0	0	0
	0.5	9	0	0	0
	1.0	9	0	0	0
Control	(no added antibiotic)	9	9	9	9

a The antibiotics were added to SKDM-2 at the concentrations indicated.
b Bacterial cells were incubated with antibiotics at 10 °C, washed free of the antibiotics in sterile peptone-saline and individual drops inoculated onto selective charcoal agar (SKDM-C) plates that contained no added antibiotics. Plates were incubated at 15 °C.
c 3 - 25 µL drops from triplicate culture tubes were dropped onto the SKDM-C plates. Numbers indicate the number of drops from which viable R.s. cells were recovered.

DISCUSSION

The results indicate that injection of any one of the antibiotics into mature female coho salmon is effective in reducing the prevalence of infections of *Renibacterium salmoninarum* within the progeny.

Cephradine and rifampicin had an inhibitory effect on *R.salmoninarum* by the time the eggs had reached the eyed stage, while there was no observed effect, at this stage, with erythromycin phosphate, penicillin G, or oxytetracycline. All five of the antibiotics demonstrated a significant effect against the pathogen by the time the hatch stage was reached. One explanation for the delay in the action of erythromycin phosphate, penicillin G, and cephradine may be differences in the pharmacokinetics of each antibiotic within this particular environment, as discussed below.

The results of the *in vitro* experiment indicate that erythromycin phosphate, penicillin G, and oxytetracycline all require a longer time of exposure to the bacterial cells to be effective. The environment within the developing salmonid egg is different than that of the *in vitro* experiment (SKDM-2 broth). The high lipid and protein content of the yolk material (Riazi & Fremat 1988; Shcherbina et.al. 1988) may contribute to the delay in the action of the antibiotics. Inhibitory binding of antibiotics by proteins has been reported (Craig & Suh 1986), although the complete mechanism for this phenomenon is not known. Austin (1985) indicated that surfactants and lipids in the form of "Tween" (registered trademark, Rhom & Haas) and liposomes markedly

decrease the efficacy of certain antibiotics, particularly macrolides (such as erythromycin phosphate), when added to the feed of juvenile rainbow trout (*O.mykiss*). Again, the mechanism of this effect is not yet known. The lipid content of salmonid egg yolk may have the same effect on the antibiotics examined here.

Another factor to be considered is the incubation temperature. The eggs were incubated at 10 ^{O}C. This is a lower temperature than in the experiments described by Wolf & Dunbar (1959) and Austin (1985). *R.salmoninarum* is a slow-growing organism, its optimal growth temperature *in vitro* being 15 to 18 ^{O}C (Ordal & Earp 1956; Smith 1964; Sanders & Fryer 1980). The bacterial cells multiply very slowly *in vitro* at temperatures below 10 ^{O}C (Smith 1964). The antibiotics selected affect different stages of the metabolism of the bacterial cells (Table 1) (Lancini & Parenti 1982). It may be that the slow bacterial growth at low temperatures would account for the delay of the inhibitory effect of the antibiotics.

The bacterial cells were microinjected into one location within the yolk material of the unfertilized, non-water-hardened eggs. The *R.salmoninarum* cells would not be likely to multiply extensively at 10 ^{O}C (Sanders & Fryer 1980). Also, they would not likely have spread evenly throughout the developing egg because the bacterium is non-motile (Sanders & Fryer 1980). Clumps of bacterial cells probably therefore occurred within the yolk. This is likely, since *R.salmoninarum* has been shown to have a hydrophobic cell surface (Daly & Stevenson 1987; Bruno 1988) and to be auto-aggregating (Evelyn et.al. 1973; Bruno 1988). Thus, the antibiotics within the egg may not have

contacted all cells of the pathogen immediately, resulting in the delayed effect.

It must be noted that there was considerable variation in the results obtained from the progeny of different fish. Antibiotic concentrations and prevalences of infection due to *R.salmoninarum* showed greater variation between groups of eggs or alevins from different fish than within a group from a single fish. This difference may be due, in part, to variations in the technique used when the broodstock were injected with the antibiotics. Inadvertant injection of antibiotic into the muscle could lead to a lower concentration of the drug being deposited within the egg. The difference may also be explained by possible physiological, cellular, or metabolic differences among fish. These could result in variations in the amount of antibiotic deposited within the eggs. Also, there could be some differences in the composition of the eggs within different fish, resulting in variations on the effectiveness of the antibiotics, or the viability of the bacterial cells within the egg.

Most of this "fish-to-fish" variation is beyond the control of the investigator. To overcome the difficulties in interpreting the data obtained that were presented by this variable, several fish were used for each experimental group. In further experiments it may be necessary to use an even larger number of fish for each treatment.

None of the five antibiotics was effective in eliminating *R.salmoninarum* from all of the eggs or alevins. The reasons for this may be the same as described above: uneven dispersal of the bacteria, inhibition of the activity of the drugs because of low temperatures, or deleterious effects on the antibiotics due to the nature of the yolk

material. It should be noted also that each egg was injected with approximately 10 *R.salmoninarum* cells. This may be a much higher concentration of bacterial cells than would be encountered in a natural infection situation (Evelyn et.al. 1986c). It has been postulated that only one or two cells of the pathogen are initially present in infected eggs (Evelyn et.al. 1986c). Thus, prophylactic injection into broodstock salmon of any one of the antibiotics tested may be more effective in reducing the prevalence of natural intra-ovum infections due to *R.salmoninarum* than was seen in this experimental infection. In a natural situation the same amount of antibiotic (given that the same concentration of drug was injected at the same time into the pre-spawning female salmon as was done for this study) would be deposited within the egg as was seen in this study, but the drug would act against fewer cells of *R.salmoninarum*. Therefore the antibiotic could be more effective in eliminating the pathogen from the egg.

The concentration of each of the antibiotics within the developing egg declined as the alevin reached the hatch stage. Only erythromycin phosphate and rifampicin persisted within the alevins at detectable levels. The fact that the prevalence of infection was reduced within the alevins for all of the experimental groups indicates that each drug was present in quantities sufficient to inhibit *R.salmoninarum*.

Rifampicin was injected into one group of pre-spawning broodstock female salmon at the lowest concentration of the five antibiotics. However, the average concentration detected within the eggs taken at spawning was the highest of all the experimental groups, and furthermore, as stated, the drug persisted within the developing embryo.

The *in vitro* experiment showed that rifampicin was the most effective bactericidal antibiotic against *R.salmoninarum*, with regards to minimum lethal concentration and time of exposure.

The above-mentioned data suggests that rifampicin could be the most effective antibiotic for use in controlling vertical transmission of BKD, possibly at higher concentrations. Two questions must be addressed, however, if the use of rifampicin in hatcheries is to be considered. One is the possible toxic effect of rifampicin on the pre-spawning salmon itself, or on the developing embryos (this latter point is discussed in greater detail later in this thesis). Second is the possibility of hazards to human health. Rifampicin is used in medicine as an anti-tubercular drug (Lancini & Parenti 1982). The possibility of inadvertently selecting for antibiotic-resistant strains of a human pathogen, if rifampicin were exposed to the environment, must be considered when addressing the question of widespread use of rifampicin.

However, when examining the results expressed in Table 4 it can be seen that there was no significant difference in the effect of the five antibiotics by the time the hatch stage was reached. Therefore the apparent advantage of rifampicin is not significant in considering the first objective of this study, examining the effectiveness in reducing the intra-ovum infections due to *R.salmoninarum*. Other antibiotics, such as erythromycin phosphate or oxytetracycline may be used with equal effectiveness, and with less possible risk to human health.

The histological examination of the alevins suggests that the *R.salmoninarum* cells are located within the yolk sac of the developing embryo. It should be noted that serial sections of the whole animal were

not done. This would be necessary in order to determine more accurately whether or not there were any bacterial cells within the embryonic tissues themselves.

There was no inflammatory response observed within the alevin tissues, nor any obvious abnormalities in cell morphology. Again, to more clearly examine the effect of the antibiotics on the developing embryos, serial sections of the whole embryos would be desirable. Also, if the alevins had been incubated for a longer time, to beyond the stage when the yolk sac is totally absorbed, inflammatory responses to the presence of the pathogen may have been observed, because by this stage, a more competent immune system may have developed. It is postulated that the immune system is not fully developed in juvenile salmonids until the fish reach a weight of approximately 4 g. - long after the yolk sac is absorbed (Paterson & Fryer 1974; Khalifa & Post 1976).

DeCew (1972) has reported teratogenic effects of penicillin G procaine, dihydrostreptomycin sulfate, and oxytetracycline on the progeny of fish that were injected before spawning with a complex of the above antibiotics. The effects included deformities of the fins and mandibles. Other investigators have reported deleterious effects, including destruction of the liver and kidney tissues, of erythromycin when administered to juvenile rainbow trout in feed (Hicks & Geraci 1984), and of oxytetracycline when injected into lake trout (*Salvelinus namaycush*) (Marking et.al. 1988). The possibility of adverse effects of the antibiotic injection procedure should be examined, including any long-term effects on smolting, osmoregulation, and growth.

There are other factors to be considered when using antibiotics, whatever the method of application. One of these is the risk of generating, or selecting for antibiotic-resistant strains of the pathogen. *R.salmoninarum* can develop resistance to erythromycin when exposed to the antibiotic *in vitro* (Bell et.al. 1988). The mechanism for erythromycin-resistance in *R.salmoninarum* has not yet been described. No plasmids for antibiotic-resistance have been isolated from cells of *R.salmoninarum* (Toranzo et.al. 1983), The mechanism for this resistance may therefore involve mutational events (Tanaka et.al. 1968). In light of these findings, the possibility of the development of strains of *R.salmoninarum* that are resistant to the other four antibiotics, as well as to erythromycin phosphate must be noted.

The results of this study illustrate some areas that should be considered for further research, in addition to those already discussed. In addition to the examination of the long-term effects of the antibiotics on the juvenile salmonids, these young fish from broodstock injected with the drugs should be maintained in pathogen-free water until after smolting. This would ensure an examination of the efficacy of the injection procedure as it impacts on the later stages of the life cycle of the salmon. The salt-water phase would be of particular interest, because epizootics of BKD are more likely to occur at this time (Klontz 1983, Austin & Rayment 1985; Evelyn 1988).

Studies should also be done to determine the time of injection and the concentration of injected antibiotic required to achieve maximum intra-ovum concentrations. A greater amount of drug within the eggs could lead to a greater reduction in the prevalence of infection.

Different types of antibiotics act on different aspects of bacterial metabolism (Lancini & Parenti 1982). Complexes of antibiotics have been reported to be successful in reducing pre-spawning mortalities due to BKD (DeCew 1972). Consideration should therefore be given to determining whether the injection of a combination of antibiotics may be more effective in controlling vertical transmission of BKD than the injection of single antibiotics.

CONCLUSIONS AND SUMMARY

The following conclusions and statements can be made from the results of the studies discussed in this thesis:

First, the cells of *R.salmoninarum* were killed upon *in vitro* exposure to each of the five antibiotics: erythromycin phosphate, penicillin G, oxytetracycline cephradine or rifampicin. The minimum inhibitory concentration and minimum time of exposure required to kill the bacterium varied with each antibiotic.

Second, injection of any one of the five antibiotics into maturing female coho salmon (*Oncorhynchus kisutch*) resulted in deposition of detectable concentrations of the antibiotic within the egg. Erythromycin phosphate and rifampicin persisted within the developing embryos at least until the hatch stage.

Third, the injection procedure resulted in a significant reduction in the percentage of alevins that contained viable *R.salmoninarum*. However, none of the antibiotics was successful in eliminating the pathogen from all of the eggs or alevins. Prophylactic injection of antibiotics into broodstock female salmon should prove to be an effective method for controlling, but not eliminating, vertical transmission of *R.salmoninarum*, the causative agent of BKD.

APPENDICES

Appendix A. Antibiotic concentration within each egg taken at spawning, from all fish.

Antibiotic	Fish	Antibiotic concentration (µg mL^{-1})									
		Egg #									
		1	2	3	4	5	6	7	8	9	10
Eryth.	1	0.2	0.2	-[a]	-	-	-	0.2	-	-	0.2
	2	2.1	2.9	0.4	0.9	1.7	0.9	0.4	0.4	0.4	0.9
	3	0.4	-	-	-	-	-	-	-	-	-
	4	3.3	1.2	3.3	2.5	3.3	3.3	3.3	3.3	2.5	1.2
Pen.G	1	-	0.2	0.3	0.3	0.2	0.2	-	0.2	0.3	-
	2	0.2	0.6	0.3	0.3	0.3	0.2	0.2	-	0.2	0.2
	3	-	0.2	-	-	0.3	0.3	-	-	0.3	0.2
	4	-	-	0.2	0.2	0.3	0.2	0.2	-	-	-
Oxytet.	1	1.2	1.2	1.2	1.2	1.2	1.2	1.2	-	1.2	1.2
	2	-	-	-	-	-	1.2	1.2	-	-	-
	3	1.2	-	1.2	1.2	1.2	-	1.2	1.2	1.2	1.2
Ceph.	1	-	-	-	-	-	0.5	0.5	0.5	0.5	-
	2	-	-	-	-	-	0.5	-	-	-	-
Rifamp.	1	-	-	4.0	-	4.0	-	-	-	-	4.0
	2	-	2.0	-	2.0	2.0	-	-	-	-	2.0
	3	-	-	-	2.0	-	-	-	-	-	2.0

[a] - = antibiotic not detected.

Appendix B. Antibiotic concentration within each alevin at hatch, from all fish.

Antibiotic	Fish	Antibiotic concentration ($\mu g\ mL^{-1}$)				
		Alevin #				
		1	2	3	4	5
Eryth.	1	0.1	0.1	0.1	0.1	0.1
	2	0.1	0.1	0.1	0.1	-[a]
	3	0.1	0.2	0.1	0.2	0.25
	4	1.0	0 1	0.3	[b]	
Pen.G	1	-	-	-	-	-
	2	-	-	-		
	3	-	-	-		
	4	-	-	-	-	-
Oxytet.	1	-	-	-		
	2	-	-	-	-	
	3	-	-	-		
Ceph.	1	-	-	-	-	-
	2	-	-	-	-	
Rifamp.	1	0.25		0.3	0.2	0.25
	2	0.25	0.25	0.1	0.25	0.25
	3	0.3	0.3	0.25		

a - = antibiotic not detected

b blank space indicates fewer than 5 alevins were sampled from that particular fish.

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