IN VITRO EFFICACY OF SIX ANTIBIOTICS AND PLASMA CONCENTRATIONS OF TWO QUINOLONES IN ATLANTIC SALMON (*Salmo salar*)

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ABSTRACT

The minimum inhibitory concentrations (MICs) of six antibiotics were determined for *Aeromonas salmonicida, Vibrio anguillarum* and *Vibrio ordalii* isolated from disease outbreaks in fish on the Atlantic and Pacific coasts. MICs were performed at three incubation temperatures (10, 20, 30°C). Incubation temperature influenced the MIC for some antibiotics. The most pronounced case was a two-fold or greater increase in the MIC of oxytetracycline against *Vibrio* spp. with increasing temperature. Changes in resistance patterns were examined in the isolates collected from 1985 to 1990. The *in vitro* efficacy of the antibiotics examined ranged from the potent quinolones (oxolinic acid and sarafloxacin) to ormetoprim-sulfadimethoxine and oxytetracycline, to the relatively ineffective drugs erythromycin and streptomycin.

The plasmid profiles of oxytetracycline and streptomycin resistant and sensitive bacterial isolates were examined by agarose gel electrophoresis. Resistant isolates were examined when grown in the presence and in the absence of antibiotic. Resistance to these antibiotics did not appear to be plasmid-mediated.

Plasma concentrations of oxolinic acid and sarafloxacin in Atlantic salmon in 10°C seawater were determined during and following the recommended oral treatment regime to assess whether concentrations were sufficient for the treatment of furunculosis and vibriosis. Antibiotic concentrations were assayed via a microbiological assay using *V. anguillarum* ATCC 19264 as the test organism. Plasma concentrations of 3-5 times the MIC for *Vibrio* spp. were present for 48 h post-treatment with sarafloxacin and for 72 h post-treatment with oxolinic acid. Plasma concentrations of oxolinic acid were 3-5 times the MIC for *A. salmonicida* for 72 h post-treatment; such concentrations were observed only in the 6 and 12 h post-treatment samples for sarafloxacin. Plasma concentrations of sarafloxacin were 2-3 times the MIC for *A. salmonicida* for 24 h post-treatment.

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ATCC - American Type Culture Collection

CFU - colony forming units

Da - daltons

d - day

GLM - General Linear Model

h - hour

kb - kilobase

kDa - kilodalton

Md - megadalton

MIC - minimum inhibitory concentration

min - minute

mL - millilitre

sec - second

TSA - tryptic soy agar

TSB - tryptone soya broth

μg - microgram

μL - microlitre

1. GENERAL INTRODUCTION

1.1 Bacterial diseases of salmonids

The major bacterial diseases of salmonids are caused by Gram negative bacteria, such as *Aeromonas salmonicida, Aeromonas hydrophila, Vibrio anguillarum, Vibrio ordalii, Yersinia ruckeri* and *Flavobacterium* sp. The most important Gram positive pathogen is *Renibacterium salmoninarum.* These bacteria infect both wild and cultured populations and can result in massive mortalities amongst cultured salmonids. Disease outbreaks are usually associated with a stress component, such as low dissolved oxygen concentrations, high ammonia, overcrowding and increased water temperatures, all of which frequently occur in the farmed fish industry (Trust, 1986). The immune system of salmonids consists of cell-mediated immunity and an antibody-mediated response. Only one class of immunoglobulin is produced, IgM, which activates the classical complement pathway. IgM exists in blood, tissue fluids, lymph and mucus of the gut and skin. In salmonids held at 10°C, the primary antibody response takes 10-15 days, peaks at 30-50 days and persists for 100-150 days (Trust, 1986). Bacterial diseases such as furunculosis and vibriosis manifest predominantly as acute forms of disease; thus, early antibiotic therapy is crucial for effective causal treatment.

1.1.1 Furunculosis

The etiological agent of furunculosis is *Aeromonas salmonicida*, a Gram negative rod-shaped bacterium. Furunculosis was first recorded in a 1894 outbreak in farmed brown trout (*Salmo trutta*) in Germany. The first reported outbreak in North America occurred in Michigan in 1902 in Rainbow trout (*Oncorhynchus mykiss*). Furunculosis now occurs worldwide, predominantly in salmonids (McCarthy, 1975).

The acute form of furunculosis is most common and manifests as a septicemia with extensive haemorrhages and tissue necrosis. Mortalities are high and occur within a few days after the onset of disease. Outbreaks are linked to stress conditions such as overcrowding and to an increase in water temperature above 13°C. The chronic form of furunculosis is characterized by focal lesions called furuncles in the musculature. Mortalities are low in the chronic form and asymptomatic carrier states may develop (McCarthy, 1975).

Furunculosis is transmitted from fish to fish by direct contact or the consumption of infected fish tissues. Vertical transmission via eggs has also been suggested (McCarthy, 1975). Although furunculosis is generally considered a disease problem in freshwater, transmission from fish to fish has also been reported in full seawater (Scott, 1968) and the disease has been observed in sable fish

(*Anoplopoma fimbria*), a strictly marine species (McCarthy, 1975).

1.1.2 Vibriosis

The term vibriosis generally refers to a haemorrhagic septicemia caused by either *Vibrio anguillarum* or *Vibrio ordalii*. *V. anguillarum* and *V. ordalii* are Gram negative, rod-shaped, halophilic bacteria, which cause disease in both marine and freshwater fish, and in shellfish. Vibriosis was described as early as 1868 as a disease of eels referred to as 'red pest', but the etiologic agent was not isolated until 1893 (Post, 1987). *Vibrio ordalii* was previously classified as *V. anguillarum* biotype II (Horne, 1982).

Vibriosis due to *Vibrio anguillarum* manifests as a haemorrhagic septicemia. *Vibrio ordalii* can produce a bacteremia similar to that of *V. anguillarum*, but tends to produce aggregates in tissues rather than being evenly dispersed throughout the tissues. Also, the number of organisms in the blood were reported to be lower than with *V. anguillarum* infections (Ransom *et al*, 1984).

The main routes of entry of the pathogen are skin, lateral line, anus, wounds and, to a lesser extent, gills (Kanno *et al*, 1989). Infection by *V. anguillarum* through the gut can be demonstrated experimentally, but this is not likely the case in naturally acquired infections (Evelyn, 1984). Gut isolates of *V. anguillarum* are not generally pathogenic and

are believed to be part of the normal flora of marine fish (Horne, 1982).

1.2 Antibiotic therapy

The only antibiotics currently licensed for use in aquaculture in Canada are oxytetracycline and Romet-30® (5:1 sulfadimethoxine + ormetoprim) (Bennett, 1991). Antibiotics can be administered via bath immersion, injection or, more commonly, by medicated feed (Post, 1987). Certain criteria need be examined in the selection of antibiotics, including palatability of oral formulation, antibiotic efficacy, safety to the host and to the administrator, and the pharmacokinetic processes of the drug (absorption, distribution, metabolism, elimination). For the effective treatment of bacterial diseases of homeotherms, plasma or serum concentrations of 3 to 5 times the minimum inhibitory concentration (MIC) have been recommended (Washington, 1985). The use of antibiotics of importance in human medicine (streptomycin, ampicillin, bacitracin, kanamycin, chloramphenicol, erythromycin) and those known to possess plasmid-mediated resistance, should be avoided or restricted in aquaculture (Austin, 1984).

1.2.1 History of antimicrobial agents

The first antibiotic used clinically was gramicidin discovered by Rene Dubos in 1930. Although Fleming first discovered penicillin in 1929, it was not purified until 1940 (Crease, 1989). The synthetic sulfonamides were developed in

the 1930s. By the 1950s, chloramphenicol, erythromycin and tetracycline were available. Vancomycin, cephalosporins and the first-generation quinolones, namely nalidixic acid, were produced in the 1960s. In the 1970s, potentiated sulfonamides, such as trimethoprim-sulfamethoxazole, and the second-generation quinolone, oxolinic acid, were available. The second-generation fluoroquinolones (norfloxacin, ciprofloxacin) (Norris and Mandell, 1988) and third-generation fluoroquinolones (lomefloxacin, difloxacin, sarafloxacin) were developed in the 1980s. The third generation quinolones possess two or more fluorine substitutions (Siporin, 1989). The main emphasis in the development of new antibiotics has been on those that are efficacious, yet non-toxic at therapeutic concentrations and depleted efficiently and completely from the body. Depletion is of particular concern in the treatment of fish for human consumption.

1.3 Bacterial drug resistance

Continual use of antibiotics often results in the selection of resistant strains of bacteria. The genes encoding for resistance to antibiotics can be carried on bacterial chromosomes, on plasmids or on transposons. Plasmids are extrachromosomal, self-replicating units which consist typically of 30-100 genes (Richmond, 1972). Transposons are mobile genetic units which are capable of inserting into various nonhomologous sequences of DNA, such as

into chromosomes or plasmids. Transposons consist of one or more drug resistance genes flanked by inverted repeats, DNA sequences that are homologous yet inverted with respect to their direction. The variability seen in many resistance plasmids (R-plasmids) is due to the loss or insertion of transposons (Mitsuhashi, 1977).

The acquisition of genes by one bacterium from another can occur by one of three methods: transformation, transduction or conjugation. Transformation is the direct acquisition of naked DNA through the culture medium or environment. Transduction refers to the transmission of DNA via a carrier, such as a bacteriophage. Conjugation is the transfer of DNA from a donor to recipient cell via a specialized structure of the donor called a pilus (Richmond, 1972).

The transfer of genes encoding for antibiotic resistance may be between two pathogenic bacteria or between non-pathogens and pathogens sharing the same niche. It has been documented in a case of shigellosis in humans that antibiotic resistance genes present in commensal gut isolates of *Escherichia coli* were transferred to the *Shigella* isolates during the course of antibiotic therapy (Richmond, 1972). This scenario could theoretically exist in the aquaculture situation, particularly when antibiotic therapy is often given as prophylactic treatment when disease outbreaks most commonly occur. Transformation has been reported with *Vibrio* sp. in

seawater at a frequency of $2.7x10^{-10}$ to $1.7x10^{-6}$ transformants per recipient, but was not observed in the sediments (Paul *et al*, 1991). Transformation by a protease gene was noted in *A. salmonicida* in river sediment (Sakai, 1987).

Plasmid-mediated resistance can occur for many antibiotics and is of concern in the treatment of cultured salmonids. Transferable R-plasmids have been reported for *A. salmonicida*, conferring resistance to chloramphenicol, streptomycin and sulfonamides (Aoki *et al*, 1983). Transferable R-plasmids to chloramphenicol, tetracycline and sulfamonomethoxine were observed in isolates of *V. anguillarum* (Aoki *et al*, 1981). Quinolone resistance is not plasmid-mediated, but carried on the chromosome. Quinolones may cure plasmids, likely due to the plasmid replicating system being more sensitive than that of the chromosome (Weisser and Weidemann, 1985). Oxolinic acid resistance by *A. salmonicida* and *Y. ruckeri* has been observed as increasing over the last 5 years in Scotland (Tsoumas *et al*, 1989). Unfortunately, resistance to one quinolone frequently confers some degree of resistance to a range of quinolones (Barry and Jones, 1984; Smith and Lewin, 1988).

1.4 Thesis objectives

The objectives of this thesis can be grouped into three phases. Phase I was the determination of the minimum inhibitory concentrations (MICs) of six antibiotics against three bacterial pathogens of salmonids: *A. salmonicida, V. anguillarum* and *V. ordalii*. The MICs were determined at three incubation temperatures (10, 20, 30°C) to examine any influence of temperature on MIC which may alter interpretation of data (susceptible versus resistant). Bacterial isolates were collected from disease outbreaks on the Atlantic and Pacific coasts, over a range of years (1985 - 1990), to examine patterns in antibiotic susceptibility and resistance.

Phase II was the examination of antibiotic resistant isolates for plasmids by agarose gel electrophoresis. Plasmid profiles of resistant isolates were compared to plasmid profiles of susceptible isolates to evaluate whether there was evidence that the drug resistance was plasmid encoded.

Phase III was the determination of plasma concentrations of two quinolones, oxolinic acid and sarafloxacin, in Atlantic salmon in seawater. A microbiological assay was developed to quantify oxolinic acid and sarafloxacin concentrations in plasma. A pilot study involving single oral dosing and a single intraperitoneal injection of either oxolinic acid or sarafloxacin was performed to determine blood sampling times for the medicated feeding trial. In the medicated feeding trial, plasma antibiotic concentrations were determined during

and after the recommended treatment regime. Plasma concentrations of oxolinic acid and sarafloxacin were compared to the MICs to assess whether concentrations were sufficient for treatment of furunculosis and vibriosis.

2. MINIMUM INHIBITORY CONCENTRATIONS

2.1 Introduction

The minimum inhibitory concentration (MIC) is the lowest concentration of an antibiotic required to inhibit bacterial growth. MICs can be determined by dilution techniques, such as agar dilution or broth dilution. In these techniques, bacteria are inoculated onto agar plates or into tubes of broth which contain the antibiotic. A series of plates or tubes are prepared with two-fold dilutions of the antibiotic. Better reproducibility has been reported with agar dilution. The agar dilution technique also permits up to 37 isolates to be examined simultaneously, allows easy detection of contaminants and is quantitative (Washington, 1985). Disk diffusion techniques are generally technically easier than dilution techniques, but are qualitative, dividing isolates into three basic categories: susceptible, intermediate or moderately susceptible, and resistant (Thornsberry and Sherris, 1985). The agar dilution technique was selected in this study because it is quantitative. Also, differences in antibiotic susceptibility and resistance of a group of isolates can be observed by analysis of the MICs.

The minimum inhibitory concentrations (MICs) of six antibiotics were determined for *Aeromonas salmonicida, Vibrio anguillarum* and *Vibrio ordalii* isolated from disease outbreaks on the Atlantic and Pacific coasts. *A. salmonicida*, the

etiological agent of furunculosis, and *V. anguillarum* and *V. ordalii*, the etiological agents of vibriosis, frequently cause extensive mortalities in farmed salmonids (Trust, 1986).

The antibiotics examined in this study were oxytetracycline, ormetoprim-sulfadimethoxine (ormet-sulfa), oxolinic acid, sarafloxacin, streptomycin and erythromycin. Oxytetracycline and Romet-30® (1:5 ormetoprim-sulfadimethoxine) are the only antibiotics currently licensed for use in Canadian aquaculture (Bennett, 1991). The genes encoding for resistance to oxytetracycline and sulfonamides can be contained on plasmids or transposons (Barth *et al*, 1976; Levy, 1984). Therefore, there is concern regarding the potential for rapid spread of resistance not only to other bacterial fish pathogens, but also to human pathogens.

Oxolinic acid has been used in Japan (Endo *et al*, 1973) and the United Kingdom (Hastings and McKay, 1987; Tsoumas *et al*, 1989) for treatment of furunculosis. Sarafloxacin is a newer quinolone presently being examined for its potential use in aquaculture. Advantages of the quinolones are low MICs and lack of plasmid-mediated resistance (Smith and Lewin, 1988).

Streptomycin, an aminoglycoside antibiotic, is not used in aquaculture but is of interest because resistance to streptomycin can be carried on the same transposon or plasmid as other antibiotics, such as trimethoprim (Barth *et al*, 1976), sulfonamides and chloramphenicol (Aoki *et al*, 1983).

Erythromycin, a macrolide, is effective against Gram positive bacteria, such as *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (Austin, 1984), but generally ineffective against Gram negative bacteria, such as *Aeromonas* spp. and *Vibrio* spp.

MIC values for fish pathogens have been determined at incubation temperatures of 18-30°C (Aoki *et al*, 1983; Lewin & Hastings 1990; Nakano *et al*, 1989; Nusbaum & Shotts 1981; Stamm 1989). Although incubation temperature affects virulence (Ishiguro *et al* 1981) and biochemical reactions (Hahnel & Gould 1982) of *Aeromonas salmonicida*, limited information is available on the influence of incubation temperature on antibiotic susceptibility (Hahnel & Gould 1982). As well, farmed salmonids are subjected to seasonally fluctuating water temperatures and an increase in water temperature above 13°C has been associated with disease outbreaks (McCarthy, 1975). MICs in this study were determined at 10, 20 and 30°C to assess the influence of incubation temperature.

Two collections of isolates of *A. salmonicida, V. anguillarum* and *V. ordalii* were examined to compare antibiotic susceptibilities and resistance patterns. One group was from the Atlantic coast and the other group was from the Pacific coast. Atlantic coast isolates were collected from 1976 to

1990 and Pacific coast isolates were collected from 1968 to 1990 to assess yearly patterns in antibiotic resistance.

2.2 Materials and Methods

2.2.1 Preparation of antibiotic solutions

The antibiotics tested were: oxytetracycline-HCl, 929 μg/mg active drug (Pfizer Canada Inc., London, Ontario); a 5:1 sulfadimethoxine (99.97% active drug) : ormetoprim (100% active drug) combination (Hoffmann LaRoche, Etobicoke, Ontario); oxolinic acid, 99.58% active drug (Austin Laboratories Ltd., Joliette, Quebec); sarafloxacin-HCl, 829 μg/mg active drug (Abbott Laboratories, Chicago, Illinois); erythromycin estolate, 656 μg/mg active drug (Eli Lilly Canada Inc., Scarborough, Ontario); streptomycin sulphate, 762 μg/mg active drug (Bio Agri Mix Ltd., Mitchell, Ontario).

Stock solutions containing 1280 μg/mL active drug were prepared for each of the six antibiotics and frozen as 7 mL aliquots in polyethylene tubes at -20°C. Stock solutions of ≥ 1000 μg/mL can be held frozen without loss of activity for up to 6 months (Anhalt and Washington, 1985). Glass tubes were not used as some antibiotics adhere to glass surfaces (Anhalt and Washington, 1985). Distilled water was used as diluent, except for erythromycin which was initially dissolved in acetone then diluted with distilled water (Anhalt and Washington, 1985). Erythromycin had to be prepared fresh for each experiment. A few drops of 0.1N NaOH was required to

dissolve oxolinic acid and sulfadimethoxine (Anhalt and Washington, 1985). A small amount of 0.05N HCl was required to dissolve ormetoprim (Anhalt and Washington, 1985) and sarafloxacin needed to be heated to boiling (Stamm, 1989). The sulfadimethoxime-ormetoprim combination were frozen as separate 1280 μg/mL stocks and mixed 5:1 prior to use. A series of 2-fold dilutions of the initial 1280 μg/mL stock were made in distilled water immediately before addition to agar.

2.2.2 Preparation of antibiotic plates

Mueller-Hinton agar (pH 7.4, Oxoid Ltd.) for *Aeromonas salmonicida* or Mueller-Hinton agar plus 2% sodium chloride (NaCl) for *Vibrio anguillarum* and *Vibrio ordalii* was prepared as directed by the manufacturer but made up to 900 mL rather than 1 L to compensate for dilution upon the addition of antibiotic solution, and autoclaved. While still molten, 54 mL quantities of agar were aseptically dispensed into sterile 125 mL Wheaton bottles. The bottles of agar were labelled to indicate the appropriate antibiotic and dilution and placed in a water bath set at 50 - 52°C. Six millilitres of antibiotic solution were added to the 54 mL of agar, giving a further 1:10 dilution of the antibiotic. Twenty millilitres of antibiotic containing agar were poured aseptically into appropriately labelled sterile petri plates (100mm by 15mm); 3 plates were poured from each bottle of agar. Control bottles of Mueller-Hinton or Mueller-Hinton plus 2% NaCl

received 6 mL of sterile de-ionized water in place of the antibiotic solution. Agar plates were allowed to solidify, inverted and left at room temperature overnight to dry. Oxytetracycline is light sensitive; thus precautions were taken to prepare and store plates in minimal light. After drying overnight, plates were bagged and kept at 4°C up to 1 week without loss of antibiotic activity, as recommended by Washington (1985).

2.2.3 Bacterial isolates

A group of Atlantic coast isolates of *Vibrio anguillarum, Vibrio ordalii* and *Aeromonas salmonicida* collected between 1976 and 1990 was kindly supplied by the Department of Fisheries and Oceans, Halifax, Nova Scotia and the Fish Health Unit, Atlantic Veterinary College, University of Prince Edward Island. Isolates were from disease outbreaks in salmonids (predominately Atlantic salmon) from cage sites, hatcheries and rivers mainly in Atlantic Canada. Two isolates were from Quebec and four were from Maine, U.S.A. A similar but less comprehensive collection of Pacific coast isolates was supplied by the Department of Fisheries and Oceans, Pacific Biological Station, Nanaimo, British Columbia and the United States Department of the Interior, Fish and Wildlife Service, Olympia, Washington. These isolates were collected between 1968 and 1990. Isolates were from disease outbreaks in salmonids (predominantly Pacific salmon species) from cage sites, hatcheries, rivers and lakes in British Columbia,

Canada and Washington State, U.S.A. All isolates were convenience sampled. American Type Culture Collection (ATCC) strains used as controls were as follows: *A. salmonicida* ATCC 14174 and ATCC 33658, *V. anguillarum* ATCC 14181 and ATCC 19264 and *V. ordalii* ATCC 33509. Stock cultures were either frozen in 2% skim milk at - 70°C or lyophilized or both.

2.2.4 Preparation of bacterial inoculum

Bacteria were grown on Tryptic soy agar (TSA; CASO®, Merck) for *A. salmonicida* or TSA plus 2% NaCl for *Vibrio* spp. and incubated at 20°C for 48 h to check for purity. Two or three isolated colonies were used to inoculate 20 mL of tryptone soya broth (TSB; Difco) or TSB plus 2% NaCl and incubated at 20°C for 15 to 30 h. Optical densities were measured on a Spectronic 20D spectrophotometer (Milton Roy Company, Rochester, N.Y.) at 620 nm. Cultures were grown to mid-log phase (10^8 CFU/mL, as confirmed by plate counts) as indicated by an optical density reading of 0.6. A reading in the range of 0.5 to 0.8 was acceptable, as confirmed by plate counts. Cultures were then diluted 1:10 in either 0.85% sterile saline for *Vibrio* spp. or sterile distilled water for *A. salmonicida*. Four hundred microlitres (μL) of each culture were added to 36 wells of a 37 well sterile replicator plate; the first well contained India ink as a marker. Plates were inoculated immediately.

2.2.5 Inoculation of antibiotic plates

As described above, bacterial suspensions were added to 36 of 37 wells of a gas-sterilized replicator plate. An autoclaved pin head was fitted on the RAM (Replitech Analysis Module; Quadra Logic Technologies Ltd., Vancouver, B.C.) replicator system. Plates were inoculated by pushing the pins down into the wells, then onto the surface of the plate. Pins were calibrated to deliver 1 μL of suspension; thus, the spot inoculum contained 10^4 CFU. A control plate was inoculated at the beginning and at the end of the procedure to check inoculum viability. The antibiotic plates were inoculated by starting at the lowest concentration to minimize any carry over effect. All plates were prepared in triplicate. Plates were inverted and each series was incubated at 10, 20, and 30°C for 48 hours. The minimum inhibitory concentration (MIC) was recorded as the lowest antibiotic concentration which inhibited bacterial growth.

2.3 Results

The MIC data for the Atlantic and Pacific coast isolates are summarized in Tables I and II. The MIC_{50} refers to the concentration of an antibiotic required to inhibit growth of 50% of the isolates examined. The MIC_{90} refers to the concentration of an antibiotic required to inhibit growth of 90% of the isolates examined. The MIC data for the American Type Culture Collection (ATCC) isolates used as control

strains are given in Table III. The statistical significance of incubation temperature, coast and year of isolation on the MIC is summarized in Table IV. Statistical analysis was performed on log transformed data using the General Linear Model (GLM) of the Statistical Analysis Systems (SAS) program. A p value of < 0.05 is significant and a p value between 0.05 and 0.1 is of borderline significance.

Numbers of Atlantic coast isolates examined were 38 (10, 20°C and 30°C) for *A. salmonicida*, 29 (10, 20°C) and 28 (30°C) for *V. anguillarum* and 47 (10°C) and 48 (20, 30°) for *V. ordalii*. Numbers of Pacific coast isolates examined were 22 (10, 20°C) and 17 (30°C) for *A. salmonicida*, 21 (10°C) and 24 (20, 30°C) for *V. anguillarum* and 3 (10, 20, 30°C) for *V. ordalii*.

Incubation temperature had a significant effect on the MIC for most of the antibiotics and bacterial species examined (Table IV). The effect, however, is most prominent with oxytetracycline. The MIC of oxytetracycline against *Vibrio* spp. displayed a 2-fold or greater increase as incubation temperature increased (Tables I and II and Figure 1). Although a pronounced shift to oxytetracycline resistance (MIC >4.0 μg/mL) was not observed, the percentage of moderately susceptible isolates increased (MIC 2.0-4.0 μg/mL as defined by Tsoumas *et al*, 1989) (Table V). The percentages of moderately susceptible *V. anguillarum* Atlantic coast isolates

were 6.9%, 65.5% and 89.6% at 10, 20 and 30°C, respectively. All Pacific coast isolates were susceptible at 10 and 20°C, but 16.7% were moderately susceptible at 30°C. The percentages of Atlantic coast *V. ordalii* isolates designated as moderately susceptible were 0%, 43.75% and 100% at 10, 20 and 30°C, respectively. Control plates revealed visible increase in growth as incubation temperature increased. This pattern of increasing MIC was not noted for the other antibiotics; thus, the effect was not due to variations in growth rates alone.

Although incubation temperature had a statistically significant effect on the MIC of oxytetracycline against *A. salmonicida*, the effect was not as pronounced upon the examination of MIC_{50} data (Tables I and II and Figure 2). The percentage of resistant isolates did not appreciably increase with increasing incubation temperature (refer to Table V). However, the percentage of moderately susceptible isolates did vary: 36.8%,28.9% and 75% for the Atlantic coast isolates at 10, 20 and 30°C respectively and 0%, 4.5% and 47% for the Pacific coast isolates at 10, 20 and 30°C respectively. Figure 2 demonstrates the range of sensitivities of the Atlantic coast isolates of *A. salmonicida*, from susceptible to moderately susceptible to highly resistant.

Table I. Minimum inhibitory concentrations of six antibiotics against Atlantic coast isolates of *Aeromonas salmonicida* [a], *Vibrio anguillarum* [b] and *Vibrio ordalii* [c]

	MIC_{50} (µg/mL)			MIC_{90} (µg/mL)			Range (µg/mL)		
	10°C	20°C	30°C	10°C	20°C	30°C	10°C	20°C	30°C
Oxytetracycline									
A. salmonicida	1.0	1.0	2.0	32	64	64	0.25-128	0.5->128	0.5->128
V. anguillarum	0.25	1.0	2.0	0.5	2.0	4.0	0.125-8.0	0.125-16	0.5-32
V. ordalii	0.125	0.5	2.0	0.5	1.0	4.0	0.0625-0.5	0.25-1.0	1.0-16
Oxolinic Acid [d]									
A. salmonicida	0.0625	0.03125	0.03125	4.0	1.0	1.0	0.03125-16	0.0156-32	0.0156-32
V. anguillarum	0.0156	0.0156	0.03125	0.03125	0.0625	0.0625	0.0156-0.125	0.0156-0.25	0.0156-0.25
V. ordalii	0.0156	0.0156	0.0156	0.0156	0.0156	0.0156	0.0156-0.25	0.0156-0.03125	0.0156-0.03125
Sarafloxacin [d]									
A. salmonicida	0.25	0.0625	0.03125	0.25	0.0625	0.03125	0.0156-2.0	0.0156-2.0	0.0156-2.0
V. anguillarum	0.0156	0.0156	0.03125	0.125	0.125	0.125	0.0156-0.25	0.0156-0.5	0.0156-0.25
V. ordalii	0.0156	0.0156	0.0156	0.0156	0.0156	0.0156	0.0156-0.125	0.0156-0.25	0.0156-0.125

Table I (con't). Minimum inhibitory concentrations of six antibiotics against Atlantic coast isolates of *Aeromonas salmonicida* [a], *Vibrio anguillarum* [b] and *Vibrio ordalii* [c]

	MIC_{50} (μg/mL)			MIC_{90} (μg/mL)			Range (μg/mL)		
	10°C	20°C	30°C	10°C	20°C	30°C	10°C	20°C	30°C
Ormet-sulfa									
A. salmonicida	2.0	1.0	1.0	4.0	2.0	2.0	0.5-8.0	0.5-4.0	0.125-8.0
V. anguillarum	0.25	0.25	0.25	0.5	0.5	0.5	0.125-4.0	0.125-4.0	0.25-4.0
V. ordalii	0.25	0.25	0.25	0.5	0.5	0.5	0.125-0.5	0.125-1.0	0.125-1.0
Erythromycin									
A. salmonicida	8.0	8.0	4.0	8.0	8.0	8.0	2.0-8.0	2.0-16	1.0-16
V. anguillarum	8.0	16	16	32	32	32	1.0-32	2.0-32	2.0-128
V. ordalii	8.0	16	16	16	32	32	0.5-16	8.0-32	8.0-32
Streptomycin									
A. salmonicida	8.0	8.0	4.0	8.0	16	16	4.0- >128	4.0- >128	0.5- >128
V. anguillarum	32	32	64	32	64	64	4.0-64	16-128	8.0-128
V. ordalii	8.0	16	16	16	32	32	1.0-32	16-64	16-64

[a] 38 isolates

[b] 29 (10, 20°C), 28 (30°) isolates

[c] 47 (10°C), 48 (20, 30°C) isolates

[d] a MIC of 0.0156 refers to ≤0.0156μg/mL

Table II. Minimum inhibitory concentrations of six antibiotics against Pacific coast isolates of *Aeromonas salmonicida* [a], *Vibrio anguillarum* [b] and *Vibrio ordalii* [c]

	MIC_{50} (μg/mL)			MIC_{90} (μg/mL)			Range (μg/mL)		
	10°C	20°C	30°C	10°C	20°C	30°C	10°C	20°C	30°C
Oxytetracycline									
A. salmonicida	0.5	1.0	2.0	32	64	64	0.5-128	0.5-128	0.5-64
V. anguillarum	0.5	1.0	2.0	0.5	1.0	4.0	0.125-1.0	0.5-8.0	2.0-8.0
V. ordalii	0.5	0.5	1.0	0.5	0.5	1.0	0.125-0.5	0.5	1.0
Oxolinic Acid [d]									
A. salmonicida	0.03125	0.03125	0.03125	0.0625	0.0625	0.0625	0.0156-1.0	0.0156-1.0	0.0156-0.125
V. anguillarum	0.03125	0.03125	0.0625	0.0625	0.0625	0.125	0.0625-0.125	0.0625-1.0	0.0625-2.0
V. ordalii	0.0156	0.03125	0.03125	0.0156	0.03125	0.03125	0.0156	0.0156-0.03125	0.03125
Sarafloxacin [d]									
A. salmonicida	0.0625	0.0625	0.0156	0.125	0.0625	0.03125	0.0156-1.0	0.0156-1.0	0.0156-0.0625
V. anguillarum	0.125	0.125	0.125	0.125	0.125	0.125	0.0156-0.25	0.0156-0.25	0.0156-0.5
V. ordalii	0.0156	0.0156	0.0156	0.0156	0.0156	0.0156	0.0156	0.0156	0.0156

Table II (con't). Minimum inhibitory concentrations of six antibiotics against Pacific coast isolates of *Aeromonas salmonicida* [a], *Vibrio anguillarum* [b] and *Vibrio ordalii* [c]

	MIC_{50} (μg/mL)			MIC_{90} (μg/mL)			Range (μg/mL)		
	10°C	20°C	30°C	10°C	20°C	30°C	10°C	20°C	30°C
Ormet-sulfa									
A. salmonicida	1.0	1.0	1.0	1.0	2.0	2.0	1.0	0.5-4.0	1.0-2.0
V. anguillarum	0.25	0.25	0.5	0.5	0.5	0.5	0.125-0.5	0.125-0.5	0.125-0.5
V. ordalii	0.25	0.25	0.5	0.25	0.25	0.5	0.125-0.25	0.125-0.5	0.5
Erythromycin									
A. salmonicida	4.0	4.0	2.0	4.0	4.0	4.0	2.0-4.0	2.0-4.0	0.5-8.0
V. anguillarum	8.0	16	16	16	32	32	4.0-16	4.0-32	4.0-32
V. ordalii	2.0	4.0	4.0	2.0	4.0	4.0	2.0	2.0-4.0	2.0-8.0
Streptomycin									
A. salmonicida	8.0	8.0	4.0	8.0	8.0	16	8.0	8.0-16	0.5-16
V. anguillarum	32	32	64	32	32	64	8.0-32	16-64	8.0-64
V. ordalii	4.0	8.0	8.0	4.0	8.0	8.0	4.0	8.0	4.0-8.0

[a] 22 (10, 20°C), 17 (30°) isolates

[b] 21 (10°), 24 (20, 30°) isolates

[c] 3 isolates

[d] a MIC of 0.0156 refers to ≤0.0156μg/mL

Table III **Minimum inhibitory concentrations for six antibiotics against ATCC control isolates of *Aeromonas salmonicida, Vibrio anguillarum* and *Vibrio ordalii***

	MIC(μg/ml)		
	10°C	20°C	30°C
Oxytetracycline			
ATCC 14174[a]	0.5	1.0	2.0
ATCC 33658[a]	0.5	1.0	2.0
ATCC 19264[b]	0.5	0.5	2.0
ATCC 14181[b]	0.5	2.0	4.0
ATCC 33509[c]	NG[d]	128	64
Ormet-sulfa			
ATCC 14174	1.0	1.0	1.0
ATCC 33658	0.5	1.0	1.0
ATCC 19264	0.25	0.25	0.5
ATCC 14181	0.25	0.25	0.25
ATCC 33509	NG	128	128
Oxolinic acid			
ATCC 14174	0.06	0.03	0.03
ATCC 33658	0.06	0.03	0.03
ATCC 19264	0.016	0.016	0.016
ATCC 14181	0.016	0.016	0.016
ATCC 33509	NG	1.0	1.0
Sarafloxacin			
ATCC 14174	0.06	0.06	0.03
ATCC 33658	0.06	0.03	0.03
ATCC 19264	0.016	0.016	0.016
ATCC 14181	0.016	0.016	0.016
ATCC 33509	NG	0.5	0.5
Erythromycin			
ATCC 14174	4.0	4.0	4.0
ATCC 33658	8.0	8.0	8.0
ATCC 19264	8.0	16.0	16.0
ATCC 14181	8.0	16.0	16.0
ATCC 33509	NG	64	64
Streptomycin			
ATCC 14174	8.0	8.0	8.0
ATCC 33658	4.0	8.0	8.0
ATCC 19264	32	32	32
ATCC 14181	4.0	16.0	16.0
ATCC 33509	NG	128	128

[a] = *A. salmonicida*
[b] = *V. anguillarum*
[c] = *V. ordalii*
[d] = no growth

Table IV Statistical significance of the variables of incubation temperature and year and coast of isolation on the MICs of six antibiotics

	Pr > F		
	Temperature	Coast	Year
Oxytetracycline			
A. salmonicida	0.0011**	0.8506	0.0001**
V. anguillarum	0.0001**	0.2731	0.0255**
V. ordalii	0.0001**	0.9097	0.0458**
Ormet-sulfa			
A. salmonicida	0.4703	0.7206	0.0766*
V. anguillarum	0.6944	0.0792*	0.0001**
V. ordalii	0.0070**	0.1273	0.0001**
Oxolinic acid			
A. salmonicida	0.0001**	0.0001**	0.0001**
V. anguillarum	0.0002**	0.0001**	0.0001**
V. ordalii	0.7454	0.0313**	0.6440
Sarafloxacin			
A. salmonicida	0.0001**	0.1296	0.0001**
V. anguillarum	0.7703	0.0001**	0.0001**
V. ordalii	0.7898	0.8182	0.0183**
Erythromycin			
A. salmonicida	0.0861*	0.0011**	0.0001**
V. anguillarum	0.0008**	0.0211**	0.0288**
V. ordalii	0.0001**	0.0101**	0.0695*
Streptomycin			
A. salmonicida	0.0001**	0.1763	0.0001**
V. anguillarum	0.0001**	0.9310	0.0001**
V. ordalii	0.0001**	0.0001**	0.3879

** = significant ($p<0.05$)
* = borderline significant ($0.05<p<0.1$)

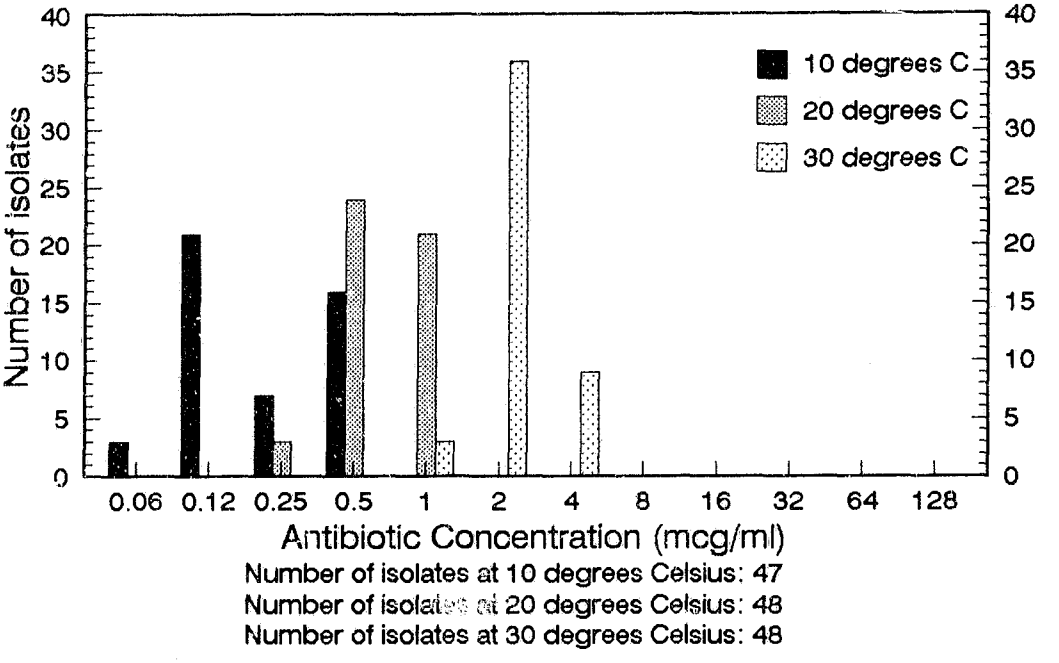


Figure 1: Minimum inhibitory concentrations of oxytetracycline against *Vibrio ordalii*

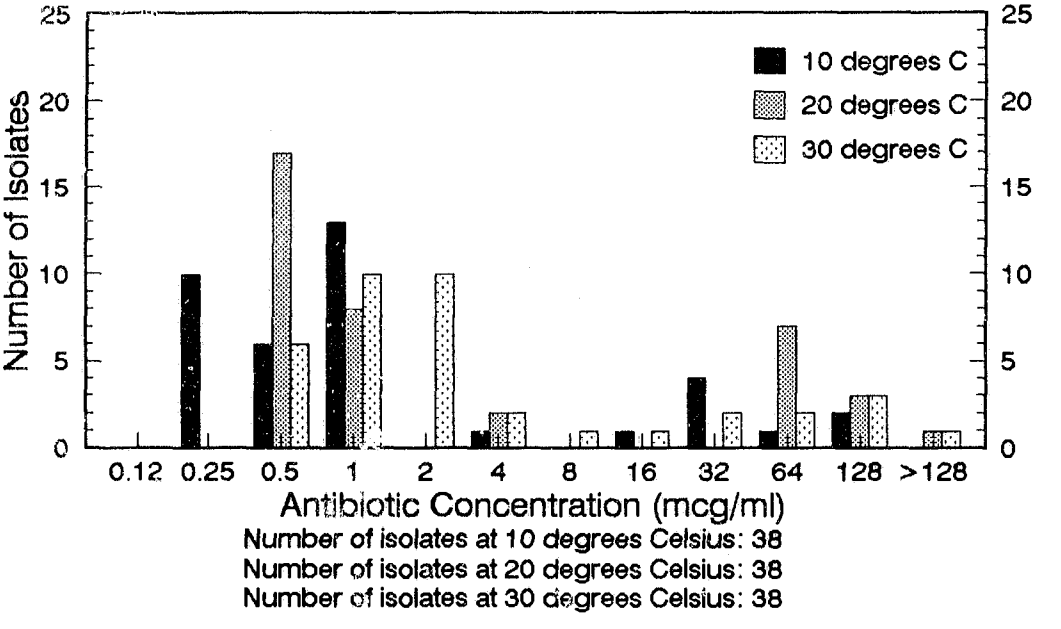


Figure 2: Minimum inhibitory concentrations of oxytetracycline against *Aeromonas salmonicida*

Statistically significant differences were seen in MICs at different incubation temperatures for the quinolones, oxolinic acid and sarafloxacin, against *Aeromonas salmonicida* (Table IV). For the quinolones, susceptible is defined as a MIC < 0.125 μg/mL, moderately susceptible as a MIC of 0.125 - 0.5 μg/mL and resistant as a MIC ≥ 1.0 μg/mL, by Tsoumas *et al* (1989). Aoki *et al* (1983) has a more stringent definition of resistance, a MIC ≥ 0.4 μg/mL. For the Atlantic coast collection, the MIC_{50} for oxolinic acid was one concentration higher at 10°C than at 20°C or 30°C, but remained within the susceptible range of < 0.125 μg/mL as defined by Tsoumas *et al* (1989) (Figure 3). MICs for sarafloxacin against *A. salmonicida* were more variable. A 2-fold or greater increase in MIC was noted with decreasing incubation temperature (Figure 4). With Atlantic coast isolates (Table I), the MIC_{50} was in the range of moderate susceptibility (0.125-0.5 μg/mL) as defined by Tsoumas *et al* (1989) and was not resistant by the definition of Aoki *et al* (1983).

The MIC_{50} of oxolinic acid against *V. anguillarum* at 30°C was double the MIC_{50} at 10 or 20°C. MICs remained constant for oxolinic acid against *V. ordalii* and for sarafloxacin against *V. anguillarum* and *V. ordalii*.

MICs for ormet-sulfa against Atlantic coast isolates of *A. salmonicida* at 10°C were double the MICs at 20°C and 30°C. MICs for Pacific coast *A. salmonicida* isolates and Atlantic

Table V. Percentage of resistant isolates of *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*

	Atlantic coast			Pacific coast		
	10°C	20°C	30°C	10°C	20°C	30°C
Oxytetracycline[a]						
A. salmonicida	21.1	28.9	23.7	13.6	13.6	17.6
V. anguillarum	6.9	3.4	3.6	0	4.2	4.2
V. ordalii	0	0	4.2	0	0	0
Oxolinic Acid						
A. salmonicida						
≥0.4µg/mL	26.3	28.9	21.0	4.5	4.5	0
≥1.0µg/mL	23.7	23.7	18.4	4.5	4.5	0
V. anguillarum						
≥0.4µg/mL	0	0	0	0	4.2	4.2
≥1.0µg/mL	0	0	0	0	4.2	4.2
Sarafloxacin						
A. salmonicida						
≥0.4µg/mL	15.8	26.3	2.6	4.5	4.5	0
≥1.0µg/mL	7.9	5.3	2.6	4.5	4.5	0
V. anguillarum						
≥0.4µg/mL	3.4	3.4	0	0	0	0
≥1.0µg/mL	0	0	0	0	0	4.2

[a] >4.0µg/mL

Table V (con't). Percentage of resistant isolates of *Aeromonas salmonicida, Vibrio anguillarum* and and *Vibrio ordalii*

	Atlantic coast			Pacific coast		
	10°C	20°C	30°C	10°C	20°C	30°C
Streptomycin[b]						
A. salmonicida	7.9	10.5	7.9	0	0	0
V. anguillarum	58.6	89.7	96.4	71.4	87.5	87.5
V. ordalii	19.1	85.4	97.9	0	0	0
Erythromycin[b]						
A. salmonicida	0	0	0	0	0	0
V. anguillarum	24.1	24.1	14.3	0	39.1	39.1
V. ordalii	0	12.5	4.2	0	0	0

[b] >16μg/mL

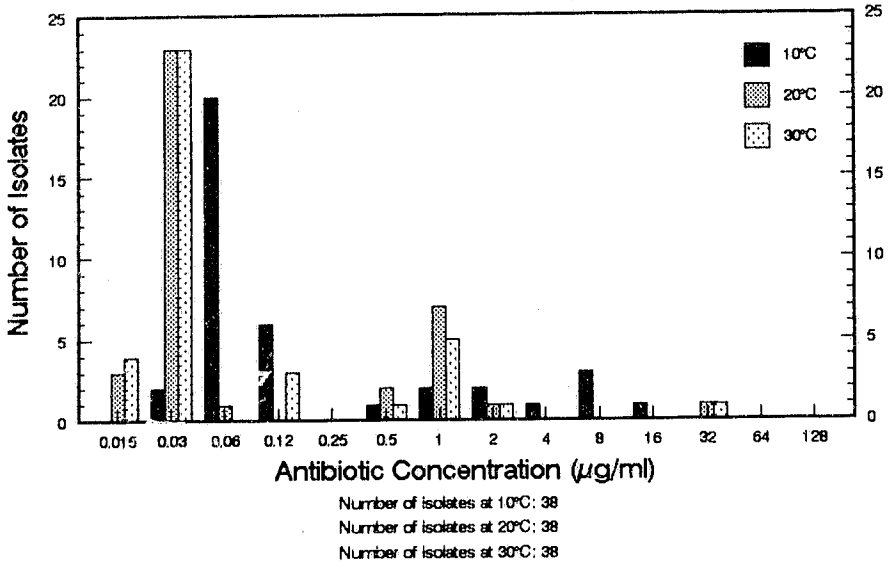


Figure 3: Minimum inhibitory concentrations of oxolinic acid against *Aeromonas salmonicida*

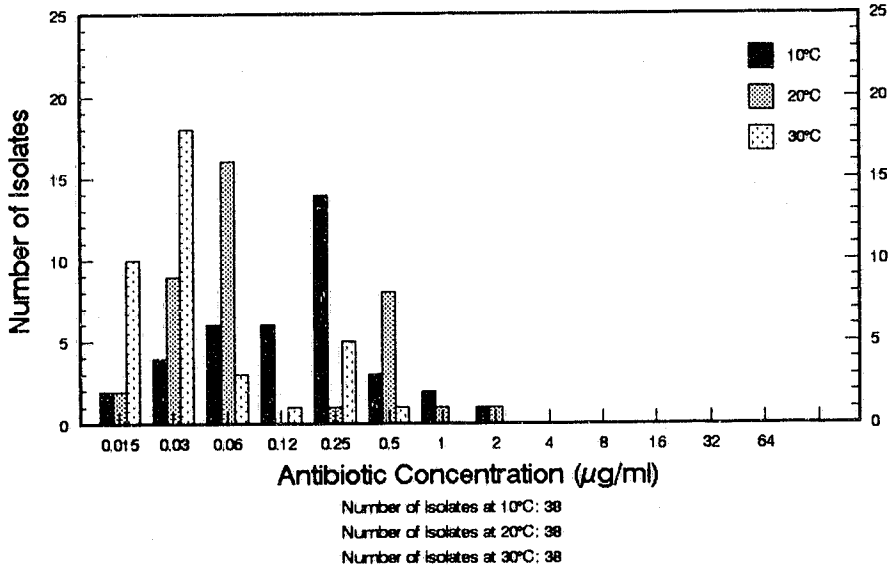


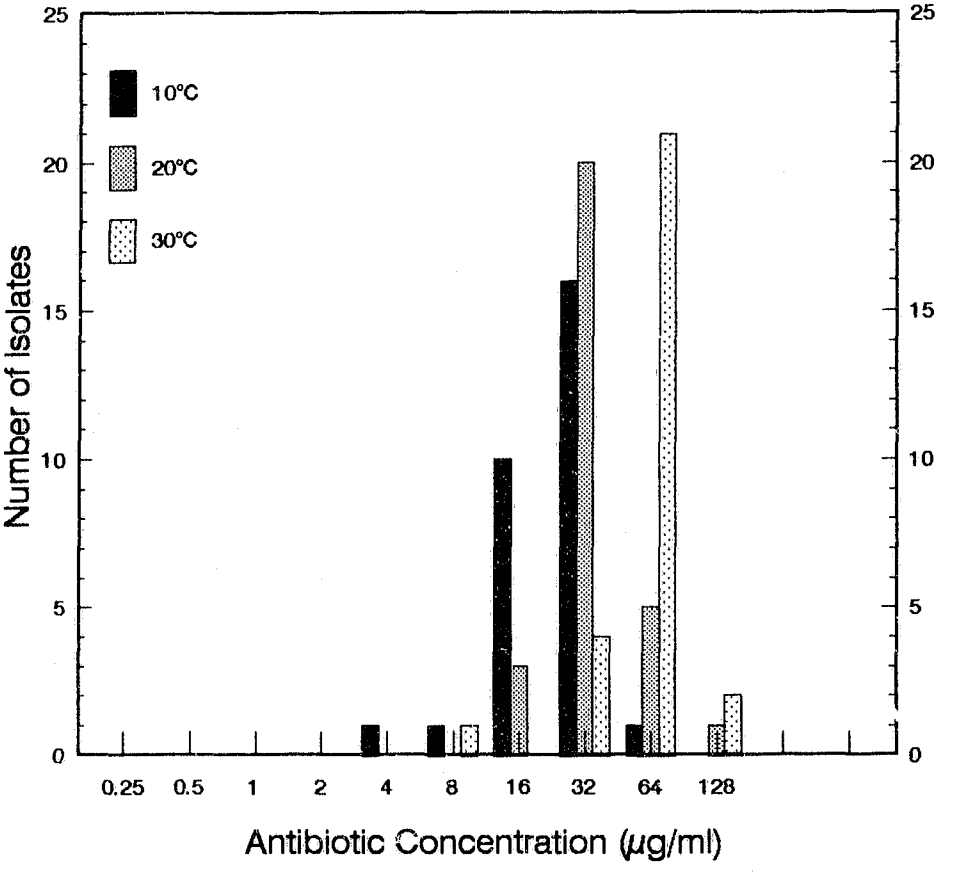
Figure 4: Minimum inhibitory concentrations of sarafloxacin against *Aeromonas salmonicida*

coast isolates of *Vibrio* spp. were not influenced by incubation temperature. However, the MIC of ormet-sulfa at 30°C was double the MIC at 10° and 20°C for the Pacific coast isolates of *Vibrio* spp. None of the isolates were resistant to ormet-sulfa.

MICs for erythromycin against *Vibrio* spp. differed statistically although the range was moderately susceptible (8-16 μg/mL) to resistant (≥32 μg/mL) at all three incubation temperatures.

Streptomycin was relatively ineffective against all three bacterial species at the three incubation temperatures. *Vibrio* spp. showed an increase in the percentage of resistant isolates at 20°C and 30°C (Table V), and an increase in level of resistance for *V. anguillarum* (MIC_{50} of 32 μg/mL at 20°C and 64 μg/mL at 30°C) (Table I and II and Figure 5). Most *Vibrio* spp. isolates were resistant to streptomycin (Table V), although multiple antibiotic resistance was uncommon (Table VI).

The statistical significance of geographical origin (Atlantic versus Pacific coast) of isolates on the MICs is summarized in Table IV. A valid comparison of the MICs for *Vibrio ordalii* is not possible because only three isolates were obtained from the Pacific coast. MICs for oxolinic acid against *A. salmonicida* and *V. anguillarum* from Atlantic and Pacific coasts were significantly different. The MIC_{50} values



Number of isolates at 10°C: 29

Number of isolates at 20°C: 29

Number of isolates at 30°C: 28

Figure 5: Minimum inhibitory concentrations of streptomycin against *Vibrio anguillarum*

were comparable, but the MIC_{90} values were higher for Atlantic coast isolates of *A. salmonicida* (Table I and II), suggesting a greater percentage of resistant isolates amongst the Atlantic coast collection: 23.7% of Atlantic coast isolates versus 4.5% of Pacific coast isolates (MIC ≥1.0 μg/mL at 20°C) (Table V). The number of isolates exhibiting multiple antibiotic resistance was higher in the Atlantic coast collection, as shown in Table VI. Multiple antibiotic resistance patterns observed in the Atlantic coast *A. salmonicida* isolates were oxytetracycline + streptomycin, streptomycin + erythromycin, oxolinic acid + sarafloxacin, and oxolinic acid + sarafloxacin + oxytetracycline. The only multiple resistance pattern observed in the Pacific coast isolates was oxolinic acid + sarafloxacin. The MIC_{50} for oxolinic acid against *V. anguillarum* was greater for the Pacific coast isolates than for the Atlantic coast isolates. None of the Atlantic coast *V. anguillarum* isolates were resistant to oxolinic acid, where as 4.2% of the Pacific coast isolates were oxolinic acid resistant (MIC ≥1.0 μg/mL). The only multiple resistance pattern in *Vibrio* spp. was streptomycin + erythromycin (Table VI). The percentage of Pacific coast isolates (33.3%) with this pattern was somewhat higher than the Atlantic coast isolates (20.7%).

The MIC_{50} of sarafloxacin against Pacific coast *V. anguillarum* was significantly higher than MIC_{50} for Atlantic

Table VI. Percentage of isolates of *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii* displaying multiple resistance patterns at 20°C

	Atlantic coast	Pacific coast
OTC[a] + SM[b]		
A. salmonicida	7.9	0
SM + ERY[c]		
A. salmonicida	2.6	0
V. anguillarum	20.7	33.3
V. ordalii	10.6	0
OA[d] + SA[e]		
A. salmonicida		
≥0.4μg/mL	26.3	4.5
≥1.0μg/mL	5.3	4.5
OA + SA + OTC		
A. salmonicida[f]	7.9[g]	0

[a] OTC = oxytetracycline, >4.0μg/mL

[b] SM = streptomycin, >16μg/mL

[c] ERY = erythromycin, >16μg/mL

[d] OA = oxolinic acid

[e] SA = sarafloxacin

[f] ≥0.4μg/mL for OA + SA

[g] 5.3% moderately susceptible to ormet-sulfa (MIC of 2-16μg/mL)

coast isolates. No Atlantic coast isolates were resistant to sarafloxacin (MIC ≥1.0 μg/mL) and only one Pacific coast isolate was classified as resistant at 30°C. A higher percentage of Pacific coast isolates were classified as moderately susceptible (MIC 0.125 - 0.5 μg/mL) to sarafloxacin: 85.7%, 79.2% and 91.7% at 10, 20 and 30°C respectively, as compared to 31%, 27.6% and 28.6% of Atlantic coast isolates incubated at 10, 20 and 30°C respectively.

The year of isolation was found to be a statistically significant variable for all antibiotics and bacterial species with the exceptions of oxolinic acid and streptomycin against *V. ordalii* (Table IV). The majority of the Atlantic coast isolates were collected between 1985 and 1990. The range of years examined for the Atlantic coast isolates was 1976-1990 for *A. salmonicida*, 1980-1990 for *V. anguillarum* and 1982-1990 for *V.ordalii*. The range of years examined for the Pacific coast isolates was 1969-1990 for *A.salmonicida*, 1968-1990 for *V. anguillarum* and 1978-1990 for *V. ordalii*. The GLM statistical package analyzes both Pacific and Atlantic coast isolates together. Tables VII, VIII and IX give the percentages of resistant strains per year for Atlantic and Pacific coast isolates separately.

Table VII shows the variation in percentage of resistant isolates of *Aeromonas salmonicida* collected from 1985-1990 at an incubation temperature of 20°C. Oxytetracycline resistance was high in 1985 (66.7%), declined to a low in 1988 and

increased again in 1989 (44.4%) and 1990 (33.3%) for the Atlantic coast isolates. A similar pattern was noted with the quinolones, oxolinic acid and sarafloxacin, against the Atlantic coast isolates. In 1985, 33.3% of the isolates examined were resistant to the quinolones. Resistance declined in 1986 and 1987, and has increased to 66.7% in 1990. The percentage of resistance to oxytetracycline, sarafloxacin and oxolinic acid was lower for the Pacific coast isolates (12.5% in 1990).

Table VIII shows the yearly variation in the percentage of resistant isolates of *Vibrio anguillarum*. Oxytetracycline resistance in *V. anguillarum* was uncommon, only one Atlantic coast isolate in 1987 and one Pacific coast isolate from 1990. Erythromycin resistance of isolates from both coasts was variable throughout the years examined. Streptomycin resistance was prominent in both Atlantic and Pacific coasts isolates of *V. anguillarum* in all years examined.

A high percentage of Atlantic coast *Vibrio ordalii* isolates collected between 1982-1989 were streptomycin resistant, as shown in Table IX. Erythromycin resistance was variable and none of the isolates were resistant to oxytetracycline. Only 3 Pacific coast *V. ordalii* isolates were obtained; therefore, resistance trends could not be examined.

Table VII. Yearly percentage of resistant isolates of *Aeromonas salmonicida* at 20°C

	Atlantic coast				Pacific coast			
Year	n	OTC[a]	OA[b]	SA[c]	n	OTC[a]	OA	SA
1990	3	33.3	66.7	66.7	8	12.5	12.5[c]	125[b]
1989	9	44.4	55.6	44.4[d],11.1[c]	3	33.3	0	0
1988	3	0	33.3	33.3[b]	0	0	0	0
1987	6	12.5	16.7	0	2	0	0	0
1986	7	14.3	0	0	2	0	0	0
1985	6	66.7[e]	33.3[c]	33.3	0	0	0	0

[a] OTC = oxytetracyline, >4.0 μg/mL

[b] OA = oxolinic acid, ≥1.0 μg/mL

[c] SA = sarafloxacin, ≥1.0 μg/mL

[d] SA = sarafloxacin, ≥0.4 μg/mL

[e] 50% streptomycin resistant

Table VIII. Yearly percentage of resistant isolates of *Vibrio anguillarum* at 20°C

	Atlantic coast				Pacific coast			
Year	n	OTC[a]	SM[b]	ERY[c]	n	OTC	SM	ERY
1990	6	0	83.3	50	7	14.3	85.7	0
1989	11	0	100	18.2	1	0	100	100
1988	2	0	50	0	1	0	100	0
1987	2	50	100	50	1	0	100	0
1986	1	0	100	0	1	0	100	100
1985	4	0	100	0	2	0	100	100
1982	1	0	100	100	0	0	0	0
1980	1	0	100	100	0	0	0	0
1975	0	0	0	0	1	0	0	100
1974	0	0	0	0	1	0	0	100
1969	0	0	0	0	2	0	100	0
1968	0	0	0	0	1	0	100	100

[a] OTC = oxytetracycline, >4.0μg/mL
[b] SM = streptomycin, >16μg/mL
[c] ERY = erythromycin, >16μg/mL

Table IX. Yearly percentage of resistant isolates of *Vibrio ordalii* at 20°C

	Atlantic coast			
Year	n	OTC[a]	SM[b]	ERY[c]
1989	18	0	94.4	16.6
1988	8	0	62.5	0
1987	7	0	85.7	0
1986	7	0	85.7	0
1985	5	0	100	60
1983	1	0	100	0
1982	2	0	100	0

[a] OTC = oxytetracycline, >4.0μg/mL

[b] SM = streptomycin, >16μg/mL

[c] ERY = erythromycin, >16μg/mL

2.4 Discussion

The comparison of MIC data from various sources is difficult due to failure of other researchers to include ATCC control strains and lack of standardization of type of media used, of incubation temperature and of inoculum size. The presence of divalent cations (Ca^{2+}, Mg^{2+}) affects tetracycline and aminoglycoside activity. Thymidine can decrease trimethoprim and sulfonamide activities. As well, pH can alter antibiotic activities (Anhalt and Washington, 1985). An increase in inoculum size by 10^3 CFU/mL increases broth MIC 5-fold (O'Grady, 1987). An inoculum equivalent to a McFarland standard of 0.5 - 1 (10^8 CFU/mL) has been recommended (Anhalt and Washington, 1985). The inoculum in this study was standardized to 10^8 CFU/mL using a spectrophotometer reading at 620 nm of 0.6, as described in sections 2.2.4 and 2.2.5, eliminating human error from reading McFarland standards. Mueller-Hinton agar was the medium of choice because its formulation considers the above mentioned media-related variables; ATCC controls were included on every plate (MIC data given in Table III).

A statistically significant difference by the GLM SAS program can be the result of a change in MIC by one drug concentration. Thus, to assess the influence of the variables of incubation temperature, coast and year of isolation, the MIC data need be examined more closely. A statistically significant difference in MIC in the susceptible range for

that particular antibiotic may not be considered a difference of practical concern. Examination of the MIC data in this study revealed that the MIC_{50} provided a more representative value of the required therapeutic concentration of antibiotic than the MIC_{90}. In the calculation of the MIC_{90}, the more resistant isolates need be considered, thus, indicating a higher MIC than is necessary to inhibit bacterial growth in most instances. For example, the MIC_{50} of oxytetracycline against *A. salmonicida* was 1.0 μg/mL, compared to a MIC_{90} of 64 μg/mL. By examining the MIC_{90} alone, an incorrect assumption is made regarding the percentage of oxytetracycline resistant isolates (Tables I, II and V).

The MIC of oxytetracycline against *Vibrio* spp. displayed a 2-fold or greater increase as the incubation temperature increased (refer to Tables I and II and Figure 1). Although the control plates showed a visible increase in the amount of growth as incubation temperature increased, this pattern was not noted for other antibiotics; thus, the effect could not be explained by variations in growth rates alone. The outer membrane of other Gram negative bacteria contains proteins called porins, which are believed to form water-filled channels. Porins range in size from 33,000 to 45,000 daltons (Da) and facilitate the entry of hydrophillic compounds (Darveau *et al*, 1983). In *Escherichia coli*, oxytetracycline passes through 'Ia' porins, which allow diffusion of particles up to 650 Da. The tetracycline antibiotics are hydrophillic

and less than 500 Da in molecular mass. Resistance to the tetracyclines in *E. coli* involves a 15,000 Da cell envelope carrier protein, necessary for efflux of antibiotic from the cell. Incorporation of these proteins likely requires alteration of the physical state of the lipid domain. Resistant bacteria held at 5°C could no longer exclude or remove tetracycline from the cell, possibly due to transition from a fluid to a crystalline phase of the membrane area where the carrier protein would normally reside (Chopra & Howe, 1978). Although the mechanism of oxytetracycline resistance in *V. anguillarum* and *V. ordalii* has not been identified, a similar situation may occur where proteins required for the efflux of oxytetracycline from the cell are more numerous at higher incubation temperatures. Although the percentage of oxytetracycline resistant isolates (MIC > 4.0 μg/mL) did not increase markedly with increasing incubation temperature (Table V), the percentage of moderately susceptible isolates (MIC 2.0 - 4.0 μg/mL) compared to susceptible isolates (MIC ≤ 2.0 μg/mL) was greater at the higher incubation temperature (Figure 1).

The influence of incubation temperature on the MIC of oxytetracycline against *A. salmonicida* was not as dramatic as with the *Vibrio* spp. The percentage of resistant isolates did not appreciably increase with increasing incubation temperature. However, the percentage of moderately susceptible isolates did increase (Figure 2).

Atlantic coast isolates of *A. salmonicida* displayed an increasing MIC for sarafloxacin with decreasing incubation temperature (Table I and Figure 4). MIC_{50} at 10°C were within the moderately susceptible range (0.125-0.5μg/mL) as defined by Tsoumas (1989), whereas the MIC_{50} at 20 and 30°C were in the susceptible range. MICs for oxolinic acid remained relatively constant at the three incubation temperatures. Martinsen *et al* (1991) examined broth MICs for sarafloxacin at 4°C and at 15°C. Although a statistical difference was not reported, mean MICs at 4°C were greater than mean MICs at 15°C for *A. salmonicida*. As with this study, MICs were constant for sarafloxacin against *V. anguillarum*. The influence of incubation temperature on MIC was further verified by Inglis and Richards (1991). MICs determined by agar dilution for enrofloxacin and sarafloxacin against *A. salmonicida* were at least four times greater at 10°C than at 22°C.

Sarafloxacin is of relatively low solubility (1 g/L) (Markwardt & Klontz, 1989). Stock solutions required boiling to solubilize and were observed to precipitate from solution upon freezing and thawing or when stored at 4°C. Perhaps the antibiotic was not as uniformly diffused throughout the agar in plates incubated at 10°C, but this would not explain why this effect of increasing MIC with decreasing temperature was not observed for *Vibrio* spp. or the Pacific coast isolates of *A. salmonicida*.

Sarafloxacin and oxolinic acid are DNA gyrase inhibitors. Sarafloxacin has a molecular mass of 420.87 Da (Abbott Laboratories), compared to 261.2 Da for oxolinic acid (Hirai *et al*, 1986). In studies with *E. coli* K-12, the outer membrane protein (Omp), OmpF, allows the diffusion of quinolones into the cell. Hydrophobic quinolones, such as oxolinic acid, flumequine and nalidixic acid can diffuse through the phospholipid bilayer directly. Hydrophillic quinolones, such as norfloxacin, ciprofloxacin and enoxacin diffuse through porin pores such as OmpF (Hirai *et al*, 1986). A major outer membrane porin of 42,000 Da has been described for *A. salmonicida* (Darveau *et al*, 1983), which could be involved in the entry of antibiotics into the cell. Multiple antibiotic resistance in *E. coli* to structurally unrelated hydrophobic and hydrophillic antibiotics, such as tetracycline, chloramphenicol, some β-lactams and quinolones, is related to a decrease in the amount of OmpF (Hirai *et al*, 1986). OmpF production was highest under conditions of low temperature and low osmolarity (Cohen *et al*, 1988); therefore, the MIC should decrease at lower incubation temperatures. The opposite shift in susceptibility was shown for sarafloxacin against *A. salmonicida* (Table I and Figure 3). Perhaps the lower temperature influenced the fluidity of the lipid bilayer, as discussed previously, but this hypothesis does not explain the specificity of the effect.

Multiple antibiotic resistance has been reported for *A. salmonicida* to low molecular mass (≤635 Da) antibiotics, including oxolinic acid, flumequine, nalidixic acid, penicillin, ampicillin, tetracycline, chloramphenicol and novobiocin, but not to antibiotics >700 Da, such as erythromycin, streptomycin and polymyxin B. A change in the Omp composition was noted, with the appearance of a 37 kDa Omp and the absence of a 38.5 kDa Omp. The result was a decrease in permeability to the lower molecular mass antibiotics (Wood *et al*, 1986). The multiple resistance patterns at an incubation temperature of 20°C are given in Table VI. *A. salmonicida* strains showing multiple resistance were relatively infrequent, and a possible link between oxytetracycline and quinolone resistance was not demonstrated.

Resistance in *E. coli* to norfloxacin, a fluoroquinolone structurally similar to sarafloxacin (Appendix A), is caused by a single nucleotide change in the *gyrA* gene encoding for subunit A of DNA gyrase. Resistance was reported to be present for cultures incubated at 30°C, but not at the organism's optimum incubation temperature of 37°C (Crumplin, 1990). The MIC of sarafloxacin was greater at 10°C than at the optimum incubation temperature of 20°C for *A. salmonicida*. Outbreaks of furunculosis are observed as water temperatures increase

above 13°C (McCarthy, 1975); thus, the practical significance of these results are debateable.

The proportion of oxolinic acid resistant isolates of Atlantic coast *A. salmonicida* was greater than the proportion of sarafloxacin resistant isolates (Figures 3 and 4). Oxolinic acid is bactericidal only to dividing cells, whereas the third-generation fluoroquinolones, such as ciprofloxacin and norfloxacin and potentially sarafloxacin, are bactericidal to non-dividing cells as well (Lewin & Hastings, 1990).

Streptomycin was ineffective against all three bacterial species at the three incubation temperatures. The majority of *Vibrio* spp. isolates were resistant to streptomycin (Table V) and an increase in the level of resistance with increasing incubation temperature was noted for *V. anguillarum* (Figure 5). High-level, stable mutants to streptomycin can occur at a high frequency due to a single-step mutation. Replacement of a single amino acid in either of two positions in the S12 protein of the 30s ribosomal subunit results in a decrease in binding of the drug to the ribosome (Franklin & Snow, 1989). Resistance can also be the result of modifying enzymes which phosphorylate, adenylate or acetylate the drug, or due to decreased permeability. The amount of visible growth on the plates increased with increasing incubation temperature. It is plausible that an increase in the amount of modifying enzymes coincided with increased growth at higher incubation temperatures.

Streptomycin resistance has been reported to be carried on the same transposon as trimethoprim resistance (Barth *et al*, 1976) or on the same plasmid as sulfonamides and chloramphenicol (Aoki *et al*, 1983). Although ormetoprim and sulfadimethoxine were not examined individually, no isolates were resistant to ormet-sulfa. Five *V. anguillarum* isolates resistant to streptomycin were examined for chloramphenicol resistance using 30 μg disks. All isolates were susceptible to chloramphenicol, as defined by a zone diameter of ≥ 18 mm (Barry and Thornsberry, 1985), with zone diameters of 30.9, 35.0, 39.2, 43.3 and 44.9 mm. Resistance to both streptomycin and erythromycin was present in some *Vibrio* spp. isolates (Table VI).

MICs of the quinolones showed significant differences according to geographical origin (Atlantic versus Pacific coasts) of the isolate. MICs for oxolinic acid and sarafloxacin against *A. salmonicida* were higher for the Atlantic coast isolates (Tables I and II), with a corresponding increase in the percentage of resistant or moderately susceptible isolates (Table V). The opposite was true for *V. anguillarum*; MICs were higher for the Pacific coast isolates (Table I and II). The proportion of oxytetracycline resistant isolates of *A. salmonicida* was greater for the Atlantic coast isolates (Table V). Variations in MICs, particularly increases in resistance, due to

geographical location suggest possible differences in the amount of drug use by the aquaculture industries. Unfortunately, isolates came from a variety of areas on the two coasts; thus, resistance patterns more likely reflect differences in the localities sampled than differences in the two coasts.

The yearly variation in MICs reflects trends in resistance patterns. Resistance to oxytetracycline, oxolinic acid and sarafloxacin by the Atlantic coast isolates of *A. salmonicida* was greatest in 1985 (Table VII). Resistance declined from 1986-1988, but then increased again in 1989 and 1990. Resistance patterns more likely reflect differences in localities of sampling sites, than an overall pattern for the Atlantic coast. Resistance patterns often reflect the amount of antibiotic use and is of particular concern in the case of plasmid-mediated drug resistance. Although oxytetracycline resistance can be plasmid encoded or regulated by transposons (Levy, 1984), quinolone resistance is chromosomally-mediated (Cohen *et al*, 1988). Also, quinolones may act as plasmid curing agents (Weisser and Wiedemann, 1985). Interpretation of the MICs for Pacific coast isolates is more difficult due to the fewer numbers of isolates per year.

The *in vitro* efficacy of the antibiotics examined ranged from the potent quinolones (oxolinic acid and sarafloxacin) to ormet-sulfa and oxytetracycline, to the relatively ineffective drugs erythromycin and streptomycin. The variables of

incubation temperature, year and coast of isolation did have a significant effect on the MIC in some instances. The effect of incubation temperature is of practical concern in producing reproducible and meaningful results, as well as for comparisons of MIC data reported elsewhere. Temperature effects could be of concern in treatment of fish with antibiotics which are housed in systems of seasonally fluctuating water temperatures. Other variables, such as type of medium used and inoculum size, and inclusion of widely accessible control strains need to be examined and standardized before inter-laboratory comparisons are possible.

3. PLASMID PROFILES

3.1 Introduction

The basic structure of deoxyribose nucleic acid (DNA) is a double stranded helix, usually right handed in orientation, consisting of sugar (deoxyribose) molecules linked together by phosphodiester bonds. Covalently linked to the sugar molecule is a purine (adenine [A] or guanine [G]) or pyrimidine (cytosine [C] or thymine [T]) base. Hydrogen bonding between the bases (A-T or G-C) holds the strands together and base stacking maintains the helical structure. There are three configurations of DNA: linear, open circular and covalently closed circular (or supercoiled) (Freifelder, 1987). Based on the general composition and structure of DNA, techniques have been developed for extraction and differentiation of DNA configurations by centrifugation and agarose gel electrophoresis.

In bacteria, the genes encoding for resistance to antibiotics may be located on the chromosome, on plasmids or on transposons. Plasmids are extrachromosomal, self-replicating, supercoiled units of DNA that can be transferred between bacteria (Richmond, 1972). Transposons are mobile elements of DNA that are capable of inserting into several nonhomologous regions of plasmids or of the chromosome (Mitsuhashi, 1977). Frequent usage of antibiotics often results in selection pressures within a bacterial population

for antibiotic resistant strains. Antibiotic resistance genes may be acquired from nonpathogenic commensals sharing the same niche as pathogenic bacteria, as was the scenario in shigellosis outbreaks in humans (Richmond, 1972).

Plasmid-mediated antibiotic resistance has been reported in fish pathogenic isolates of *Aeromonas salmonicida* and *Vibrio anguillarum* (Aoki *et al*, 1971; Aoki *et al*, 1981; Aoki *et al*, 1983; Bast *et al*, 1988; Hedges *et al*, 1985; Toranzo *et al*, 1983). Transferable R-plasmids conferring resistance to chloramphenicol, streptomycin and sulfonamides were reported in *A. salmonicida* (Aoki *et al*, 1983). Transferable R-plasmids have been reported from *V. anguillarum* isolates resulting in resistance to chloramphenicol, tetracycline and sulfamonomethoxine (Aoki *et al*, 1981).

The objectives of this study were to examine the plasmid profiles of oxytetracycline and streptomycin resistant isolates of *A. salmonicida, V. anguillarum* and *V. ordalii* by agarose gel electrophoresis and to compare them to the plasmid profiles of antibiotic susceptible isolates, to assess whether antibiotic resistance is plasmid-mediated.

3.2 Materials and Methods

3.2.1 Growth of bacteria

Aeromonas salmonicida isolates were grown on Mueller-Hinton agar plates containing 32 μg oxytetracycline-HCl/mL.

Vibrio anguillarum and *Vibrio ordalii* isolates were grown on Mueller-Hinton agar plates plus 2% NaCl containing 32 μg streptomycin sulphate/mL. All cultures were grown at 20°C for 48 h.

3.2.2 DNA extraction protocols

3.2.2.1 Ethidium bromide/high salt extraction

The procedure used was a modification of the 20 minute ethidium bromide/high salt extraction of Stemmer (1991). The bacterial growth was removed from agar plates with a disposable inoculating loop and suspended in 250 μL of TELT lysing solution (Appendix B) in a 1.5 mL microcentrifuge tube. Samples were mixed thoroughly with a vortex (FisherBrand Vortex Genie 2) for 15 sec and stored at 4°C overnight. Subsequently, 15 μL of 10 mg/mL ethidium bromide and 140 μL of 7.5 M ammonium acetate were added to each sample and mixed thoroughly by inverting the tubes. DNA was extracted by the addition of 420 μL of 1:1 (v/v) phenol: chloroform, mixed with a vortex for 15 sec and centrifuged at 14,000 rpm (Eppendorf microcentrifuge model 5415) for 5 min at 4°C. The upper aqueous layer was transferred to a clean microcentrifuge tube and the extraction repeated with 400 μL of 1:1 (v/v) phenol: chloroform. DNA was precipitated by the addition of 1 volume cold (-20°C) isopropanol at room temperature for 2 min. Samples were centrifuged (14,000 rpm) at 4°C for 5 min and the pellet washed with 200 μL of cold (-20°C) 100% ethanol. The pellet was allowed to air dry at room temperature and

resuspended in 100 μL of TE (Tris-EDTA) buffer (Appendix C).

3.2.2.2 Rapid mini-prep

The procedure used was a modification of the protocol of Brent (1990). The bacterial growth was removed from the agar plates using a disposable inoculating loop, suspended in 500 μL of TELT lysing solution (Appendix B) in a 1.5 mL microcentrifuge tube. The samples were mixed with a vortex for 15 sec and stored at 4°C overnight. The next morning, DNA was extracted by the addition of an equal volume of 1:1 (v/v) phenol: chloroform, mixed with a vortex 15 sec and centrifuged at 14,000 rpm for 5 min at 4°C. The upper aqueous phase was transferred to a clean microcentrifuge tube and the phenol: chloroform extraction repeated. The DNA was precipitated by the addition of 1 volume of cold (-20°C) isopropanol for 2 min at room temperature. The DNA was pelleted by centrifuging (14,000 rpm) for 5 min at 4°C and washed with 200 μL of cold (-20°) 100% ethanol. The pellet was allowed to air dry at room temperature, resuspended in 50 μL of TE buffer (Appendix C) plus 2 μL ribonuclease A (RNase A) (Appendix D) and left on ice for 30 min.

3.2.2.3 Rapid mini-prep with heat

The procedure used was a modification of the protocol of Kado and Liu (1981). The bacterial growth was removed from the agar plates using a disposable inoculating loop and suspended in 500 μL of sodium dodecyl sulphate (SDS) lysing solution (Appendix B) in a 1.5 mL microcentrifuge tube.

Samples were mixed thoroughly with a vortex for 15 sec and heated for 30 min at 65 - 70°C in a water bath. While in the water bath, 2 volumes of 1:1 phenol:chloroform were added to each sample. The solution was emulsified by shaking and then centrifuged (14,000 rpm) for 5 min at 4°C. The upper aqueous phase was transferred to a clean microcentrifuge tube and samples drawn for electrophoresis.

3.2.3 Agarose gel electrophoresis

Gel electrophoresis was performed using the Horizon™ 11-14 horizontal gel electrophoresis apparatus (Bethesda Research Laboratories). A 0.7% agarose gel was prepared by the addition of 0.42 g of reagent grade agarose to 60 mL of Tris-acetate/EDTA (TAE) electrophoresis buffer (Appendix E) and heating to boiling to dissolve the agarose. The agarose was allowed to cool to 48 - 50°C in a water bath. The well-forming comb (14-tooth) was inserted into the slots of the UVT (ultra violet transmitting) gel tray and approximately 50 - 55 mL of the molten agarose was poured onto the UVT tray, enough to produce a 4 mm thick gel. After the agarose had solidified, approximately 20 - 30 min, the comb was removed and the gel covered with TAE electrophoresis buffer to a depth of 2 mm (approximately 700 mL). The entire unit was stored at 4°C until the DNA samples were ready to load onto the gel.

In a clean microcentrifuge tube, 18 μL of the DNA sample was mixed with 2 μL of 10X sample loading buffer (Appendix F). The entire mixture was transferred to the gel wells using an

automatic pipette. Supercoiled DNA marker was diluted according to the manufacturer's instructions (10 μL in 65 μL of TE buffer) and 7 μL of the dilution mixed with 2 μL of 10X sample loading buffer were included on each gel. Electrophoresis was carried out at 68 V at 4°C for 5 h. The gel was stained with 0.5 μg of ethidium bromide per mL of distilled water (Appendix G) at room temperature for 30 min and destained in distilled water for 30 min.

3.2.4 Photography

Gels were viewed with a UV transilluminator and photographed with a Polaroid MP4 camera fitted with a 23A red filter. Kodak Plus X Pan professional film was exposed for 30 sec at an aperture of F8. The film was developed for 6 min using HC-100 Dilution B at room temperature.

3.2.5 Estimation of molecular size

A standard curve was generated with Lotus Freelance version 3.01 by plotting the log of molecular size (kb) against the distance of migration (mm) for the supercoiled DNA ladder (Bethesda Research Laboratories) and two plasmids, pBR322 (Bethesda Research Laboratories) and pGEM-3Z (Fotodyne Inc.). The distance of migration was measured from the bottom of the well to the leading front of the plasmid band. Migration distances were not measured directly from the gel, but from photographs of the gels. All measurements were normalized to a standard gel length of 14 cm to compensate for differences in magnification during photography. Normalizing

the measurements was performed by dividing 14 by the gel length measured from the photograph and multiplying by the distance of migration measured from the photograph. Normalized measurements for the supercoiled DNA ladder were taken from seven different gels and the means used in the standard curve.

3.3 Results

The mean distances of migration of the supercoiled DNA ladder and the plasmids pBR322 and pGEM-3Z are presented in Table X. Figure 6 is the standard curve generated on Lotus Freelance (version 3.01) by plotting the log molecular size against the distance of migration. The coefficient of determination (r^2) was 0.996. Measurements from the supercoiled DNA ladder had to be averaged because of double banding, probably due to degradation of the markers. Bands that were consistent in all gels examined and were of greater intensity were selected as the bands corresponding to the molecular sizes for the markers (Figure 7). The smallest supercoiled DNA marker (2.07 kb) was omitted from the fitting of the curve because it shifted the entire curve away from the two plasmids of known molecular size. The supplier of the supercoiled DNA ladder recommended using a 0.9% agarose gel. The gels in this study were 0.7% agarose. A 0.9% agarose gel was examined, but the same problem was apparent with the

Table X Distance of migration of supercoiled DNA ladder and of the plasmids pBR322 and pGEM-3Z

Molecular size (kb)	Mean distance of migration (mm)
Supercoiled DNA ladder[1]	
16.21	37.1 ± 2.2
14.17	40.4 ± 2.8
12.14	43.8 ± 2.1
10.10	52.6 ± 2.2
8.07	58.4 ± 2.2
7.05	64.0 ± 1.7
6.03	69.7 ± 2.5
5.01	75.8 ± 2.8
3.99	86.3 ± 2.3
2.97	101.5 ± 3.9
2.07	113.0 ± 3.0
pBR322[2]	
4.36	80.0
pGEM-3Z[2]	
2.74	110.0

[1] n = 7

[2] n = 1

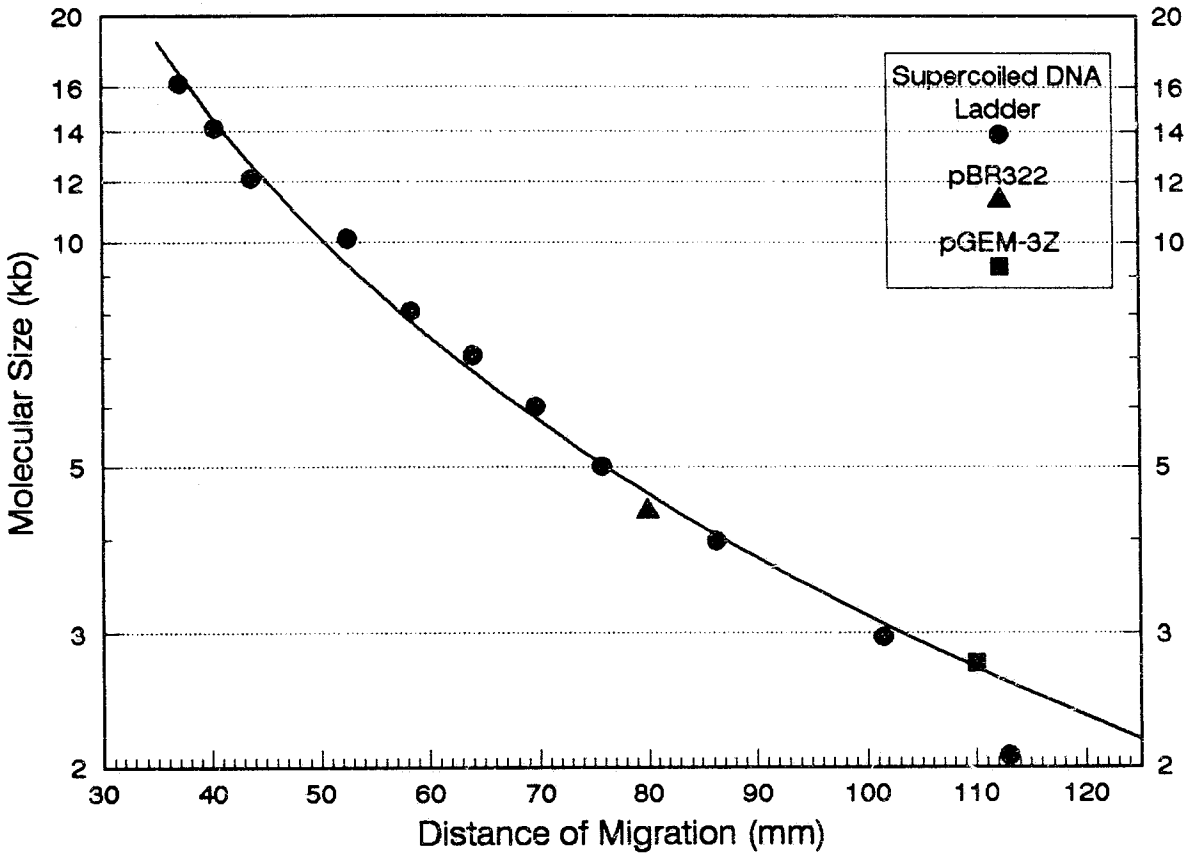


FIGURE 6: Standard curve for supercoiled DNA ladder and two plasmids

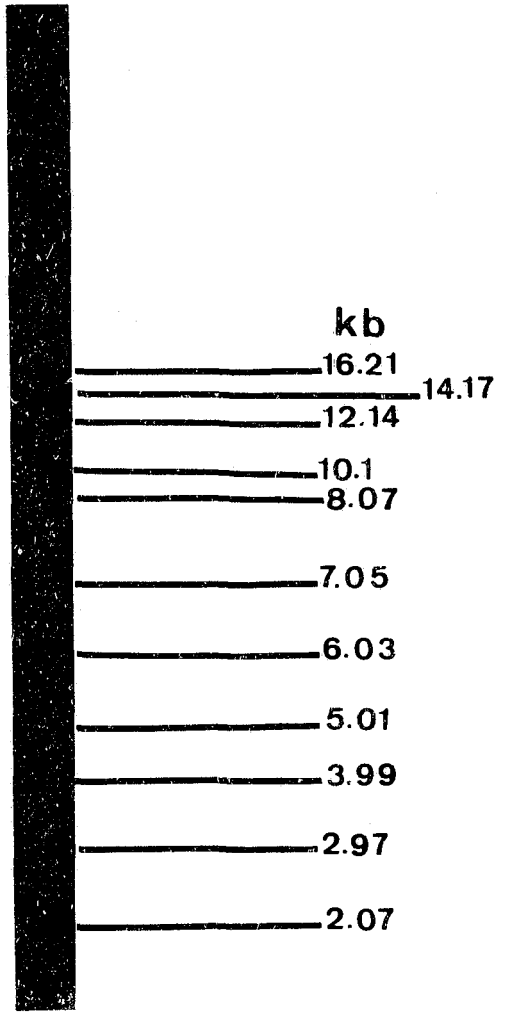


FIGURE 7 Supercoiled DNA marker

supercoiled DNA ladder. Therefore, the plasmids pBR322 and pGEM-3Z were also used in the generation of the standard curve.

Figure 8 is photographs of gels comparing the ethidium bromide/high salt extraction (section 3.2.2.1), the rapid mini-prep (section 3.2.2.2) and the rapid mini-prep with heat (section 3.2.2.3) for *A.salmonicida* and *Vibrio spp.* Both the ethidium bromide and the rapid mini-prep were generally agreeable in the number of bands observed from isolates of *A. salmonicida*. In one instance, the ethidium bromide/high salt extraction did recover a high molecular size plasmid from ATCC 14174, which was not isolated using the rapid mini-prep (Figure 8). The size of this plasmid, which was located above the chromosome band, could not be determined with the molecular size markers used in this study. As well, the recovery of this plasmid was not consistent, likely due to its size being more susceptible to shearing during the extraction procedure. The ethidium bromide/high salt protocol was selected for *A. salmonicida* plasmid extractions based upon this finding and the general reproducibility and cleanness of the preparations after repeating the protocols several times.

The comparison of three extraction protocols for *Vibrio* spp. is shown in Figure 8. Only one band was present in the *V. ordalii* ATCC 33509 with the ethidium bromide/high salt extraction, whereas two or three bands were observed with the rapid mini-prep. As well, the high molecular weight plasmid

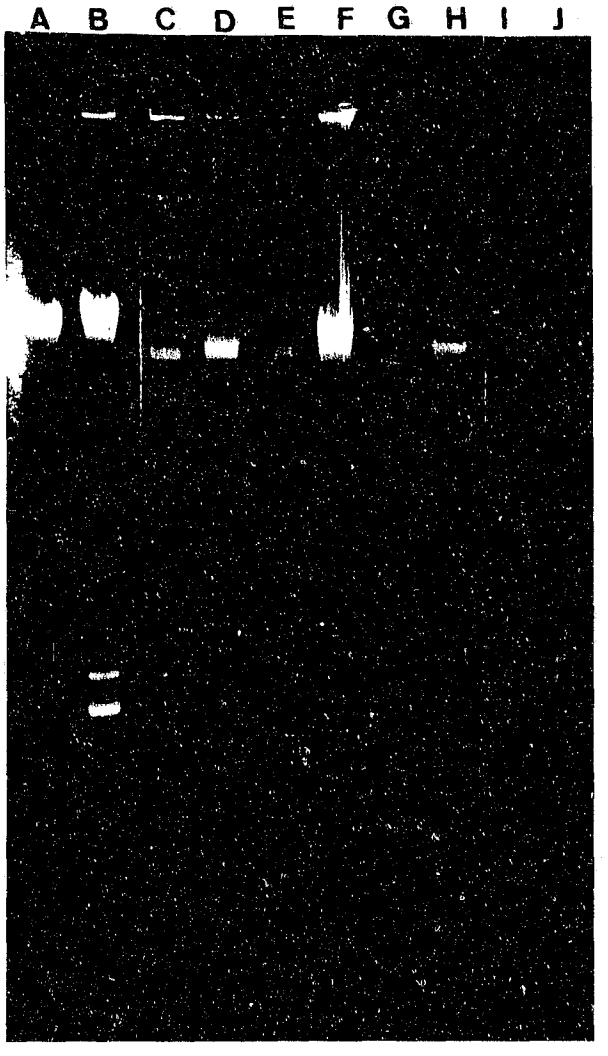


FIGURE 8 **Comparison of the ethidium bromide/high salt extraction and the rapid mini-prep for the extraction of plasmids from *Aeromonas salmonicida* (ATCC 14174) and *Vibrio* spp. (*V. anguillarum* 775 and ATCC 19264; *V. ordalii* ATCC 33509)**
Lanes: (A) ATCC 14174 rapid mini-prep;
(B) ATCC 14174 ethidium bromide/high salt;
(C) 775 ethidium bromide/high salt;
(D) 775 rapid mini-prep; (E) ATCC 19264 ethidium bromide/high salt; (F) ATCC 19264 rapid mini-prep;
(G) ATCC 33509 ethidium bromide/high salt;
(H) and (I) ATCC 33509 rapid mini-prep;
(J) ATCC 33509 rapid mini-prep with heat

of *V. anguillarum* 775 was detected with the rapid mini-prep, but not with the ethidium bromide/high salt extraction (Figure 8). Thus the ethidium bromide extraction was judged not suitable for *Vibrio* isolates. No bands were observed when ATCC 33509 was subjected to the rapid mini-prep with heat. Thus, the rapid mini-prep without a heat treatment was selected as the extraction protocol.

The number of bands and their estimated molecular sizes from the gel electrophoresis of isolates of *Aeromonas salmonicida* from the Atlantic and Pacific coasts are summarized in Table XI. Both oxytetracycline resistant and sensitive strains were examined. Resistant isolates were examined when grown on Mueller-Hinton agar with and without the addition of oxytetracycline (32 μg/mL active drug). Photographs of the gel electrophoresis of the Atlantic coast and Pacific coast isolates of *A. salmonicida* are given in Figures 9 and 10, respectively. ATCC 33658 and 14174 were included as controls on every gel. The plasmid profiles for the seven Atlantic coast isolates of *A. salmonicida* were relatively constant (4 to 6 plasmids observed) and 4 smaller plasmids were present in all isolates (size range of 4.3 or 4.4 to 8.1 or 8.4 kb) (Table XI and Figure 9). Double bands in close proximity to one another were recorded as one plasmid, such as 7.1/6.9 kb. Six of the seven isolates also exhibited a band equivalent to approximately 15 kb (14.5, 15 or 15.8 kb).

Table XI Number and estimated molecular size of DNA bands from *Aeromonas salmonicida*

	OTC	Number of bands	Estimated molecular size (kb)
ATCC 33658	S	4	8.1, 7.1/6.9, 4.8, 4.4
ATCC 14174	S	5	*, 8.1, 7.1/6.9, 4.8, 4.4
Atlantic coast isolates			
AS89-10A + OTC	R	5	15, 8.1/7.9, 6.9, 4.8, 4.4
AS89-3A	R	5	15, 7.9, 6.9, 4.8, 4.4
AS89-2A	R	4	8.1, 6.9, 4.8, 4.4
AS89-2A + OTC	R	5	15, 8.1/7.9, 6.9, 4.8, 4.4
AS87-2A	R	6	15.8, 11.9, 8.1/7.8, 6.8, 4.7, 4.3
AS87-2A + OTC	R	5	15.8, 8.1/7.8, 6.8, 4.7, 4.3
AS86-4A	R	5	14.5, 8.4, 7.3/6.8, 4.7, 4.3
AS86-4A + OTC	R	5	14.5, 8.4, 7.3/6.8, 4.7, 4.3
AS85-2A	R	6	15, 8.1/7.9, 7.1, 6.9, 4.8, 4.4
AS85-2A + OTC	R	6	15, 8.1/7.9, 7.1, 6.9, 4.8, 4.4
AS84-1A + OTC	R	4	8.1, 6.7, 4.7, 4.3
AS84-1A + SM	R	4	8.1, 6.7, 4.7, 4.3
Pacific coast isolates			
AS90-8P	R	6	8.9, 7.8/7.5, 6.7, 5.0, 4.5, 4.2
AS90-8P + OTC	R	6	8.9, 7.8/7.5, 6.7, 5.0, 4.5, 4.2
AS89-1P	R	8	*, 14.5, 9.8, 8.9, 7.9, 7.5, 5.1, 4.5
AS89-1P + OTC	R	8	*, 14.5, 9.8, 8.9, 7.9, 7.5, 5.1, 4.5
AS87-2P	S	5	7.9, 6.8/6.6, 5.9, 4.7, 3.8
AS86-2P	S	4	6.8, 6.2, 4.2, 3.9
AS78-1P	S	6	15.8, 8.6, 8.1, 7.5/7.3, 5.0, 4.5
AS71-1P	S	4	8.9, 7.8/7.5, 5.0, 4.5
AS69-1P	S	4	8.9, 7.8/7.5, 5.0, 4.5

S = oxytetracycline sensitive, MIC ≤ 4.0 μg/mL
R = oxytetracycline resistant, MIC > 4.0 μg/mL
* = band too large to determine molecular size from standard curve

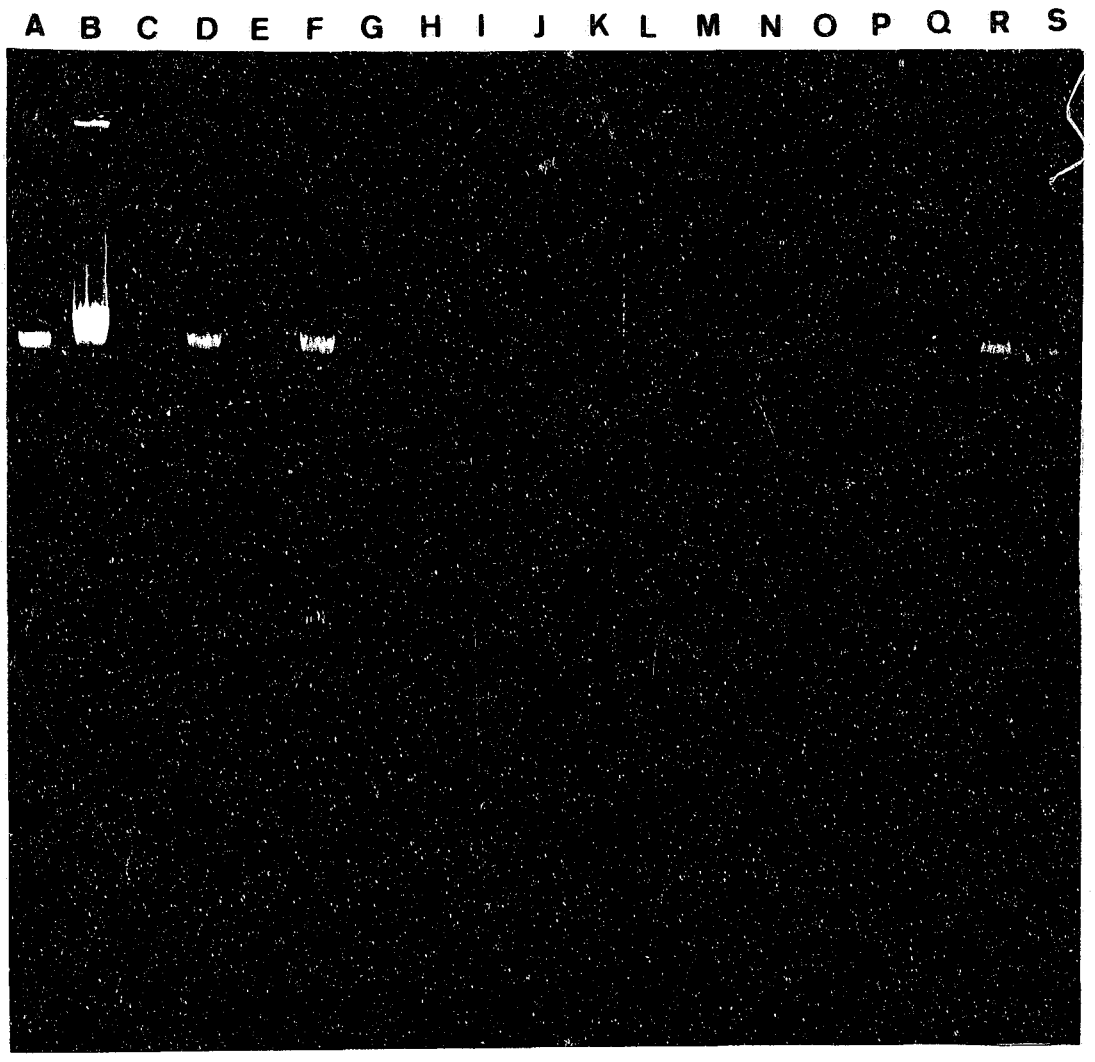


FIGURE 9 **Plasmid profiles of Atlantic coast isolates of *Aeromonas salmonicida***
Lanes: (A) ATCC 33658; (B) ATCC 14174; (C) AS89-2A; (D) AS89-2A + OTC; (E) AS89-10A; (F) AS89-10A + OTC; (G) supercoiled DNA ladder; (H) AS89-3A; (I) AS89-3A + OTC; (J) AS85-2A; (K) AS85-2A + OTC; (L) AS87-2A; (M) AS87-2A + OTC (N) AS86-4A; (O) AS86-4A + OTC; (P) supercoiled DNA ladder; (Q) AS84-1A; (R) AS84-1A + OTC; (S) AS84-1A + SM

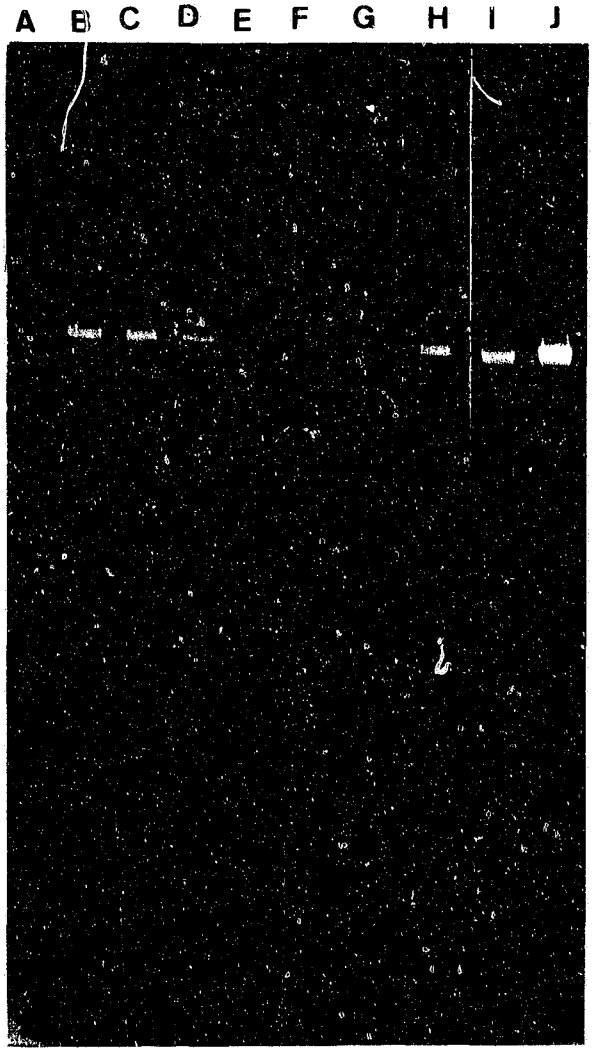


FIGURE 10 Plasmid profiles of Pacific coast isolates of *Aeromonas salmonicida*
Lanes: (A) AS69-1P OTC^S; (B) AS71-1P OTC^S;
(C) AS90-8P; (D) AS90-8P + OTC (E) AS89-1P;
(F) AS89-1P + OTC; (G) supercoiled DNA ladder;
(H) AS78-1P OTC^S; (I) AS86-2P OTC^S; (J) AS87-2P OTC^S

Plasmid profiles were similar, if not identical, to that of the ATCC control strains 33658 and 14174. Plasmid profiles of oxytetracycline resistant isolates grown in the presence and absence of the antibiotic were examined. Plasmid profiles were relatively consistent and comparable to the two oxytetracycline sensitive ATCC strains. One oxytetracycline and streptomycin resistant strain, AS84-1A, was examined when grown in the presence of oxytetracycline and in the presence of streptomycin. The plasmid profiles were identical and consisted of the 4 small plasmids observed with the other isolates.

The plasmid profiles of the Pacific coast isolates of *A. salmonicida* differed slightly from the plasmid profiles of the Atlantic coast isolates and the ATCC strains. The two oxytetracycline resistant isolates (AS90-8P and AS89-1P) possessed 6 small plasmids, 4.2, 4.5, 5.0 or 5.1, 6.7, 7.5/7.8 and 8.9 kb (Table XI and Figure 10). One of these isolates (AS89-1P) had three additional plasmids, 9.8 and 14.5 kb and one large plasmid of molecular size that could not be determined, but did not have plasmids at 6.8/6.6, 5.9 and 3.8 kb found in oxytetracycline sensitive isolates. The plasmid profiles of resistant isolates grown in the presence and absence of the antibiotic were identical. The plasmid profiles of oxytetracycline sensitive isolates were similar or identical to ATCC strains and both Atlantic and Pacific coast

isolates, with the addition of a small plasmid at 3.8 or 3.9 in two of the five isolates.

Table XII summarizes the number of bands and their estimated molecular size from *Vibrio anguillarum* and *Vibrio ordalii* isolates from the Atlantic and Pacific coasts. Streptomycin resistant isolates were examined when grown on Mueller-Hinton agar plus 2% NaCl with the addition of streptomycin (32 μg/mL active drug). Figures 8 and 11 are photographs of the gel electrophoresis of *Vibrio* spp. using the rapid mini-prep protocol. ATCC 33509 had three plasmids at 7.9, 4.5 and 3.0 kb, although the 7.9 kb plasmid was not always recovered. It is worth noting that this isolate was resistant to all of the antibiotics tested (Table III). No plasmids were detected in streptomycin resistant *V. ordalii* VO87-6A or in the streptomycin sensitive *V. anguillarum* ATCC 19264. The streptomycin resistant *V. anguillarum* isolates varied in the presence of a high molecular weight plasmid, including 775 which is known to carry a 65 kb plasmid (pJM1). The failure to isolate the larger plasmid was likely due to shearing during the extraction procedure. The molecular size of the large plasmid could not be estimated from the standard curve. Only one of the *V. anguillarum* strains (VA69-2P), isolated from the Pacific coast, had smaller molecular size plasmids of 6.7 and 4.0 kb.

Table XII Number and estimated molecular size of DNA bands from *Vibrio* spp.

	SM	Number of bands	Estimated molecular size (kb)
Vibrio ordalii			
ATCC 33509	R	3	7.9, 4.5, 3.0
VO87-6A	R	0	
Vibrio anguillarum			
ATCC 19264	S	0	
Atlantic coast			
VA89-12A	R	0	
VA89-11A	R	1	*
VA89-8A	R	0	
VA89-3A	R	0	
VA89-2A	R	0	
VA89-1A	R	0	
VA88-1A	R	1	*
VA87-1A	R	1	*
VA85-6A	R	0	
VA85-2A	R	1	*
Pacific coast			
VA90-1P	R	1	*
VA75-1P	R	0	
VA69-2P	R	2	6.7, 4.0

S = streptomycin (SM) sensitive, MIC ≤ 16 μg/mL
R = streptomycin (SM) resistant, MIC > 16 μg/mL
* = band too large to determine molecular size from standard curve

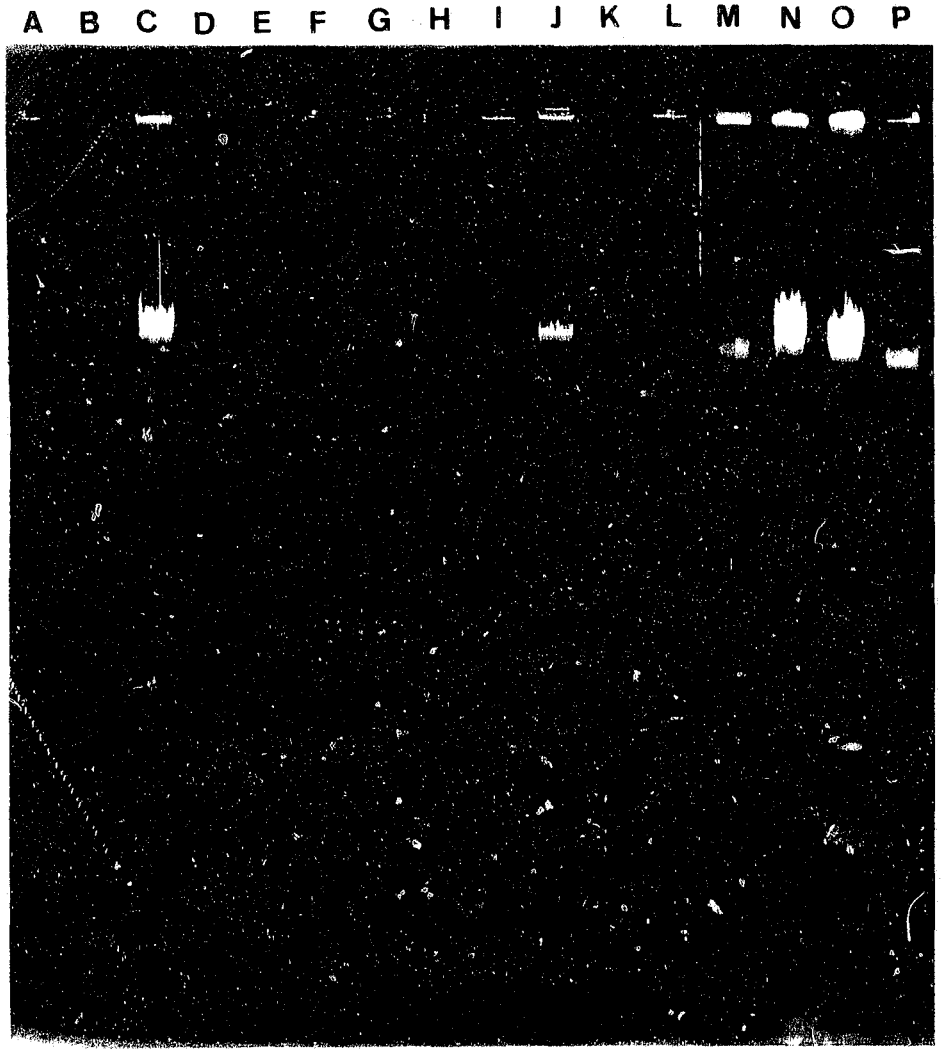


FIGURE 11 Plasmid profiles of Atlantic and Pacific coast isolates of *Vibrio anguillarum* (VA) and *Vibrio ordalii* (VO)
Lanes: (A) ATCC 19264; (B) VA89-12A; (C) VA89-11A; (D) VA89-8A; (E) VA89-3A; (F) supercoiled DNA ladder; (G) VA89-2A; (H) VA89-1A; (I) VA83-1A; (J) VA87-1A; (K) VA85-6A; (L) VA85-2A; (M) VA90-1P; (N) VA75-1P; (O) VA69-2P; (P) VO87-6A

3.4 Discussion

DNA extraction procedures utilize knowledge of the basic composition, configuration and properties of DNA. The basic steps in plasmid DNA extraction are cell lysis, selective denaturation of chromosomal DNA, neutralization, phenol:chloroform extraction and alcohol precipitation of plasmid DNA. A description of these procedures and the theories involved is warranted for the understanding of the techniques.

The first step in any DNA extraction procedure is cell lysis. Various formulations have been used, including lysozyme and detergents. The TELT buffer used in this study consists of the chelating agent ethylenediaminetetraacetic acid (EDTA), which removes the bacterial envelope, the detergent Triton X-100 to weaken the cell wall and disrupt the inner membrane (Leal *et al*, 1990) and lithium chloride to precipitate RNA (Kibenge *et al*, 1991). Lysis was performed at 4°C overnight. Low temperatures during lysis decreases endonucleolytic activity which can degrade DNA (Hansen and Olsen, 1978). A 3% sodium dodecyl sulphate (SDS) alkaline (pH 12.6) lysing solution was also used in this study in the rapid mini-prep with heat. SDS acts in the same manner as Triton X-100.

Alkaline conditions during or following lysis result in the selective denaturation of chromosomal DNA. Chromosomal

DNA is sheared into linear fragments during lysis and extraction (Dillion *et al*, 1985). At pH > 11.3, the charges on the bases are changed, eliminating hydrogen bonding; thus, the DNA is denatured into single strands (Freifelder 1987). A pH of 12 - 12.5 selectively denatures linear DNA, such as chromosomal DNA. If the pH of the solution is > 13, supercoiled DNA, such as plasmid DNA, is irreversibly denatured (Birnboim and Doly, 1979).

The ethidium bromide/high salt extraction (section 3.2.2.1) was used for *A. salmonicida* isolates. Ethidium bromide intercalates between base pairs causing the DNA molecule to unwind. The result is decreased density of the molecule. Supercoiled or covalently closed circular DNA has no free ends. As the DNA unwinds, the entire molecule must twist in the opposite direction to relieve the stress, resulting in a figure 8 configuration. The ethidium bromide continues to bind to the DNA until the molecule is unable to twist any further. Linear and open circular DNA do not have such topological constraints; therefore, they can bind more ethidium bromide. Supercoiled DNA can be separated from linear and open circular DNA based on density by centrifugation. In the ethidium bromide/high salt extraction, chromosomal DNA binds more ethidium bromide, which is extracted in the organic phase during the phenol:chloroform extraction.

The ethidium bromide/high salt extraction protocol also utilizes the properties of DNA in a high salt solution. Ammonium acetate neutralizes the alkaline pH, resulting in precipitation of protein-detergent complexes and high molecular mass RNA (Birnboim and Doly, 1979). As well, a salt concentration of > 0.05M prevents electrostatic repulsion of phosphates; thus, hydrogen bonding between bases and base stacking is restored and the chromosomal DNA renatures (Freifelder 1987).

Extraction protocols may use heat to optimize plasmid DNA purification, as with the modification of the rapid mini-prep with heat (section 3.2.2.3). Hansen and Olsen (1978) used a 5 minute 50°C heat treatment. They explained enhanced plasmid recovery as follows. Chromosomal DNA can be described as consisting of domains that are folded on one another. Heat treatment results in unfolding of these domains. Plasmids were speculated to be in association with but not integrated with the domains of the chromosome. Thus, the unfolding of the chromosome results in the release of the plasmid into solution (Hansen and Olsen, 1978). Kado and Liu (1981) used prolonged heat treatments (up to 72 min at 65°C) to reduce the amount of linear DNA and RNA by denaturation, while retaining intact plasmid DNA.

Phenol:chloroform (1:1, v:v) is commonly used in DNA extractions. Kado and Liu (1981) found the phenol:chloroform mixture minimized the formation of brown oxidation pigments

that can occur with phenol alone. Their mixture was unbuffered and lowered the pH of the solution to 9.3, eliminating the need for a neutralization step and resulting in the renaturation of chromosomal DNA. The phenol:chloroform mixture used in this study was Tris-buffered, pH 8. Tris-buffered phenol has a decreased solubility in the aqueous phase, thus, a better separation of the phases is possible (Dillion *et al*, 1985). In addition, phenol denatures and extracts proteins, extracts nucleases and selectively removes denatured DNA (Dillion *et al*, 1985). Centrifuging (14,000 rpm) separates the sample into an upper aqueous phase and a lower organic phase. The upper phase is decanted and the plasmid DNA precipitated with either ethanol (2 volumes) or isopropanol (1 volume). Alcohol concentrates the DNA by removing the water (Dillion *et al*, 1985).

The DNA pellet is resuspended in TE (Tris-EDTA) buffer. EDTA is a chelating agent which removes metal ions necessary for nuclease activity. Tris has a high buffering capacity between pH 7 - 9 and little interaction with metal ions (Dillion *et al*, 1985). Protocols may include the addition of RNase at this point, such as RNase A from bovine pancreas, to remove contaminating RNA which binds ethidium bromide and creates a haziness when photographed (Dillion *et al*, 1985).

Two extraction protocols for *A. salmonicida* were compared (Figure 8), the ethidium bromide/high salt extraction and the rapid mini-prep. Both protocols recovered the 4 small

plasmids of the ATCC strains 33658 and 14174. In one instance, a high molecular size DNA band was observed above the chromosomal DNA from ATCC 14174 using the ethidium bromide/high salt extraction. The ethidium bromide/high salt protocol was not selected for use with the *Vibrio* isolates due to a failure to recover the plasmid bands of ATCC 33509 (Figure 8). The rapid mini-prep occasionally produced smeared lanes. The smearing could be a result of overloading the gel with too much DNA or due to DNA degradation (Leal *et al*, 1990). If samples were quantified for DNA content (absorbance at 260 nm) and subsequently diluted, generally no bands were observed. To diminish the problem of smearing without diluting the sample, a heat treatment was performed to eliminate linear DNA (section 3.2.2.3). Three bands were observed with *V. ordalii* ATCC 33509 with the rapid mini-prep, but none were present with the heat treated samples (Figure 8). One interpretation could be that the bands were actually linear DNA, and hence, removed by the heat treatment, but the distance of migration and molecular sizes (7.9, 4.5, 3.0 kb) would suggest a supercoiled configuration. Thus the rapid mini-prep was selected as the protocol for subsequent DNA extractions for *Vibrio* sp.

Estimation of the molecular size of the DNA or plasmid bands was complicated by double banding with the supercoiled DNA ladder. A nuclease may have been present in the DNA ladder, resulting in its degradation. Macrina *et al* (1978)

reported a similar problem with an *E. coli* strain with plasmids suitable for use as molecular size markers. Open circular (OC) forms accumulated in the plasmid preparation with prolonged storage at 4°C and with repeated freezing and thawing. The OC forms migrated 10-15% slower than the corresponding supercoiled forms. The supercoiled DNA ladder used in this study was stored at 4°C.

Plasmid profiles for the Atlantic coast isolates of *A. salmonicida* were relatively constant and comparable to the ATCC strains, with 4 small plasmids being recovered (Table XI and Figure 9). Plasmid profiles of the Pacific coast isolates of *A. salmonicida* differed slightly from the plasmid profiles of the Atlantic coast isolates and the ATCC strains, with 6 small plasmids being recovered in the resistant isolates (Table XI and Figures 9 and 10). Slight differences in molecular sizes between the 2 groups of isolates could result from variations in the electrophoresis, such as in the distance of migration of the tracking dye front or due to size polymorphism of plasmids. Resistance plasmids can vary in size due to the insertion or deletion of transposons (Mitsuhashi, 1977). Gels were electrophoresed for 5 h at 68 V in a 4°C environmental room to minimize variations between gels. The term "distance of migration" was selected over the more commonly used term "relative mobility" (Rm). The relative mobility is the ratio of the distance of migration of the unknown molecule to that of a marker substance, namely the

tracking dye (Dillion *et al*, 1985). In the present study, the distance of migration of the tracking dye was not recorded and measurements were made from the photograph and normalized to a 14 cm gel length.

A larger molecular size plasmid of ATCC 14174 was not always recovered, likely due to shearing during transfer of the sample with an automatic pipette. Double bands were frequently observed, such as 7.1/6.9 or 8.1/7.9, but probably do not represent 2 plasmids. Therefore, they were reported as one plasmid band in Table XI. Birnboim and Doly (1979) addressed the problem of the interpretation of gels due to the presence of open circular (OC) forms of DNA and dimers of covalently closed circular (supercoiled) DNA. Such bands are less intense when stained with ethidium bromide, do not migrate as far as supercoiled DNA and may not consistently appear on gels. If supercoiled DNA is subjected to UV radiation, the open circular form is produced. Bands on a gel can be distinguished as OC by treating the sample with UV light and observing bands of increased intensity. Zasloff *et al* (1978) reported that OC forms could be removed via several acid phenol extractions, although the theory behind this finding was not known. Linear DNA may also be present. Linear DNA runs before OC when TAE electrophoresis buffer is used. The band corresponding to chromosomal DNA consists of linear fragments from the shearing of the DNA during lysis and extraction. A more diffuse band results because fragments are

of different sizes. Linear DNA can be distinguished from OC and supercoiled forms by heating to 100°C for 3-7 min to eliminate linear DNA (Dillion *et al*, 1985). Understanding the relative mobilities of the different forms of DNA is needed for interpretation of the gel electrophoresis results.

Plasmid profiles corresponded reasonably well with the results by other researchers, except that the higher molecular mass plasmid of ATCC 14174 was isolated only on one occasion (Figure 8). Other sources often report plasmid sizes as molecular mass (megadaltons, Md) and not in terms of kilobase pairs (kb). For comparative purposes, masses were converted to the estimation in molecular size in kb, where 1 kb is equivalent to 0.66 Md. These values are presented in brackets.

Toranzo *et al* (1983) reported 9 plasmids from ATCC 14174, ranging in size from 3.4 to 55 Md (5.2 to 83.3 kb). Bast *et al* (1988) reported 5 plasmids from ATCC 14174 with molecular masses of 3.3, 3.5, 3.6, 4.2 and 50-56 Md (5.0, 5.3, 5.5, 6.4 and 75.8-84.8 kb). Multiple faint bands were reported in the 8 to 11.2 Md (12.1 to 17 kb) range, but could be removed by an acid phenol extraction. Thus, it was concluded that these bands were open circular forms of DNA and not plasmids (Bast *et al*, 1988). Two bands in oxytetracycline resistant AS89-1P (14.5, 9.8 kb) that were not present in sensitive isolates are within size ranges of OC DNA. A 90 Md (136 kb) transferable plasmid conferring resistance to

tetracycline and sulfonamides was also reported in one of the 4 resistant isolates (Bast *et al*, 1988). This study reported 5 bands from ATCC 14174, although the high molecular size plasmid could not be recovered every time. Bast and co-workers (1988) also reported problems in the consistent isolation of larger plasmids, likely due to shearing during the extraction procedure. Freeze drying has been reported to affect the resistance patterns of *E. coli*, whereas storage in glycerol at -20°C did not result in the loss of resistance (Tripathi *et al*, 1989). Frequent subculturing of bacteria also causes loss of plasmids.

Ishiguro *et al* (1981) reported that the plasmid profiles of 3 *A. salmonicida* strains and their attenuated counterparts were the same: 4.8, 6.2 and 91 Md (7.3, 9.4, 138 kb). Thus, loss of virulence upon culturing at higher than optimum temperatures (26-27°C) was not due to loss of a virulence plasmid, but rather due to loss of the A-layer. Aoki *et al* (1983) reported 2 plasmids in *A. salmonicida* from cultured salmonids. A 29 Md (44 kb) transferable plasmid conferred resistance to chloramphenicol, streptomycin and sulfamonomethoxine. A 7.6 Md (11.5 kb) non-transferable plasmid encoded for tetracycline resistance. In this study, a 11.9 kb plasmid band was recovered from one strain (AS87-2A) when grown in the absence of oxytetracycline, but not in the presence of the antibiotic (Table XI and Figure 9). This band

was faint (Figure 9) and could possibly represent OC or linear DNA.

Hedge *et al* (1985) observed a 100 Md (151.5 kb) plasmid of Incompatibility group C (Inc C) encoding for resistance to streptomycin, tetracycline and chloramphenicol. Inc C plasmids are the most common in *Vibrio cholerae* and the potential exists for antibiotic resistance plasmids to be transferred from fish pathogens to human pathogens existing in the same microcosm. Aoki *et al* (1971) reported transferable plasmids for sulfonamide, tetracycline and chloramphenicol resistance in 3 out of 25 isolates of *A. salmonicida*. Resistance transfer was shown by mating experiments with an *E. coli* strain but were not confirmed by plasmid profiles. In this study, the plasmid profiles of the streptomycin and oxytetracycline resistant *A. salmonicida* isolate AS84-1A were identical.

The plasmid profiles between oxytetracycline resistant and sensitive isolates and between resistant isolates grown in the presence and absence of the antibiotic were similar. Thus, based on plasmid profiles resistance to oxytetracycline does not appear to be plasmid-mediated. Mating experiments with a sensitive strain of *E. coli* would determine if the antibiotic resistance was transferable. Plasmid profiles of the *E. coli* would assess which plasmids were transferable. Transposons are also responsible for antibiotic resistance and

their presence in plasmids or chromosomes could be confirmed by extensive restriction enzyme mapping. Antibiotic resistant isolates could also be treated with plasmid curing agents, such as acridine dyes, ethidium bromide or possibly some quinolone antibiotics (Michel-Briand *et al*, 1986; Weisser and Wiedemann, 1985) and subsequent gels examined for changes in the plasmid profiles. The MICs of these plasmid cured isolates would indicate their antibiotic sensitivity.

Plasmids were uncommon in the Atlantic and Pacific coast isolates of *Vibrio* spp. *V. ordalii* ATCC 33509 was resistant to all antibiotics tested (Table III) and possessed 3 plasmid bands (3.0, 4.5, 7.9 kb), although the 7.9 kb band was not always recovered. No plasmids were isolated from streptomycin resistant VO87-6A. A 20 Md (30 kb) plasmid has been reported as responsible for virulence in *V. ordalii* (Trust *et al*, 1981). Larsen and Olsen (1991) noted that environmental isolates did not possess plasmids, whereas those isolates from fish varied in their plasmid content. The majority of the isolates from fish had plasmids in the 5-10 kb range; a 68-70 kb and a 190 kb plasmid were also recovered in some isolates. Wiik *et al* (1989) do not isolate any plasmids in *V. ordalii*.

Recovery of a high molecular size plasmid from *V. anguillarum* isolates was variable (Table XII and Figure 11). The plasmid pJM1 was recovered only on one occasion from type strain 775. This 65 kb plasmid encodes for a siderophore and iron uptake system which allows the bacterium to compete for

iron which is normally bound to proteins in serum. Thus, presence of the plasmid has been correlated to virulence (Tolmasky and Crosa, 1984). As well, curing of the plasmid by culturing the bacterium at 37°C results in attenuation of virulence (Crosa *et al*, 1980). Trust *et al* (1981) reported a 47 Md (71 kb) pJM1-like plasmid in 3 out of 8 isolates. Wiik *et al* (1989) also recovered a 47 Md plasmid that was similar, but not identical to pJM1 upon restriction enzyme mapping. Larsen and Olsen (1991) reported the pJM1 plasmid (67 kb) in 63 out of 74 strains of *V. anguillarum* isolated from diseased fish. Nine strains also possessed a 98 kb plasmid and only one strain was plasmidless. The situation was different in *V. anguillarum* strains isolated from the mucus and gills of healthy fish. Plasmids were recovered in 29 of 50 isolates, but 21 isolates did not possess plasmids (Larsen and Olsen, 1991). Transferable resistance to chloramphenicol, tetracycline and sulfamonomethoxine was reported in 165 out of 250 *V. anguillarum* isolates from cultured ayu (*Plecoglossus altivelis*). Mating experiments were performed, but the plasmid profiles were not examined (Aoki *et al*, 1981). Transferable resistance to streptomycin, sulfonamides, chloramphenicol and tetracycline was noted in 65 of 68 isolates from ayu. Resistance was transferable to *V. cholerae* in mating experiments. The isolates were not examined by gel electrophoresis for their plasmid profiles (Aoki *et al*, 1974).

The inability to recover the large molecular size plasmid pJM1 or a similar plasmid may be a result of its large size, which is more susceptible to shearing during pipetting. No plasmids were found in the streptomycin sensitive ATCC 19264, a finding in contrast with Toranzo *et al* (1983) who reported 2 plasmids of 2.6 and 5.5 Md (3.9, 8.3 kb). Only one strain possessed small plasmids of 4.0 and 6.7 kb (VA69-2P).

The plasmid profiles of *Vibrio* spp. suggest that streptomycin resistance is not plasmid-mediated. Streptomycin resistance is frequently carried on transposons (Barth *et al*, 1976). Although a 29 Md plasmid conferring resistance to streptomycin and chloramphenicol has been reported in *A. salmonicida* (Aoki *et al*, 1983) and transferable resistance to streptomycin has been observed in *V. anguillarum* (Aoki *et al*, 1974), the presence of resistance plasmids has not been confirmed in *Vibrio* spp.

In conclusion, from examination of the plasmid profiles of oxytetracycline resistant and sensitive strains of *A. salmonicida* and streptomycin resistant and sensitive strains of *V. anguillarum* and *V. ordalii*, resistance to these antibiotics does not appear to be plasmid-mediated. Plasmid curing experiments would help verify these results. As well, mating experiments were not performed to determine whether the resistance is transferable to other fish pathogens or to human pathogens. Transferable antibiotic resistance is not

necessarily under the control of plasmids, but may be a result of transposons integrated within plasmids or within the chromosome.

4. MICROBIOLOGICAL ASSAY

4.1 Introduction

The measurement of antibiotic concentrations in body fluids is important to ensure sufficient therapeutic concentrations are achieved without attaining potentially toxic concentrations. Information relating blood concentrations to drug efficacy is limited, but concentrations of three to five times the MIC have been suggested as desirable for homeotherms (Washington, 1985).

The basic principle of a microbiological assay is that the degree of inhibition of growth of a test organism is related to the concentration of antibiotic in the blood or tissue sample. Inhibition of growth of the test organism is most frequently measured by zones of inhibition in diffusion assays. In the selection of a microbiological assay one must consider the accuracy, the precision and the sensitivity of the assay for the antibiotic to be measured. Accuracy refers to the ability of the assay to measure a concentration correctly and precision refers to maintaining a small standard deviation. Both accuracy and precision are determined by the repeated assaying of internal controls, such as the reference concentration included in triplicate on each plate in the Association of Analytical Chemists (AOAC) protocol (Helrich, 1990). Sensitivity refers to the lowest concentration that can be accurately measured and may not correspond to the limit

of detection of the assay (Reeves and Wise, 1978). The concentration that designates the limit of detection of an assay is called the critical concentration. Concentrations below the critical concentration do not produce zones of inhibition in diffusion assays. The critical concentration is generally in the order of three to four times higher than the MIC for the test organism (Cooper, 1963).

A microbiological assay was developed for the determination of sarafloxacin-HCl and oxolinic acid concentrations in Atlantic salmon plasma. The assay was a modification of the AOAC method for antibiotic concentrations in feeds (Helrich, 1990) using disk diffusion and a lawn of *V. anguillarum* ATCC 19264 as the test organism. With these modifications, sensitivities of 0.04 μg/mL for sarafloxacin and 0.125 μg/mL for oxolinic acid were obtained. Other bacteria, such as *B. subtilis* or *E. coli*, are more commonly used as test organisms. Both of these bacteria failed to provide the degree of sensitivity required for an antibiotic depletion phase study.

4.2 Materials and Methods

4.2.1 Preparation of antibiotic stock solutions

Antibiotic stock solutions containing 1280 μg/mL of active drug were prepared in distilled water, dispensed as 1 mL aliquots into 1.5 mL Eppendorf tubes and stored at -20°C. Oxolinic acid (99.58% active drug) required 0.1 N NaOH to

dissolve and sarafloxacin-HCl (829 μg/mg active drug) required boiling.

4.2.2 Investigation of optimum conditions for a microbiological assay

4.2.2.1 Preparation of *Bacillus subtilis* and *Escherichia coli* seeded plates

Escherichia coli ATCC 25922 was grown in tryptone soya broth (TSB) at 37°C. A bacterial suspension equivalent to a McFarland barium sulphate standard (McFarland nephelometer) (Anhalt and Washington, 1985; Hendrickson, 1985) of 0.5 to 1.0 was prepared with an optical density at 620 nm of 0.08 to 0.1 and a plate count of approximately 10^8 CFU/mL. Seeded plates were prepared by the addition of 2 mL of this suspension to 250 mL of molten agar (50-55°C).

Bacillus subtilis ATCC 6633 was obtained as a spore suspension (Difco) consisting of 10^5 spores per mL. A volume of 0.1 mL of spore suspension was added to 250 mL of molten agar (50-55°C), to produce a final spore concentration of 2.5×10^8 spores per mL.

Three different forms of plates were prepared for both test organisms. The first form of plate consisted of a 5 mL unseeded base agar layer (BBL Medium #2) and a 8 mL seeded agar overlay (Difco Antibiotic Medium #8). The base agar layer was allowed to solidify before pouring the seeded agar overlay. While the seeded overlay was still molten, a well-forming mould was set in place. The mould consisted of 6

pieces of 6.5 mm diameter teflon rod in a hexagonal array attached to a nylon template. The mould was removed after the agar solidified. The moulds were autoclavable and produced wells in the arrangement specified by the Association of Official Analytical Chemists (AOAC), as described in section 4.2.6.

The second form of plate consisted of 5 mL of seeded base (BBL Medium #2) and 8 mL of seeded agar overlay (Difco Antibiotic Medium #8). Wells were made using the mould, as described above.

The third form of plate consisted of a 8 mL of seeded agar (Difco Antibiotic Medium #8) and no base layer. Wells were cut into the solidified agar with a 6.5 mm cork borer.

Wells were filled to the top with either 0.25, 0.5, 1.0, 2.0 or 4.0 μg/mL of oxolinic acid diluted with Atlantic salmon plasma (Fish Health Unit, A.V.C., U.P.E.I.). Control wells containing only plasma were included. All plates were prepared in duplicate for both test organisms. *E. coli* seeded plates were incubated at 37°C for 24 h and *B. subtilis* seeded plates were incubated at 32°C for 24 h. Zones of inhibition were measured to the nearest 0.05 mm using dial callipers.

4.2.2.2 Preparation of low agar concentration and alkaline agar plates

Isotonic Sensi agar (BDH, Inc.), pH 7.4, was adjusted to pH 8.15 by the addition of 1.7 mL of sterile 1 N NaOH to 250 mL of autoclaved molten agar. Mueller-Hinton agar (Oxoid

Ltd.), pH 7.4, was adjusted to pH 8.13 by the addition of 3.1 mL of sterile 1 N NaOH to 250 mL of autoclaved molten agar. The agar concentration altered plates were prepared by the addition of 1% (w/v) agar (BBL) to Mueller-Hinton broth (Oxoid Ltd., pH 7.4). Mueller-Hinton agar consists of 1.7% (w/v) agar.

4.2.2.3 Effects of pH, agar concentration, diluent and pre-incubation on bioassay sensitivity

Agar content and pH adjusted plates were prepared as described in section 4.2.2.2 and seeded with 0.1 mL of *B. subtilis* spore suspension per 250 mL molten agar (50-55°C). The three types of plates, pH adjusted Isotonic Sensi and Mueller-Hinton agars and 1% agar Mueller-Hinton, were divided into three pre-incubation groups for antibiotic diffusion: 0 h, 2 h at 4°C and 2 h at 20°C. Within these pre-incubation groups, two diluents for sarafloxacin were examined. Two-fold dilutions of antibiotic ranging from 0.06 to 2.0 μg/mL were prepared in both sterile distilled water and Atlantic salmon plasma.

Well diffusion and disk diffusion techniques were also compared. Wells were prepared using the mould device as described previously (section 4.2.2.1) and were filled with the antibiotic solution. Sterile blank 6.5 mm diameter disks (Becton-Dickinson) were aseptically placed on the agar plates, in the hexagonal arrangement specified by the AOAC protocol (section 4.2.8). Twenty microlitres of the antibiotic stock

were added to each disk using a calibrated automatic pipette. After the specified pre-incubation period, plates were incubated at 32°C for 24 h. Zones of inhibition were measured to the nearest 0.05 mm using dial callipers.

4.2.3 Selection of fish isolates as test organisms

The bacterial isolates tested were *Aeromonas salmonicida* ATCC 14174 and *Vibrio anguillarum* ATCC 19264. Suspensions in saline (0.85% NaCl) for *V. anguillarum* and distilled water for *A. salmonicida* equivalent to a McFarland standard of 0.5 were used to swab agar plates. Isotonic Sensi agar (pH 7.4) and Antibiotic Medium #2 (pH 6.6) were used for *A. salmonicida* and Mueller-Hinton agar supplemented with 2% NaCl was used for *V. anguillarum*. Two-fold dilutions ranging from 0.0156 to 0.25 μg/mL of sarafloxacin and 0.0156 to 1.0 μg/mL of oxolinic acid were prepared in Atlantic salmon plasma. Sterile blank disks (6.5 mm diameter) were aseptically applied to the swabbed plates and 20 μL of antibiotic solution were applied to each disk using a calibrated automatic pipette. Plates were inverted and incubated at 30°C for 48 h. Zones of inhibition were measured to the nearest 0.05 mm using dial callipers.

4.2.4 Standardization of amount and method of application of antibiotic to disks

A suspension of *V. anguillarum* ATCC 19264 of an optical density (620 nm) of 0.1 was prepared in sterile saline. The suspension was diluted 1:10 in saline and used to swab 4 mm

thick Mueller-Hinton plus 2% NaCl plates. A sarafloxacin solution of 0.15 μg/mL and an oxolinic acid solution of 0.5 μg/mL were prepared in Atlantic salmon plasma. Sterile blank disks were aseptically positioned on the swabbed plates and either 10, 15 or 20 μL of antibiotic were applied using a calibrated automatic pipette. Another group of disks was dipped into the antibiotic solutions and pressed against the inside of the tube to remove excess fluid. The dipped disk was then placed onto the swabbed plate. The procedure was done in duplicate, with one set of plates incubated at 20°C for 48 h and an identical set of plates incubated at 30°C for 24 h.

4.2.5 Standardization of bacterial inoculum

A suspension of *Vibrio anguillarum* ATCC 19264 equivalent to an optical density (620 nm) of 0.6 was prepared in saline. The suspension was diluted in saline by 1:300, 1:325, 1:350 and 1:400. The following quantities of the diluted suspensions were swabbed onto Mueller-Hinton plus 2% NaCl plates (pH 7.4): 250, 300 and 350 μL. Disks dipped into a solution of 0.2 μg/mL sarafloxacin were placed on the plates. Plates were incubated at 30°C for 24 h and zones of inhibition measured with dial callipers.

4.2.6 Modification of the AOAC method

The AOAC microbiological method for the detection of antibiotics in feeds (Helrich, 1990) was adapted for use in this study. The AOAC method employs cylinders placed on the

agar plate surface and filled with test solutions. The method was modified to use disks which have been dipped into the test solutions. Six disks are applied to each plate in a hexagonal array. Three alternate disks contain the reference concentration and the other 3 disks contain the test solution. Three plates are set up for each test solution, producing 9 values for each solution tested.

The AOAC method uses agar plates seeded with an appropriate test organism. In this study the test organism, *V. anguillarum* ATCC 19264, was swabbed onto the surface of the agar plates. The bacterium was grown up in Mueller-Hinton broth plus 2% NaCl at 20°C for 48 h. A bacterial suspension in saline with an optical density (620 nm) of 0.6 was diluted to 1:350 and 250 μL were swabbed onto each Mueller-Hinton plus 2% NaCl plates.

4.2.7 Design and plotting of the standard response curve

Plates were prepared as described in section 4.2.6. Five concentrations of the antibiotic are required for a standard response curve: a reference concentration and 2 concentrations above and 2 concentrations below the reference concentration. On triplicate plates, the reference concentration is applied to three alternate disks and one of the other concentrations to the remaining three disks. This design is repeated for the 4 non-reference antibiotic concentrations, for a total of 12 plates. The average of the 36 readings of the zone diameters for the reference concentration from the 12 plates is called

the correction point for the standard response curve. For each antibiotic concentration a corrected zone diameter is calculated. The corrected value is calculated by subtracting the average of the 9 readings of the reference for those 3 plates from the correction point. The difference is added to the average of the 9 readings of the concentration being tested. This value is the corrected value for that concentration (Helrich, 1990). The corrected values for the zone diameters and the correction point are plotted on a semi-log graph using a logarithmic scale for the antibiotic concentration and an arithmetic scale for the corrected zone diameters. Graphs were done on Lotus Freelance Version 3.01.

Antibiotic concentrations used in the sarafloxacin standard response curve were 0.05, 0.1, 0.15, 0.2 and 0.25 μg/mL, with 0.15 μg/mL as the reference concentration. Plates were incubated at 30°C for 24 h. A second standard response curve for sarafloxacin was used for samples with antibiotic concentrations <0.05 μg/mL. The antibiotic concentrations used were 0.04, 0.1, 0.15, 0.2 and 0.25 μg/mL, with 0.15 μg/mL as the reference concentration. Plates were incubated at 20°C for 48 h. Antibiotic concentrations for the oxolinic acid standard response were 0.125, 0.175, 0.25, 0.5 and 0.75 μg/mL, with 0.25 μg/mL as the reference concentration. Plates were incubated at 20°C for 48 h. All dilutions of the 1280 μg/mL antibiotic stocks were made with Atlantic salmon plasma.

4.2.8 Determination of antibiotic concentrations in plasma

On triplicate plates, the reference concentration is applied to 3 alternate disks and the plasma sample to the other 3 disks. The reference point for the reference concentration is determined from the standard response curve. The correction point will be the same as the reference point if the correction point falls on the plotted curve. The corrected values for the plasma samples are calculated as follows. The average of the 9 readings for the reference concentration is subtracted from the average of the 9 readings for the plasma sample from those 3 plates. This difference is added to the reference point from the standard response curve, to give the corrected value for the plasma sample. The concentration of antibiotic in the plasma sample is determined from the standard response curve using the corrected value.

4.3 Results

Plates seeded with either *B. subtilis* spore suspension or *E. coli* were prepared as described in section 4.2.2, with wells formed by a mould (with or without a base agar layer) or by cutting with a cork borer (without a base agar layer). Sensitivities of the test organisms were 1.0 μg/mL and 2.0 μg/mL of oxolinic acid for *B. subtilis* and *E. coli* respectively. Control Atlantic salmon plasma did not produce any zone of inhibition.

Adjusting the pH of either Isotonic Sensi or Mueller-Hinton agars from 7.4 to 8.1, as described in section 4.2.2.2, did not increase the sensitivity of the bioassay for sarafloxacin using *B. subtilis* as the test organism. The same results were noted for Mueller-Hinton broth plus 1% agar. A pre-incubation period to allow for antibiotic diffusion into the medium at 4°C or 20°C for 2 h did not enhance sensitivity. Well diffusion and disk diffusion techniques were equally as effective, except with 1% agar medium, where wells were prone to collapse following removal of the mould. The sensitivity was 1.0 μg/mL for sarafloxacin diluted in Atlantic salmon plasma and 2.0 μg/mL for sarafloxacin diluted in distilled water.

The sensitivity of a bioassay using bacterial isolates from fish was performed as described in section 4.2.3. The sensitivities of *A. salmonicida* ATCC 14174 for oxolinic acid on Isotonic Sensi agar, pH 7.4, and Antibiotic Medium #2, pH 6.6, were >1.0 μg/mL and 0.5 μg/mL, respectively. The *V. anguillarum* bioassay had sensitivities of 0.5 μg/mL for oxolinic acid and 0.06 μg/mL for sarafloxacin on Mueller-Hinton agar plus 2% NaCl, pH 7.4.

The amount of sample applied to the disks did influence the zone diameter (Table XIII). The zone of inhibition increased with increasing volume of sample applied. One problem with the addition of 15 and 20 μL to the disk was a variation in the apparent amount of fluid absorbed by the

Table XIII. Influence of amount of sample applied to disks on the zone of inhibition

	Amount applied to disks	Mean Zone Diameter (mm) n=3
Sarafloxacin (0.15μg/mL)		
24 h at 30°C	dipped	15.65 ± 1.06
	20 μL	15.80 ± 1.59
	15 μL	13.60 ± 1.23
	10 μL	9.70 ± 1.06
48 h at 20°C	dipped	16.40 ± 1.95
	20 μL	16.60 ± 2.52
	15 μL	14.00 ± 1.28
	10 μL	12.00 ± 2.07
Oxolinic acid (0.5 μg/mL)		
24 h at 30°C	dipped	14.20 ± 1.21
	20 μL	13.50 ± 0.86
	15 μL	13.20 ± 1.51
	10 μL	8.80 ± 0.87
48 h at 20°C	dipped	18.05 ± 0.29
	20 μL	17.90 ± 0.17
	15 μL	15.40 ± 0.05
	10 μL	11.60 ± 0.21

disk. Frequently the sample would overflow and run off the disk, resulting in irregular shaped zones. Dipping of disks into the test sample generally produced more regular shaped zones of inhibition. Determination of the amount of plasma absorbed to dipped disks revealed a mean absorption of 25.4 ± 0.002 μg (n=12).

Table XIV shows the influence of the bacterial inoculum on the zones of inhibition for 0.2 μg/mL of sarafloxacin. All plates were evaluated on the degree of variability in zone diameters between plates and the ease of swabbing the stated quantities, the appearance of the bacterial lawn and the sensitivity level attained. Based on these factors, an inoculum dilution of 1:350 and the quantity of 250 μL were selected for the bioassay.

Standard response curves for sarafloxacin were set up on three separate occasions (section 4.2.7). Concentrations of sarafloxacin used were 0.05, 0.1, 0.15, 0.2 and 0.25 μg/mL, with 0.15 μg/mL as the reference concentration. Figure 12 is a plot of the three curves. The coefficients of determination (r^2) were 0.9936, 0.9954 and 0.9884 for curve #1, 2 and 3, respectively.

Table XIV. **Influence of bacterial inoculum on the zone of inhibition for 0.2 μg/mL of sarafloxacin**

Inoculum dilution	Amount swabbed	Mean zone diameter[a] (mm)
1:300	250 μL	14.95 ± 0.75
	300 μL	14.95 ± 1.45
1:325	250 μL	15.30 ± 0.10
	300 μL	16.17 ± 0.67
	350 μL	17.75 ± 0.30
1:350	250 μL	16.05 ± 0.15
	300 μL	15.22 ± 0.07
	350 μL	14.65 ± 1.55
1:400	250 μL	15.15 ± 0.30
	300 μL	14.95 ± 0.90
	350 μL	16.47 ± 0.02

[a] n=2; on 2 separate plates

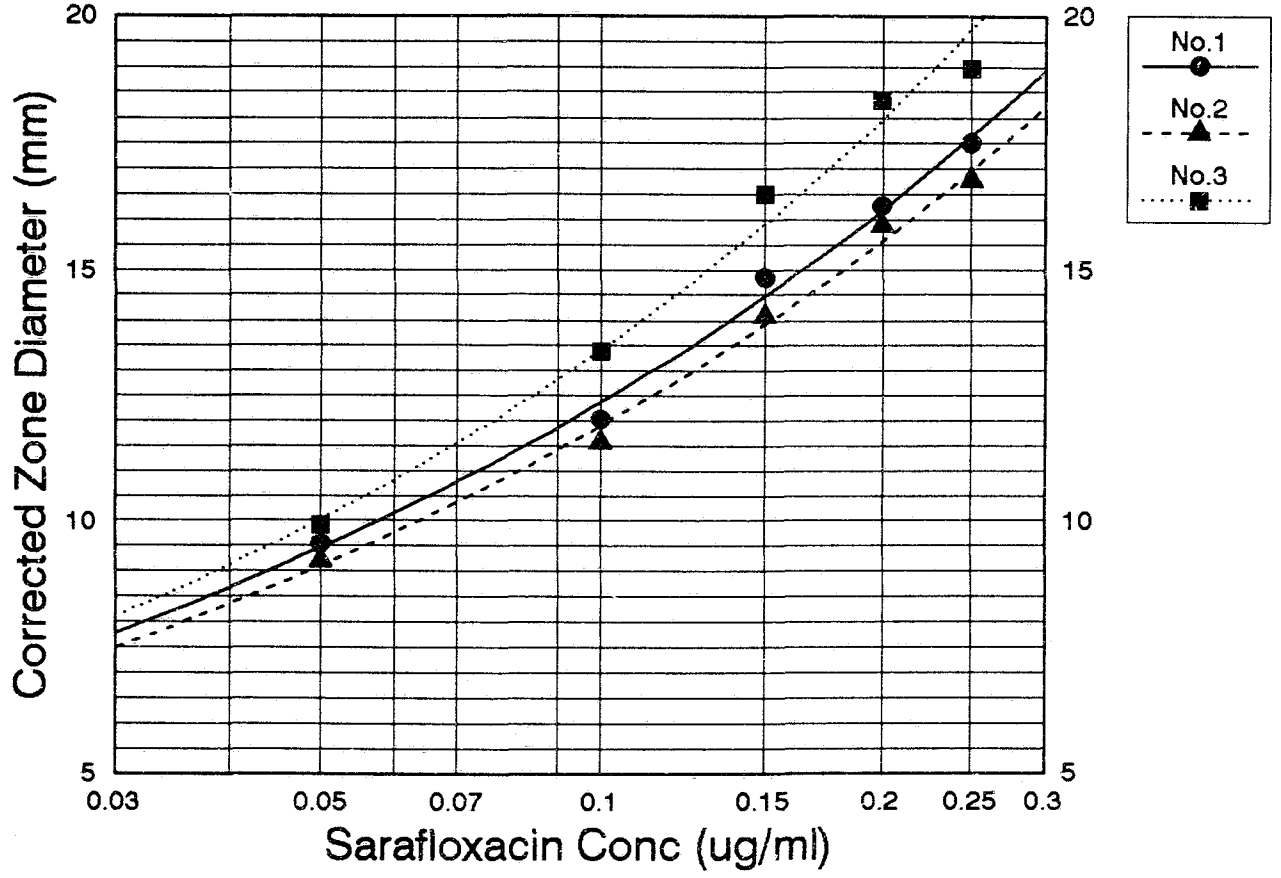


FIGURE 12: Sarafloxacin standard response curves

4.4 Discussion

B. subtilis ATCC 6633 and *E. coli* ATCC 25922 were initially selected for screening as test organisms in the bioassay. The AOAC method does not include guidelines for a microbiological assay for the detection of quinolones. *B. subtilis* is the test organism of choice for aminoglycoside assays and has the advantage of being available as a spore suspension of known concentration. Also, spores are relatively heat resistant and can be added directly to molten agar (50-55°C) in preparation of seeded plates necessary for well diffusion assays. *E. coli* ATCC 25922 is a standard control organism in MIC determinations. *E. coli* 4004 was used in a ciprofloxacin microbiological assay with a reported sensitivity of 0.03 μg/mL (Joos *et al*, 1985). The media, Antibiotic medium #8 and Base agar #2, were recommended by Difco and approved by the AOAC. The well-forming mould device proved to be a quick means of making wells in agar medium and eliminated the problem of tearing agar when using a cork borer.

Three types of plates were evaluated using both *B. subtilis* and *E. coli* as test organisms. Plates with wells produced by cutting with a cork borer contained no base layer. An initial concern was that the antibiotic solution might seep out underneath the agar layer, hence reducing sensitivity and reproducibility. This situation did not occur. Plates with wells produced using the mould contained

either an unseeded base layer or a seeded base layer. The antibiotic solution diffuses out from the well and into the agar, producing zones of inhibition of growth. As well, the solution diffuses down, as with antibiotic disks. Any decrease in assay sensitivity or distortion of the zone of inhibition would be observed in plates with a seeded base layer. The sensitivity of the assay was the same for all three plate types; 1.0 μg/mL for *B. subtilis* and 2.0 μg/mL for *E. coli*. The limits of sensitivity were not low enough for use in an antibiotic depletion phase study. *E. coli* was ruled out as a potential test organism.

Altering conditions of the culture medium was examined to enhance the sensitivity of the bioassay with *B. subtilis* as the test organism. The activity of quinolones is affected by magnesium ion (Mg^{2+}) concentration in the agar medium (Smith and Lewin, 1988). The media selected are all standardized to control for this effect. The pH of the medium was also shown to influence quinolone activity, as measured by MIC. Quinolones with a piperazine ring at carbon-7 (ciprofloxacin, norfloxacin, and ofloxacin) exhibited a decrease in activity as the pH of the medium was lowered; thus, these quinolones are least active when positively charged. Ciprofloxacin activity was enhanced 100-fold at pH 8.3, as compared to pH 5.6. Quinolones without a piperazine ring at carbon-7 (oxolinic acid, flumequine, nalidixic acid) exhibited an increase in activity as the pH of the medium was lowered; thus

activity is diminished when the drug is negatively charged (Smith and Lewin, 1988). The structures of oxolinic acid, sarafloxacin and ciprofloxacin are given in Appendix A. Sarafloxacin possesses a piperazine ring at C-7; hence, the pH of the medium was altered in attempt to increase its activity.

Isotonic Sensi agar and Mueller-Hinton agar were adjusted to pH of 8.15 and 8.13, respectively, by the addition of 1 N NaOH. Plates were also subjected to a period of pre-incubation for 0 h, 2 h at 4°C or 2 h at 20°C prior to incubation at 32°C for 24 h. The pre-incubation step permits the antibiotic to diffuse into the agar medium while growth of the test organism is limited by a less than optimum temperature; hence, larger zones of inhibition should result (Cooper, 1963). Agar content of one set of plates was adjusted to 1% from 1.7% for Mueller-Hinton agar to assess whether the size dependent inhibition of diffusion of the antibiotic was a limiting factor in bioassay sensitivity.

For all above mentioned situations, the sensitivity of the bioassay remained at 1.0 μg/mL of sarafloxacin when the drug was diluted in Atlantic salmon plasma. The sensitivity of the assay was 2.0 μg/mL when the sarafloxacin was diluted in distilled water. The fluorinated quinolones are not highly bound to serum proteins (approximately 30%) (Bergan, 1988); thus, the presence of serum or plasma should not decrease assay sensitivity. Sarafloxacin is relatively water insoluble, 1 g/L (Markwardt and Klontz, 1989) and requires

boiling to solubilize. The decrease in sensitivity of the assay when water was used to the antibiotic diluent could be related to its lower water solubility and the tendency of the drug to precipitate out when stored at 4°C and during freezing and thawing. Increasing the pH of the medium did not enhance sensitivity of the assay for sarafloxacin nor did reducing the agar concentration to 1% agar. Sarafloxacin at 420.87 Da (Abbott Laboratories) and oxolinic acid at 261.2 Da (Hirai *et al*, 1986) are relatively small molecules; thus, size should not be a limiting factor. The disc diffusion technique and well diffusion technique produced similar results. The one exception was with the 1% agar plates where wells tended to collapse after removal of the mould. Thus, it was concluded that *B. subtilis* was not sensitive enough to sarafloxacin for use in a depletion phase study.

Two bacterial isolates from fish were screened as potential test organisms. *Vibrio anguillarum* ATCC 19264 had a sensitivity of 0.06 µg/mL for sarafloxacin and 0.5 µg/mL for oxolinic acid on Mueller-Hinton agar plus 2% NaCl, pH 7.4. *Aeromonas salmonicida* ATCC 14174 had a sensitivity of 0.5 µg/mL for oxolinic acid on Antibiotic medium #2, pH 6.6, but the sensitivity was >1 .0 µg/mL on Isotoni Sensi medium, pH 7.4; thus, activity of the drug was enhanced at the lower pH. O'Grady *et al* (1988) developed a bioassay for the detection of flumequine levels in fish serum using well diffusion and plates seeded with *V. anguillarum* 775. The sensitivity of

their assay was 1.0 μg/mL. Attempting to seed agar with the fish isolates and maintain sensitivity limits required for a depletion phase study would be difficult because cells are thermosensitive. Accurate pour plates for bacterial counts of these isolates proved difficult due to the sensitivity of organisms to temperatures above 45°C, the minimum temperature to maintain most standard agar media in a molten state. Hence, plates swabbed with *V. anguillarum* ATCC 19264 were selected as the bioassay.

Table XIII shows the effect of adding various amounts of sarafloxacin solution (0.15 μg/mL) or oxolinic acid solution (0.5 μg/mL) to blank disks on the zone of inhibition. An increase in the zone diameter resulted from increasing the amount applied to the disks. Individual disks differed in the amount of sample they would readily absorb when applying 15 or 20 μL with a calibrated automatic pipette. Dipping the disk into the antibiotic solution generally produced more regular shaped zones. The incubation temperature influenced zone diameters as well. This effect was most pronounced for the oxolinic acid bioassay. Thus, incubations at 20°C for 48 h for oxolinic acid and 30°C for 24 h for sarafloxacin using disks dipped into the antibiotic solutions were selected as the assay conditions.

Plates swabbed with an inoculum standardized to an optical density (620 nm) of 0.1 exhibited great variation in uniformity of the lawn; thus, reproducibility of the assay was

compromised. To standardize the inoculum, cultures were adjusted to an optical density (620 nm) of 0.6 and several subsequent dilutions compared. Also, to minimize variation due to possible differences in the amount of inoculum absorbed to the swab when dipped into the suspension, various amounts of inoculum were applied to the plates, then swabbed over the surface of the plate. Disks dipped into a sarafloxacin solution (0.2 μg/mL) were placed onto the plates (Table XIV). Plates were evaluated on zone diameters, uniformity of the lawns, ease of spreading inoculum over the surface of the plates, and reproducibility. Subsequently, a dilution of 1:350 of the inoculum standardized to an optical density (620 nm) of 0.6 and a quantity of 250 μL applied to the plates were selected.

Standard response curves for sarafloxacin were determined on three separate occasions using the assay conditions established (Figure 12). The concentrations of sarafloxacin used were 0.05, 0.1, 0.15, 0.2 and 0.25 μg/mL, with 0.15 μg/mL as the reference concentration. Plate count data revealed $6x10^8$ CFU/mL at an optical density (620 nm) of 0.612; a further 1:350 dilution resulted in $1.7x10^6$ CFU/mL in the inoculum swabbed onto plates. A volume of 250 μL was applied to each plate; thus $4.25x10^5$ CFU were distributed over one plate. The lower limit of inoculum for diffusion bioassay has been reported as 10^3 CFU/mL (Cooper, 1963). By lowering the bacterial count of the inoculum, sensitivity is enhanced, but

relatively small variations in inoculum preparation show up as appreciable variations in the bacterial lawn and the reproducibility of the assay. Therefore, it was crucial to prepare the inoculum carefully with as little variation as possible. Based upon the variations seen in the sarafloxacin standard response curves (Figure 12), it was deemed necessary to determine a standard response curve every time a new inoculum was used in assaying the unknown samples. Samples that were below the sensitivity of this assay (<0.05 μg/mL) were re-assayed along with a second standard response curve. These plates were incubated at 20°C for 48 h and provided a limit of sensitivity of 0.04 μg/mL.

In summary, a bioassay for detection of sarafloxacin and oxolinic acid levels in Atlantic salmon plasma was developed using disk diffusion with *V. anguillarum* ATCC 19264 as the test organism. Sensitivity of the assay was 0.04 μg/mL for sarafloxacin and 0.125 μg/mL for oxolinic acid. The sensitivity level for sarafloxacin is within predicted values for the critical concentrations. Concentrations below the critical concentration do not produce zones of inhibition. This value is usually in the range of 2-4 times the MIC (Cooper, 1963). The MIC of sarafloxacin and oxolinic acid for the test organism is 0.0156 μg/mL (Table III). The critical concentration for sarafloxacin is approximately 2.6 times the MIC. The critical concentration for oxolinic acid is 8-fold the MIC for that particular isolate. The MIC_{50} at

30°C for the Atlantic and Pacific coast isolates of *V. anguillarum* is 0.0312 μg/mL (Tables I and II), 4-fold the critical concentration.

5. PILOT STUDY ON THE PLASMA CONCENTRATIONS OF OXOLINIC ACID AND SARAFLOXACIN IN ATLANTIC SALMON IN SEAWATER

5.1 Introduction

The pharmacokinetics of a drug describes the rate of processes of absorption, tissue distribution, metabolism and elimination. Knowledge of these processes is necessary to establish effective treatment regimes, to determine withdrawal times for fish to be used for human consumption and to identify metabolites which may be harmful to the environment or to the consumer. Although oxolinic acid has been used for the treatment of *A. salmonicida* and *A. hydrophila* infections in farmed fish in the United Kingdom (Tsoumas *et al*, 1989) and in Japan (Endo *et al*, 1973), limited information is available on its pharmacokinetics in fish (Endo *et al*, 1973; Hustvedt & Salte, 1991; Hustvedt *et al*, 1991; Jacobsen, 1989). The elimination half-life in rainbow trout (*Oncorhynchus mykiss*) was lower in seawater (29.1 h) than in freshwater (52.6 h) (Hustvedt and Salte, 1991) and the oral bioavailability of the drug was only 25% in Atlantic salmon in seawater (Hustvedt *et al*, 1991). The acute oral toxicity of oxolinic acid to carp was reported as >4000 mg/kg (Endo *et al*, 1973). Withdrawal times of 20 d (Jacobsen, 1989) and 30 d (Austin, 1985) have been recommended .

Sarafloxacin, a third-generation fluoroquinolone, is currently being examined for use in aquaculture. An

elimination half-life of 19.1 h was reported for rainbow trout tissues after an oral gavage of 10 mg/kg (Gingerich *et al*, 1989). Information regarding the pharmacokinetic properties of sarafloxacin is incomplete.

The objectives of this study were to determine the oral bioavailability of sarafloxacin and oxolinic acid in Atlantic salmon in seawater and to determine blood sampling times for the medicated feeding trial.

5.2 Materials and Methods

5.2.1 Fish source and acclimation

The fish species used in this study were Atlantic salmon (*Salmo salar*) smolts, St. John River strain, ranging in size from 400-600 grams. Fish were housed in a 3 tank recirculating system with 30-32 parts per thousand (ppt) salt. Flow rates were maintained to produce dissolved oxygen concentrations of >5 mg/L post-feeding and >8 mg/L during the non-feeding period. Water temperatures were maintained at 9 - 10°C and the photoperiod consisted of 12 h light and 12 h dark. Prior to moving into the 3 tank system, the fish were inventoried (weights and fork lengths recorded) and identified with a visible implant (VI) tag (Northwest Marine Technology, Inc., Olympia, Washington). Two of the 3 tanks were fitted with tank dividers. Fish were allocated to treatment group by randomizing the VI tag numbers. Fish were fed non-medicated

Atlantic salmon diet (Moore-Clark Co., Inc., Vancouver, B.C.) for 2 weeks prior to treatment.

5.2.2 Administration of antibiotics

Dosing of the fish was done either by intraperitoneal injection or by oral gavage. Treated fish received 10 mg of active drug per kg of body weight of either oxolinic acid (99.58% active drug) or sarafloxacin-HCl (829 μg/mg active drug). Dosing was performed as described in section 5.2.2.1 and 5.2.2.2.

The three tanks were identified according to treatment group. Tank 1 housed control fish. Tank 2 housed the oxolinic acid treatment group, intraperitoneal (IP) injection on side A and gavage on side B. Tank 3 housed the sarafloxacin treatment group, IP injection on side A and gavage on side B.

5.2.2.1 Intraperitoneal injection

Oxolinic acid and sarafloxacin stock solutions contained 10 mg of active drug per mL of phosphate buffered saline (PBS). Heating to boiling was necessary to dissolve sarafloxacin while a minimum amount of 0.1N NaOH was used to dissolve oxolinic acid. The dosage of 10 mg of antibiotic per kg of body weight was prepared for each fish. A 22 gauge (G) needle was fitted onto a 1 cc tuberculin syringe and the required volume of antibiotic solution was drawn into the syringe. The 22G needle was replaced with a 26G needle for injection. Fish were lightly anaesthetized with MS222 (tricaine methane sulphonate) (Atlantic Veterinary College

Teaching Hospital Pharmacy) prior to injection. The injection site was located immediately behind the pelvic fin. After injection, fish were placed in a recovery tank and observed closely until fully recovered. The time of injection was recorded for each fish to determine actual sampling time.

5.2.2.2 Oral gavage

Oxolinic acid and sarafloxacin slurries consisting of 10 mg active drug/mL were prepared as follows. The sarafloxacin slurry was prepared by the addition of 100 mg of active drug and 0.1 g of gelatin (Knox brand) to 10 mL of deionized water and heated to boiling which is required to dissolve both sarafloxacin and gelatin. Three or four drops of green food colouring were then added to the sarafloxacin slurry. The oxolinic acid slurry was prepared as follows. The gelatin (0.1 g) was dissolved in 8 mL of deionized water by heating to boiling. A few drops of 0.1 N NaOH were used to dissolve the oxolinic acid (100 mg of active drug). The dissolved oxolinic acid was then added to the cooled gelatin solution and the total volume made up to 10 mL. Three or four drops of blue food colouring were added to the oxolinic acid slurry.

Tygon™ tubing with an inner diameter of 1/8 inch (3.2 mm) and outer diameter of 1/4 inch (6.35 mm) was cut into lengths of approximately 15 cm. The tubing was connected to the end of a 1 cc tuberculin syringe and the measured amount of antibiotic slurry was drawn into the tubing. For example, a 500 g fish would receive 0.5 mL of slurry. The pieces of

tubing were labelled with the fish's VI tag number and refrigerated overnight. The tubing was allowed to warm to room temperature prior to dosing. A 3 cc syringe filled with air was attached to the end of the tubing prior to dosing. The fish were lightly anaesthetized with MS222 and the tubing inserted into the fish's esophagus. The slurry was expelled from the tubing by pushing the air out of the syringe. The fish was placed in a recovery tank and observed closely until fully recovered. The time of dosing was recorded to determine the actual sampling time.

5.2.2.3 Controls

Control fish were treated with a placebo to mimic possible handling stress effects and to examine possible inhibitory compounds in plasma. Fish were lightly anaesthetized with MS222. One-half of the control fish received a 1 mL intraperitoneal injection of PBS and the remainder received an oral gavage of 1 mL of a 1% gelatin solution. Dosing times were recorded.

5.2.3 Sampling times and plasma collection

The approximate sampling times for the treatment groups were as follows. Oral gavage groups were sampled at 3, 6, 12, 24, 36 and 60 h after treatment. Intraperitoneal injection groups were sampled at 1, 3, 6, 12, 24 and 48 h after treatment. Control fish were sampled at 6, 12 and 24 h after receiving the placebo. Times of blood sampling were recorded

and actual sampling times were determined for each fish. Usually, 2 fish were sampled per sampling time.

Blood samples were collected by either cardiac puncture or from the dorsal aorta after stunning the fish by a blow to the dorsal cranium, as specified by the Canadian Council on Animal Care (CCAC) guidelines (1984). Blood samples were collected in 3 mL lithium heparin vacutainers and fish were euthanized by exsanguination. The blood samples were centrifuged in a Beckman Model TJ-6 centrifuge at 3000 rpm for 10 min. The plasma was collected, then frozen at -70°C in 1.5 mL Eppendorf tubes identified by the fish's VI tag number. After blood collection, fish were examined for injuries at the injection site or for the presence of gelatin in the gut.

5.2.4 Microbiological assay

The microbiological assay was performed with *Vibrio anguillarum* ATCC 19264 as the test organism on Mueller-Hinton agar plus 2% NaCl as described in section 4.2.6.

5.3 Results

Plasma concentrations of sarafloxacin after a single oral dose of 10 mg sarafloxacin per kg of body weight are given in Table XV. Antibiotic concentrations were less than the limits of sensitivity of the assay (< 0.04 μg/mL) commencing at the 24 h sampling. Necropsies indicated that the gelatin was not well absorbed from the gastrointestinal (GI) tract. Gelatin could be detected in the lower stomach and upper intestine in

Table XV. Plasma antibiotic concentrations after receiving a single oral dose of sarafloxacin (10 mg/kg)

Sampling time (h,min)	Dose vol received[a] (mL)	Sarafloxacin concentration (μg/mL)
3,20	0.64	0.08
3,38	0.62	0.19
5,51	0.70	0.17
6,09	0.46	< 0.04
11,58	0.52	0.07
12,16	0.60	0.20
23,50	0.45	< 0.04
24,12	0.65	< 0.04
34,00	0.46	< 0.04
34,25	0.50	< 0.04
36,19	0.67	< 0.04
60,18	0.44	< 0.04

[a] based on 1 mL dose required

fish sampled at 3 h post-treatment and in the lower intestine at 6 and 12 h post-treatment. Fish sampled at 24, 36 and 48 h did not contain any gelatin in the GI tract. Plasma concentrations of sarafloxacin after a single IP injection of 10 mg/kg of body weight are given in Table XVI. Antibiotic concentrations were above the limit of sensitivity of the assay in all fish sampled from 1 to 48 h post-treatment.

Plasma concentrations of oxolinic acid after a single IP injection of 10 mg/kg of body weight are presented in Table XVII. Only one sample (24 h 10 min post-treatment) had antibiotic concentrations below the limit of sensitivity for the assay (< 0.125 μg/mL).

The plasma concentration of oxolinic acid after a single oral dose of 10 mg/kg of body weight was determined in only the first fish sampled (3 h 5 min post-treatment). The concentration of oxolinic acid was 0.167 μg/mL in this sample. All subsequent samples had concentrations below the limits of sensitivity for the assay (<0.125 μg/mL). Gelatin was present in the lower stomach and upper intestine at 3 h post-treatment and in the lower intestine at 6 and 12 h post-treatment. A small amount of gelatin was observed in the distal intestine at 24 h and near the vent at 36 h post-treatment. No gelatin remained in the GI tract at 60 h post-treatment.

Table XVI. Plasma antibiotic concentrations after receiving a single intraperitoneal injection of sarafloxacin (10 mg/kg)

Sampling time (h,min)	Sarafloxacin concentration (μg/mL)
1,43	1.38
2,39	4.88
3,37	0.96
5,44	1.72
6,06	8.00
12,27	2.70
12,51	1.03
23,53	0.35
23,58	0.43
24,19	0.96
48,48	1.21
49,06	1.16

Table XVII. Plasma antibiotic concentrations after a single intraperitoneal injection of oxolinic acid (10 mg/kg)

Sampling time (h,min)	Oxolinic acid concentration (μg/mL)
1,18	2.36
1,19	2.22
3,13	1.36
3,25	3.03
5,42	1.36
6,25	1.85
12,15	1.16
12,30	2.18
23,41	0.32
24,10	< 0.125
24,30	0.79
47,51	0.41
48,70	0.47

5.4 Discussion

Oral bioavailability is the fraction of dose reaching the systemic circulation and is calculated by comparing the total area under the curve (AUC) of serum concentration-time (C-T) curves for a single intravenous (IV) dose and a single oral dose. The bioavailability of an IV dose is 100% (Bergan, 1991). In the study herein, a single IP injection replaced the IV injection due to the difficulty in accurate IV injection of fish; thus, providing an estimate of bioavailability. Hustvedt *et al* (1991) reported a bioavailability of oxolinic acid of 47% in Atlantic salmon in 9°C seawater after a single IP injection of 20 mg/kg. Peak serum concentrations (C_{max}) of 25.9 μg/mL and 69.6 μg/mL were reported after IP and IV injection, respectively. The time to reach C_{max} (T_{max}) was 0.5 h after IP injection (Hustvedt *et al*, 1991), indicating an absorptive phase.

The bioavailability of both sarafloxacin and oxolinic acid appears to be low (Tables XV, XVI, XVII) when administered as described in section 5.2.2. Calculation of bioavailability is confounded by problems in dosing procedures. The gelatin used to produce a slurry for the oral gavage was poorly digested. Sufficient quantities of gelatin were present to follow its passage through the GI tract. The poor digestion and subsequent absorption of gelatin could partly explain the observed low oral bioavailability of the antibiotics, particularly if the drugs bind to gelatin.

Generally, fluorinated quinolones are not highly bound to serum proteins (approximately 30%) (Bergan, 1988) and protein binding for the third-generation fluoroquinolones has been reported as low as 10% (Siporin, 1989). Specific data on oxolinic acid and sarafloxacin are not available. Information on binding of quinolones to gelatin, a proteinaceous material, is also unavailable. Gelatin capsules are commonly used in the dosing of various drugs given as oral medication; therefore, gelatin was selected as the suspending medium. Gelatin capsules were used to administer oxytetracycline in a study on serum antibiotic concentrations in sockeye salmon (Strasdine and McBride, 1979). As well, Herman *et al* (1969) used a 5% gelatin slurry containing oxytetracycline to coat dry pellets in a study on antibiotic concentrations in tissues and plasma of rainbow, brown and brook trout (*Salvelinus fontinalis*). However, concentrations of antibiotic were not assayed in the gelatin remaining in the GI tract of the fish.

Food colouring was added to the gelatin slurry in the current study (green for sarafloxacin and blue for oxolinic acid) for easy visualization in the GI tract. Also, food colouring permitted one to observe whether fish vomited after oral gavage. No fish were observed to vomit. It is not known whether antibiotics could adhere to constituents of the food colouring, although this is unlikely to be a problem due to the water solubility of the food colouring.

An additional problem was noted with using gelatin to produce the slurry. A 1% gelatin solution was selected after testing various gelatin concentrations (0.5 to 2%) for the ability to produce a semi-solid slurry that could be drawn into Tygon™ tubing, yet not readily spill out. Although the 1% gelatin solution in water produced the desired slurry, the addition of either oxolinic acid or sarafloxacin resulted in the slurry no longer being semi-solid; thus, the slurry would easily pour out of the tubing. Therefore, the tubing containing the slurry was refrigerated overnight. After refrigeration, the tubing was allowed to warm to room temperature prior to dosing. After dosing, gelatin remained stuck to the inside surface of the tubing; thus, the fish did not receive the entire calculated dose. The tubing plus remaining gelatin was weighed, the tubing cleaned and dried and reweighed to estimate the percentage of dose delivered. The percentage of dose delivered was estimated as between 32.3% to 71.9% (mean of 57.6%) for oxolinic acid and 43.5% to 67.2% (mean of 55.9%) for sarafloxacin.

Neither sarafloxacin or oxolinic acid were readily soluble at a concentration of 10 mg/mL. The solubility of sarafloxacin has been reported as 1 g/L (1 mg/mL) (Markwardt and Klontz, 1989). Gelatin slurries were kept on a rotary shaker to keep the antibiotic as evenly distributed as possible until dispensed into the tubing. Precipitated

antibiotic was observed in the gelatin remaining in the tubing after dosing.

Sarafloxacin and oxolinic acid were prepared in phosphate buffered saline (PBS) in sterile serum vials for IP injections. The problem of drug solubility was more noticeable. Sarafloxacin produced a flocculant precipitate that was relatively easy to keep in suspension by shaking. Oxolinic acid precipitated rapidly and was difficult to keep in suspension. Also, a small amount of precipitated oxolinic acid remained in the base of the needle after injection.

Considering problems with dosing procedures, the bioavailability of sarafloxacin and oxolinic acid still appears to be low. Bioavailability calculations were not performed because of dosing problems and variability in the data set; hence, validity of the results would be questionable. Also, using IP dosing instead of IV dosing may not provide an accurate estimation of bioavailability. Hustvedt *et al* (1991) reported a bioavailability for oxolinic acid of 47% in Atlantic salmon after a single IP injection of 20 mg/kg. Limited pharmacokinetic information is available on the newer quinolone sarafloxacin. An elimination half-life ($t_{1/2}$) of 19.1 h was reported for sarafloxacin in rainbow trout tissue after a gavage of 10 mg/kg of body weight (Gingerich *et al*, 1989). Hustvedt *et al* (1991) reported a peak serum oxolinic acid concentrations of 2.5 μg/mL at 3.9 h after a single oral dose of 10 mg/kg in Atlantic salmon held in

seawater. Low bioavailability (25%) was suggested as due to either malabsorption or metabolism in the gut epithelium or liver. The suspending agent, carboxymethyl cellulose, reportedly reduced the fraction of dose available in the serum by 10 - 20%, when compared to dosing with an oxolinic acid solution (Hustvedt *et al*, 1991). In the current study, after a single oral dose of 10 mg/kg of body weight, oxolinic acid was detected in only the first fish sampled (3 h, 5 min). The appreciably lower value of 0.17 μg/mL, as compared to that of Hustvedt *et al* (1991), and failure to detect oxolinic acid in any other fish, appeared to stem from problems in the dosing procedure described previously, as well as the sensitivity of the assay (0.125 μg/mL). The first sampling time of 3,05 h may have been the peak value, but subsequent concentrations were all below the limit of sensitivity of the assay (<0.125 μg/mL). Peak serum concentrations occur within 1 to 2 h after oral administration of fluoroquinolones and, in general, a linear relationship exists between dose and serum concentration (Bergan ,1988). Assuming the same situation for oxolinic acid and considering the estimated value for the percentage of dose received (54.1%), the antibiotic concentration for 3,05 h is 0.26 μg/mL, 10-fold lower than the value reported by Hustvedt *et al* (1991). Apparently the main problem in dosing was the use of gelatin and its poor digestion and absorption in the GI tract.

Hustvedt *et al* (1991) reported an elimination half-life ($t_{1/2}$) of 60.3 h and an apparent volume of distribution at steady state (Vd_{ss}) of 1.8 L/kg for oxolinic acid after an intravenous injection of 20 mg/kg. The peak serum concentration of 25.9 μg/mL was reported at 0.5 h after an IP injection of 20 mg/kg with a bioavailability of 47%. In the current study, a plasma concentration of 2.24 μg/mL was obtained at 1 h post-injection of 10 mg/kg (Table XVII). At the last sampling time of 48 h, mean plasma concentration of 0.44 μg/mL was obtained. Problems with low solubility of the antibiotic appeared to prevent the full dose from being systemically available. An alternate explanation for the low plasma concentrations involves the Vd of oxolinic acid. Vd estimates the extent of drug distribution as the ratio of drug concentration in the body to serum drug concentration and is calculated from the C-T curve (Bergan, 1981). Thus, a Vd of 1.8 L/kg indicates a higher concentration of drug in tissues than in serum.

Plasma samples were stored at -70 °C for 7 months before assaying. A study on oxytetracycline concentrations in rainbow trout indicated that the storage of tissues for 23 weeks at -20 °C did not have a significant effect on antibiotic concentrations (Herman *et al*, 1969). Also, no difference was noted between plasma and serum (Herman *et al*, 1969), although serum may be preferable for microbiological assays due to possible interference by anti-coagulants (Reeves

and Wise, 1978). Larger quantities of plasma can be recovered than serum.

In conclusion, both sarafloxacin and oxolinic acid appear to have low bioavailabilities when prepared and administered as described herein. Detailed pharmacokinetics was not possible due to problems in the administration of required doses, namely low solubility of antibiotics and poor digestion and absorption of gelatin. As well, only 2 fish were sampled per sampling time; thus, biological variation between fish was not controlled. A sampling number of 5 per sampling time or multiple sampling from the same fish would have been preferable. Information from the pilot study was not used to determine sampling times in the feeding trial.

6. PLASMA CONCENTRATIONS OF OXOLINIC ACID AND SARAFLOXACIN IN ATLANTIC SALMON IN SEAWATER

6.1 Introduction

Vibriosis, caused by *Vibrio anguillarum* and *Vibrio ordalii*, and furunculosis, caused by *Aeromonas salmonicida*, manifest as septicemia and bacteremia in acute disease and frequently cause high mortalities in fish (McCarthy, 1975; Roberts and Shepherd, 1986). Resistance to antimicrobial agents such as oxytetracycline and sulfonamides has resulted in the pursuit of more effective drugs including quinolone antibiotics. Oxolinic acid, a second-generation quinolone, has been used extensively in Japan (Endo *et al*, 1973) and the United Kingdom (Tsoumas *et al*, 1989) for treatment of these diseases. Sarafloxacin, a third-generation fluoroquinolone, is a promising new drug still in the experimental phase of licensing for use in aquaculture. The most common and economically feasible means of administering antibiotics to fish is by medicated feed. The recommended treatment regime for oxolinic acid is 10 mg/kg body weight per day for 10 days (Roberts and Shephard, 1986). The recommended treatment regime for sarafloxacin is 10 mg/kg body weight per day for 5 days (Abbott Laboratories).

The objectives of this study are threefold. 1) To determine plasma concentrations of sarafloxacin-HCl and oxolinic acid in Atlantic salmon in 10°C seawater during the

recommended oral treatment regimes. 2) To compare plasma antibiotic concentrations to MICs for sarafloxacin and oxolinic acid against *A. salmonicida* and *Vibrio* spp. 3) To determine the length of time plasma antibiotic concentrations are maintained at 3-5 times the MIC after cessation of treatment.

6.2 Materials and Methods

6.2.1 Fish source and acclimation

Fish used in this study were Atlantic salmon (*Salmo salar*) smolts, St. John River strain, from certified disease free stock, ranging in weight from 150-300 g (average weight of 230 g).

Fish were obtained as pre-smolts, inventoried (weights and fork lengths recorded) to determine size distribution and body condition (K-factor) and tagged with V.I. (visible implant) tags (Northwest Marine Technology, Inc., Olympia, Washington). Fish were allocated to tanks in a 3 tank freshwater recirculating system (tank size of 1 metre diameter) by randomizing V.I. tag numbers. Water flow rates provided dissolved oxygen concentrations of > 5 mg/litre post-feeding and ≥ 8 mg/litre during the non-feeding period. Photoperiod was 12 h light and 12 h dark. Fish were gradually acclimated to seawater (recirculating system, salinity of 28-32 ppt, temperature 10 ± 2°C). During the acclimation period, all fish were fed a non-medicated Atlantic salmon diet (3.5 mm

pelleted New Age Extruded, Moore-Clark Co., Inc., Vancouver, B.C.). Mortalities were monitored and post-mortem examinations were performed on all fish.

6.2.2 Acclimation to seawater

Smoltification of the Atlantic salmon was achieved by adjusting photoperiod, gradually increasing salinity and altering cycles of high and low salinity. Eight days after fish were moved into the three tank freshwater recirculating system, lights were programmed to turn on 5 min earlier and turn off 5 min later each day to mimic increasing day length. The module had been previously programmed to provide 12 h and 10 min of light per day. Natural day length was reported as 13 h and 45 min at this time (information provided by Environment Canada). On Day 22, an artificial seawater mix, Instant Ocean® (Aquarium Systems Inc., Mentor, Ohio), was added to the reservoir until an initial salinity of 13 parts per thousand (ppt) was achieved. By Day 23, salinity was recorded as 19.5 ppt.

On Day 29, higher than desirable levels of ammonia and nitrite were detected (>1.0 mg/L and >10.0 mg/L respectively). Tanks were flushed with freshwater, reducing salinity to 9.5 ppt. Instant Ocean® was added to the reservoir, bringing the salinity up to 14.5 ppt. This cycle of decreasing and increasing salinity was continued up to Day 33. At this point levels of ammonia, nitrite and nitrate were within acceptable

levels (< 0.1 mg/L, < 0.1 mg/L, < 20 mg/L respectively) (Spotte, 1979).

On Day 33, lights were programmed to turn on 1 min earlier and turn off 1 min later each day. At this point the module was receiving 16 h and 20 min of light. From Day 53 to Day 64 salinity was maintained at 28.5 - 30 ppt. On Day 65 salinity was reduced to 24.75 - 26 ppt because several fish in Tanks 1 and 3 were disoriented and lying on the bottom of the tank. On Day 74, lights were programmed to maintain 17 hours and 40 minutes of light. From Day 75 onward, salinity was maintained at 28.5 - 32 ppt.

6.2.3 Administration of antibiotics

Each tank was identified according to treatment group and received 50 fish previously randomized by V.I. tag number. Test diets were prepared by Dr. S. Lall, Department of Fisheries and Oceans, Halifax, Nova Scotia, to provide a dose rate of 10 mg/kg body weight of sarafloxacin-HCl or oxolinic acid when fed at a rate of 1.5% of tank biomass per day. Feed was stored in air-tight containers and frozen at -20°C. Feed consumption rates for individual tanks were determined by recording daily consumption of the control diet for 1 week prior to commencement of treatment. The tank biomass was determined for each tank by the double bucket weighing method (Appendix H). Fish were acclimated to the control diet for 14 d at a feeding rate of 1.5% of tank biomass per day. Subsequent to acclimation, fish in treatment tanks were fed

medicated diets at a rate of 1.5% of tank biomass per day for 5 d for sarafloxacin and for 10 d for oxolinic acid and control diet. Following completion of the treatment period, control diet was fed for the remainder of depletion study. At completion of the study, any remaining feed was destroyed by incineration.

6.2.4 Sampling Times and Plasma Collection

Day 0 samples (5 fish from each of oxolinic acid and sarafloxacin treatment groups and 2 control fish) were taken 12 h before first feeding of medicated diets. During the 5 d treatment regime for sarafloxacin, fish were sampled at 0.5, 1 and 2 d, with 5 fish being sampled per sampling time. During the 10 d treatment regime for oxolinic acid, fish were sampled at 1, 2 and 5 d, with 5 fish being sampled per sampling time. Fish were fed immediately prior to sampling to ensure that the fish would consume the daily allotment of diet in one feeding. Time elapsed between feeding and blood sampling was 20-30 min. On day 5 (sarafloxacin treatment group) and day 10 (oxolinic acid treatment group), samples were collected 6 and 12 h after the medicated feeding. The remainder of the depletion phase sampling times were at 24, 48, 72 and 96 h after the last medicated feeding. Control fish were sampled at a rate of 2 per time interval.

Blood samples were obtained after stunning the fish by a blow to the head, as specified by CCAC guidelines (1984). Blood was collected from the caudal vessels with 1cc, 3cc

and/or 5cc heparinized syringes (30 mg/mL of lithium heparin solution in distilled water) with 22G needles. Fish were euthanized by exsanguination. Blood was centrifuged (2000 rpm for 10 min) in a Beckman Model TJ-6 centrifuge and the plasma collected. Plasma samples were stored at -70°C in 1.5 mL Eppendorf microcentrifuge tubes individually identified by the fish's assigned sampling number and the sampling time. After blood collection, fish were weighed and examined for presence of feed in the gastrointestinal tract. Tank biomass calculations were adjusted after each sampling time.

6.2.5 Microbiological assay

The microbiological assay was performed with *Vibrio anguillarum* ATCC 19264 as the test organism on Mueller-Hinton agar supplemented with 2% NaCl as described in section 4.2.8. The limits of sensitivity for the assays were 0.04 μg/mL for sarafloxacin and 0.125 μg/mL for oxolinic acid.

6.3 Results

Mean plasma concentrations of sarafloxacin during and following a 5 d medicated feeding regime are presented in Table XVIII. Plasma samples were collected on days 0.5, 1 and 2 during the 5 d treatment regime and for up to 96 h post-treatment. At 12 h (0.5 d) after the first sarafloxacin medicated feeding, mean plasma concentration was 0.12 μg/mL, as shown in Figure 13 and Table XVIII. Blood collection was done 24 h after feeding on days 1 and 2; therefore, these

Table XVIII. Plasma concentration of sarafloxacin during and after a 5 day medicated feeding regime.

Sampling time	Mean concentration (μg/mL)	n
During Treatment		
0.5 day	0.12 ± 0.02	4
1.0 day	0.14 ± 0.03	3
2.0 day	0.14 ± 0.06	3
Post-Treatment		
6 h	0.19 ± 0.07	5
12 h	0.18 ± 0.04	5
24 h	0.10 ± 0.03	4
48 h	0.07 ± 0.04	5
72 h	< 0.04	5
96 h	< 0.04	5

values represent the trough concentrations. Mean sarafloxacin concentrations were 0.14 μg/mL on days 1 and 2.. Figure 13 shows the trough plasma concentration of sarafloxacin or the minimum concentration of antibiotic maintained in the plasma during treatment. The mean plasma concentration at 24 h after the last medicated feeding was somewhat lower at 0.10 μg/mL. The data for this sampling time were variable, but did not permit any values to be eliminated (0.06, 0.13, 0.06 and 0.13 μg/mL). Figure 14 shows the 6 and 12 h post-feeding concentrations and initial depletion of sarafloxacin from the plasma. Mean concentrations at 6 h post-feeding (0.19 μg/mL) decreased slightly by 12 h (0.18 μg/mL). The mean plasma concentration at 48 h post-treatment was 0.07 μg/mL. Plasma sarafloxacin concentrations were below the limit of sensitivity of the assay (<0.04 μg/mL) at 72 h post-treatment.

Mean plasma concentrations of oxolinic acid during and following a 10 d medicated feeding regime are given in Table XIX. Blood samples were collected on day 1, 2 and 5 during the 10 d treatment regime and for up to 96 h post-treatment. The mean plasma concentration of oxolinic acid at 12 h after the first medicated feeding was 0.4(μg/mL, as shown in Figure 15 and Table XIX. Blood samples were collected at 24 hours after feeding on days 1, 2 and 10. Mean oxolinic acid concentrations were 0.61, 0.62 and 0.68 μg/mL on days 1, 2 and 10, respectively (Figure 15). Figure 16 shows the 6 and 12 h post-feeding concentrations and initial depletion of oxolinic

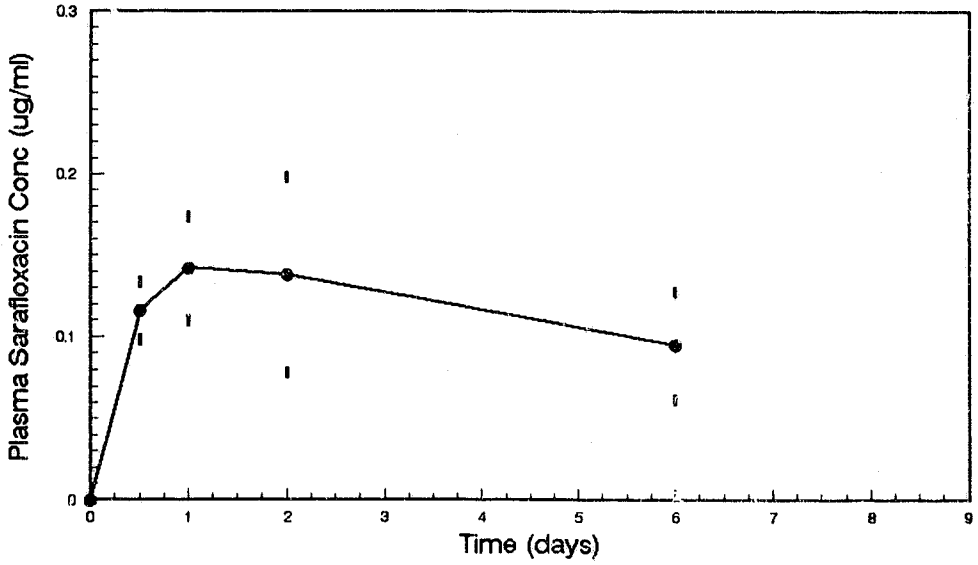


Figure 13: Mean plasma concentrations of sarafloxacin during treatment

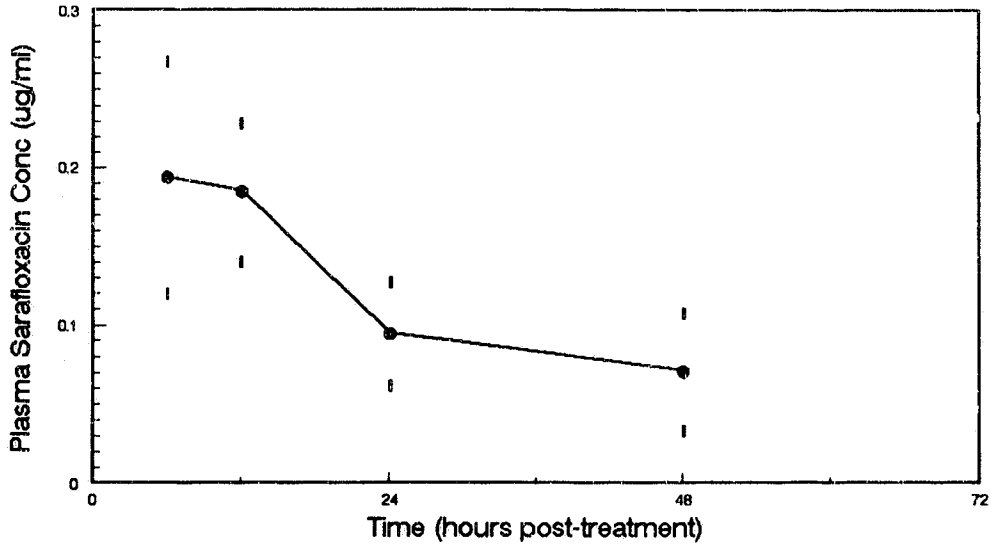


Figure 14: Mean plasma sarafloxacin concentrations at 6 and 12 h post-treatment and during depletion

Table XIX. Plasma concentration of oxolinic acid during and after a 10 day medicated feeding regime.

Sampling time	Mean concentration (µg/mL)	n
During Treatment		
1.0 day	0.49 ± 0.06	3
2.0 day	0.61 ± 0.16	4
5.0 day	0.62 ± 0.14	4
Post-treatment		
6 h	0.93 ± 0.10	5
12 h	0.92 ± 0.25	4
24 h	0.68 ± 0.16	5
48 h	0.34 ± 0.10	5
72 h	0.24	1
96 h	< 0.125	5

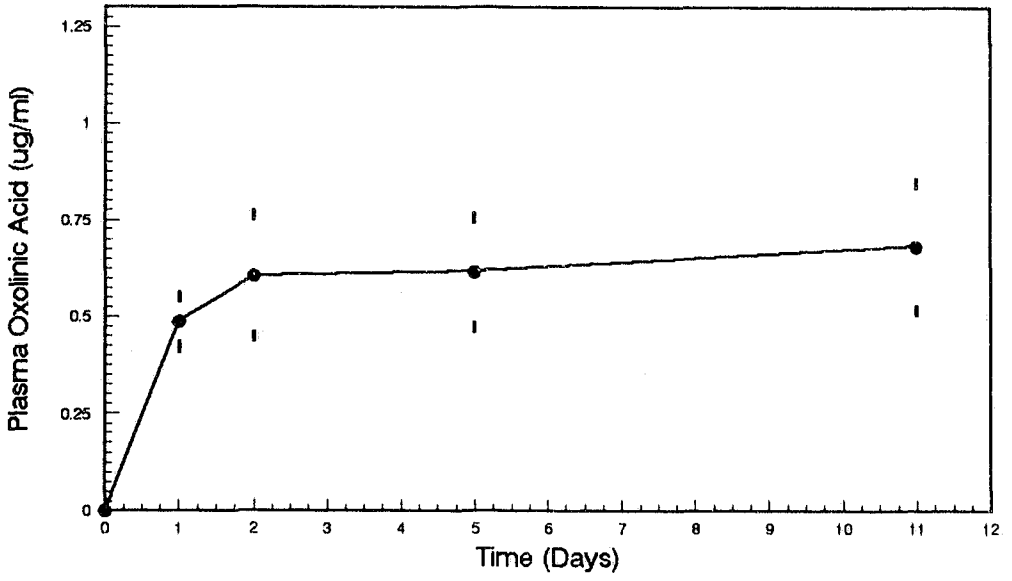


Figure 15: Mean plasma concentrations of oxolinic acid during treatment

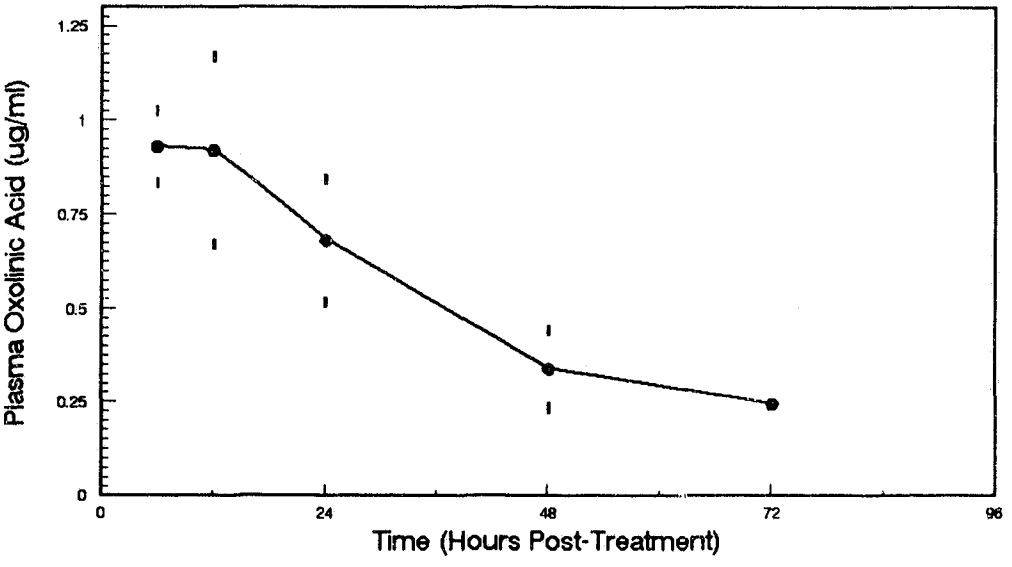


Figure 16: Mean plasma oxolinic acid concentrations at 6 and 12 h post-treatment and during depletion

acid from the plasma. Mean concentrations at 6 h post-feeding (0.93 μg/mL), decreased slightly by 12 h (0.92 μg/mL). Plasma concentrations of oxolinic acid decreased to 0.34 μg/mL at 48 h post-treatment and only one of the 5 fish sampled at 72 h had an antibiotic concentration detectable by the assay (0.24 μg/mL). Oxolinic acid concentrations were below the limit of sensitivity of the assay (<0.125 μg/mL) at 96 h post-treatment.

6.4 Discussion

Medicated feeds containing either oxolinic acid or sarafloxacin were prepared to provide a dose rate of 10 mg/kg body weight/day, based on a daily feeding rate of 1.5% of tank biomass. Tank biomasses were determined by the double bucket weighing technique. Following sampling, fish were individually weighed, and based on these weights, the actual feeding rate was 1.1-1.2% of the tank biomass. The discrepancy was due to the double bucket weighing being performed one month prior to commencing medicated feeding. Thus, the actual dose rate was 7.3-8 mg/kg body weight/day. Necropsies performed after sampling indicated that the smaller fish did receive feed as well. The fish that were not on feed and appeared to have not been eating for some time were not used in the biomass calculations. As expected, these fish did not have detectable plasma concentrations of antibiotic.

The daily allocation of diet was consumed in one feeding, to increase the probability that subordinate fish received

feed following satiation of aggressive feeders. Feeding was done immediately prior to sampling when the fish were not disturbed and willing to eat. Therefore, during the treatment phase, samples were taken 24 h after the last medicated feeding. Antibiotic concentrations in the plasma for these samples most likely represent the trough concentration because of the relatively short period of time that elapsed from feeding to blood sampling (20-30 min), coupled with the rate of absorption of quinolones. The time to peak serum concentration (T_{max}) of oxolinic acid in Atlantic salmon in seawater was 3.9 h after a single oral dose of 10 mg/kg (Hustvedt *et al*, 1991).

As a general guideline, plasma antibiotic concentrations of 3 to 5 times higher than the MIC are recommended for effective treatment (Washington, 1985). These recommendations were developed for antibiotic therapy in homeotherms, but were used as a basis of evaluation in this study. Both vibriosis and furunculosis manifest as septicemia and bacteremia in acute disease (McCarthy, 1975; Roberts and Shepherd, 1986); thus, it is important to maintain effective concentrations of antibiotics in blood.

The MIC_{50} for sarafloxacin against *Vibrio anguillarum* and *Vibrio ordalii* is 0.0156 μg/mL (Tables I and II). Based on the recommended plasma concentration of 3 to 5 times the MIC, concentrations of 0.047 to 0.078 μg/mL are required. These concentrations were surpassed during medication, with mean

trough concentrations of 0.14 and 0.10 μg/mL (Figure 13) and maintained for up to 48 h post-treatment (0.07 ± 0.04 μg/mL) (Figure 14). The MIC_{50} for sarafloxacin against *Aeromonas salmonicida* is 0.0625 μg/mL at 20°C (Tables I and II). Therefore, concentrations of 0.188 to 0.312 μg/mL are recommended for effective treatment. The lower limit of this desired range was achieved only at 6 to 12 hours after treatment (0.19 and 0.18 μg/mL, respectively). The MIC for sarafloxacin against *A. salmonicida* increased at 10°C to 0.25 μg/mL. Plasma concentrations were one-half the MIC during treatment.

However, these guidelines do not consider intracellular and interstitial fluid concentrations of antibiotics. Fluoroquinolones are noted for achieving high intracellular concentrations. These antibiotics are rapidly eliminated from serum by renal, transintestinal, biliary and metabolic elimination and by entry into blood and tissue cells. Entry into cells is achieved by passive diffusion, the rate of which depends upon the degree of ionization, the degree of protein binding and the lipid solubility of the antibiotic. Kidney and liver concentrations of the fluoroquinolones can be 5-10 times higher than the serum concentration. Also, these antibiotics can penetrate neutrophils and polymorphonuclear leucocytes (Bergan, 1988; Siporin, 1989) and third-generation quinolones appear to concentrate in phagocytes (Wolfson and Hooper, 1989). The volume of distribution (Vd) of

fluoroquinolones in humans is > 1.5 L/kg (Siporin, 1989), indicating a higher concentration of antibiotic in tissues than in serum. Hustvedt *et al* (1991) reported a Vd of 1.8 L/kg in Atlantic salmon in seawater after an IV dose of 20 mg/kg of oxolinic acid. After IV dose of 10 mg/kg of oxolinic acid, Vd of 2.6 L/kg and 2.9 L/kg were observed in rainbow trout held in seawater and freshwater, respectively (Hustvedt and Salte, 1991). Similar results were noted with enrofloxacin in fingerling rainbow trout; Vd of 2.34 L/kg after an IV dose of 10 mg/kg (Bowser *et al*, 1992). Although plasma concentrations of sarafloxacin fall below the recommended 3-5 times the MIC of *A. salmonicida*, intracellular concentrations may be considerably higher. *A. salmonicida* can be found within tissue cells and within macrophages (McCarthy and Roberts, 1980); hence, intracellular concentrations are likely as important as plasma concentrations in effective treatment of furunculosis. Also, the peak concentration of sarafloxacin was not determined in this study.

Factors other than plasma antibiotic concentrations dictate the outcome of infection. Host factors include the immune response of fish, nutritional status and genetic predisposition to disease (Groberg *et al*, 1983). Also, stress components (water temperature, water quality, overcrowding) influence disease outcome. Post-antimicrobial effects (PAE) also need consideration. PAE, observed at sub-inhibitory

concentrations, include decreased adherence and growth of the bacterium, increased autolytic enzyme production and increased phagocytosis (Brown, 1987). Second and third-generation fluoroquinolones exhibit PAE against *Staphylococcus aureus* and members of the *Enterobacteriaceae* for 1-2 h post-treatment (Wolfson and Hooper, 1989). Thus, the efficacy of sarafloxacin cannot be based on plasma concentrations alone.

Resistance to quinolones is defined as a MIC of ≥ 0.4 μg/mL by Aoki *et al* (1983) or as a MIC of ≥ 1.0 μg/mL by Tsoumas *et al* (1989). However, plasma sarafloxacin concentrations were not sufficient to be inhibitory to resistant isolates.

The MIC_{50} for oxolinic acid against Atlantic coast isolates of *Vibrio anguillarum* and *Vibrio ordalii* is 0.0156 μg/mL (Tables I and II). Based on the recommended plasma concentration of 3 to 5 times the MIC, concentrations of 0.047 to 0.078 μg/mL are required. These concentrations were surpassed during the medicated feeding, with trough concentrations of 0.606, 0.616 and 0.682 μg/mL (Figure 15) and maintained for up to 72 h post-treatment (one sample, 0.244 μg/mL) (Figure 16). The MIC_{50} for oxolinic acid against Pacific coast isolates of *Vibrio* spp. is 0.03125 μg/mL at incubation temperatures of 10 and 20°C; thus concentrations of 0.094 to 0.156 μg/mL are required. Concentrations of 3 to 5 times the MIC were achieved and maintained for up to 72 h post-treatment. At 30°C the MIC_{50} for oxolinic acid against

the Pacific coast isolates of *Vibrio* spp. is 0.0625 μg/mL. Plasma concentrations of 0.188 to 0.312 μg/mL are required. These concentrations were achieved and maintained for up to 48 h post-treatment and possibly for 72 h post-treatment.

The MIC_{50} for oxolinic acid against *Aeromonas salmonicida* is 0.03125 μg/mL (Tables I and II). Therefore, concentrations of 0.094 to 0.156 μg/mL need be achieved for effective treatment. These concentrations were achieved and maintained for up to 72 h post-treatment. Oxolinic acid should be useful in treatment of both vibriosis and furunculosis.

Resistance to oxolinic acid is defined as a MIC of ≥ 0.4 μg/mL by Aoki *et al* (1983) or as a MIC of ≥ 1.0 μg/mL by Tsoumas *et al* (1989). In either case, plasma oxolinic acid concentrations were not sufficient to be inhibitory to resistant isolates.

Achievable plasma concentrations of oxolinic acid were greater than 4 times the plasma concentrations of sarafloxacin (Tables XVIII and XIX). These data suggest that sarafloxacin is of lower oral bioavailability or undergoes a greater Vd or more rapid elimination. In general, fluorinated quinolones are not highly bound to serum proteins (10 - 30%) (Bergan, 1988; Siporin, 1989), but the solubility of sarafloxacin is low (1 g/L) (Markwardt and Klontz, 1989). The same situations were suggested by data in Chapter 5 (Tables XV, XVI and XVII) on single oral and IP dosing, although bioavailability and Vd calculations were not possible.

An alternate interpretation of the lower plasma concentrations of sarafloxacin is related to the distribution of antibiotic out of the plasma compartment and the high intracellular concentrations achieved by fluoroquinolones. Although oxolinic acid, a non-fluorinated quinolone, achieved higher plasma concentrations than sarafloxacin, a fluorinated quinolone, perhaps higher interstitial fluid or intracellular concentrations may be achieved with sarafloxacin. In treatment of fish raised for human consumption, high intracellular concentrations are of concern regarding the withdrawal time or time required for an antibiotic to be eliminated from fish. Vd is the ratio of the antibiotic concentration in the body to antibiotic concentration in the plasma; thus, a Vd > 1 signifies a higher concentration of antibiotic in the body than in plasma. Withdrawal times for sarafloxacin have yet to be established. Withdrawal times of 20 d at 18°C and ≤ 8 d at 12°C have been suggested for oxolinic acid in rainbow trout (Jacobsen, 1989).

Variability between individual fish, sensitivities of the assays (0.04 μg/mL for sarafloxacin and 0.125 μg/mL for oxolinic acid) and lack of sufficient data points during the depletion phase made detailed pharmacokinetic calculations unfeasible.

Information regarding toxicity of quinolones to aquatic species is limited. No information is available on toxicity of the newer fluoroquinolone sarafloxacin. One concern with

using MIC data to establish dosing regimes is the potential for toxicity problems to fish. Oxolinic acid has a reported acute oral toxicity (LD_{50}) of > 4000 mg/kg body weight for carp (Endo *et al*, 1973). No adverse effects were noted when brown trout (*Salmo trutta*) were subjected to a bath containing 75 mg/L of oxolinic acid for 15 minutes (Austin *et al*, 1983). Although acute oral toxicity is not likely a concern at the recommended dose of 10 mg/kg, no information is available on chronic toxicity with repeated treatments. As well, one must be careful when trying to make interpretations on the *in vivo* situation from *in vitro* MIC data.

In conclusion, both sarafloxacin and oxolinic acid are potential candidates as oral therapeutants against vibriosis based upon MIC data and achievable and maintainable plasma concentrations of these antibiotics in Atlantic salmon in seawater. Plasma concentrations of oxolinic acid were in the recommended range of 3-5 times the MIC of *A. salmonicida*. Although sarafloxacin concentrations were below this range, the fluoroquinolones are noted for achieving high intracellular concentrations. Thus, tissue concentrations need be examined as well. In the evaluation of an antibiotic, *in vivo* efficacy trials are required.

7. GENERAL DISCUSSION

The objectives of this thesis have been achieved. In Phase I, the minimum inhibitory concentrations (MICs) of six antibiotics against three bacterial pathogens of fish were determined at three incubation temperatures. In Phase II, oxytetracycline and streptomycin resistant isolates were examined for the presence of plasmids which could be responsible for the antibiotic resistance. In Phase III, plasma concentrations of oxolinic acid and sarafloxacin in Atlantic salmon in seawater were determined to assess whether concentrations were sufficient for effective disease treatment.

Certain characteristics need examination in assessment of an antibiotic as a potential candidate for use in aquaculture. These criteria include drug palatability, *in vitro* and *in vivo* efficacy, safety to the host and to the consumer and the pharmacokinetic characteristics of the drug, including withdrawal times. Direct correlation of the *in vitro* situation (MIC) to the *in vivo* situation is difficult. In the determination of MIC, an artificial scenario is established where the drug concentration, inoculum and the artificial growth medium are all controlled. In the *in vivo* situation, other factors, such as host defense mechanisms, stress, intracellular survival of the pathogen and drug bioavailability and distribution in the host all influence the

success of antibiotic therapy. As a general guideline, antibiotic blood concentrations of 3-5 times the MIC have been recommended for effective therapy in homeotherms (Washington, 1985).

Determination of MICs provides a means to quantify the of the susceptibility of bacteria to an antibiotic and to monitor resistance patterns. Comparison of MIC data from various researchers is difficult due to lack of standardization of inoculum, culture medium and incubation temperature. In this study, three incubation temperatures were examined (10, 20, 30°C) to assess effect on MICs. Potential temperature effects on the MIC are of interest for the comparison of MIC data from several sources and from the viewpoint of examining a correlation between such data and the *in vivo* situation. Farmed salmonids are subject to seasonal fluctuations in water temperatures. Disease outbreaks are related to stress factors including overcrowding, water quality and water temperature. In salmonids, acute furunculosis, a septicemic disease caused by *Aeromonas salmonicida*, occurs as water temperature increases above 13°C (McCarthy, 1975). Water temperature also influences the immune system of ectotherms. Generally, as water temperature increases within the fish's temperature tolerance range, the immune response is more rapid (Trust, 1986). In an experimental water-borne infection of coho salmon (*Oncorhynchus kisutch*) with *Vibrio anguillarum*, *in vivo* growth of the bacterium at 15°C was sufficiently rapid to overcome

host defense mechanisms. At temperatures < 12°C, growth was suppressed and at 12 - 15°C, the outcome of the challenge was related to other factors, such as nutritional status of the fish, genetic predisposition to vibriosis and other stress components (Groberg *et al*, 1983).

In this study, the MIC of oxytetracycline against *Vibrio* spp. exhibited a two-fold or greater increase with increasing incubation temperature. Oxytetracycline and Romet-30 are the only antibiotics currently licensed in Canada for use in aquaculture. Although the percentage of resistant isolates (MIC > 4.0 μg/mL) did not increase, the percentage of moderately susceptible isolates (MIC 2-4 μg/mL) did increase (Table V). An increase in MIC with increasing temperature could be of practical concern when a disease outbreak corresponds to increasing water temperature. The study by Groberg *et al* (1983) suggests that the increase in *in vivo* growth of *V. anguillarum* was responsible for the disease scenario. This observation in combination with the increase in the MIC of oxytetracycline could produce problems in the effective treatment of vibriosis at temperatures above 15°C. As well, interpretation of the MICs (sensitive or resistant) could depend upon the incubation temperature used in the study.

Bacterial isolates were collected from disease outbreaks over a range of years and from two geographical regions (Atlantic and Pacific coasts) to examine possible patterns in

antibiotic resistance. Although MICs may show statistically significant differences between the year of isolation and geographic area, a difference of practical significance constitutes a shift from antibiotic sensitivity to resistance. Resistance of *A. salmonicida* to oxytetracycline and the quinolones oxolinic acid and sarafloxacin was higher in the Atlantic coast isolates, whereas MICs for the quinolones against *V. anguillarum* was higher for the Pacific coast isolates. Differences in the MICs suggest possible differences in the amount and frequency of drug use. Similar yearly changes in the resistance profiles of these three antibiotics were also observed. The percentage of resistant *A. salmonicida* isolates were highest in 1985, declined in 1986-1988 and increased once more in 1989 and 1990 (Table VII). Although multiple resistance has been reported with *A. salmonicida* for tetracycline, oxolinic acid, nalidixic acid, penicillin and chloramphenicol (Wood *et al*, 1986), in the study herein multiple resistance was relatively uncommon and a link between oxytetracycline and quinolone resistance was not apparent (Table VI).

In Phase II, oxytetracycline and streptomycin resistant isolates of *A. salmonicida* and *Vibrio* spp. were examined by agarose gel electrophoresis for the presence of plasmids that may be responsible for the antibiotic resistance. Plasmid profiles of antibiotic resistant and sensitive strains and plasmid profiles of resistant strains grown in the presence of

antibiotic to induce resistance and grown in the absence of antibiotic were compared. The results suggested that the resistance was not plasmid-mediated. These results could be further verified by curing the plasmids (confirmed by gel electrophoresis) and by determining the MIC of the cured strains. Mating experiments would assess whether the resistance was transferable, either due to plasmids or transposons. Plasmid-mediated antibiotic resistance in fish pathogens has been reported elsewhere (Aoki *et al*, 1971; Aoki *et al*, 1981; Aoki *et al*, 1983; Bast *et al*, 1988; Hedges *et al*, 1985; Toranzo *et al*, 1983). Genes encoding for resistance to these antibiotics can be carried on plasmids or on transposons (Aoki *et al*, 1983; Barth *et al*, 1976; Levy, 1984). The potential exists for transfer of plasmids or transposons to other bacteria sharing the same niche, including human pathogens. Hedges *et al* (1985) reported plasmid conferring resistance to streptomycin, tetracycline and chloramphenicol from *A. salmonicida* that were stable in *Vibrio cholerae*. Plasmids from *V. anguillarum* coding for resistance to streptomycin, chloramphenicol, tetracycline and sulfonamides were also stable in *V. cholerae* (Aoki *et al*, 1981).

Oxytetracycline can persist in sediments under sea cages. Concentrations of oxytetracycline were above the MIC of *V. anguillarum* up to 10 weeks after treating with medicated feed (Jacobsen and Berglind, 1988). Thus, a reservoir exists for

the potential induction and transfer of plasmids or transposons within the bacterial population. Concerns over possible rapid spread of antibiotic resistance, has resulted in examination of antibiotics in which plasmid or transposon mediated resistance has not been documented, such as the quinolones. Quinolones are inhibitors of DNA gyrase, an enzyme required for DNA replication (Norris and Mandell, 1988). Resistance to quinolones is controlled by chromosomes; transferable resistance has not been reported. In contrast, quinolones can cure plasmids from members of the *Enterobacteriaceae* when used at subinhibitory concentrations (50% of the MIC) (Michel-Briand *et al*, 1986; Weisser and Wiedemann, 1985). Oxolinic acid has been used for the treatment of *A. salmonicida* in Japan (Endo *et al*, 1973) and in the United Kingdom (Tsoumas *et al*, 1989). Resistance is increasing in Scotland over the last 5 years (Tsoumas *et al*, 1989). Unfortunately, resistance to one quinolone frequently confers some degree of resistance to most quinolones (Barry and Jones, 1984; Smith and Lewin, 1988). This scenario does not occur with *nal* C mutations to subunit B of the DNA gyrase; however, mutations to the B subunit are less common than mutations to subunit A or permeability mutants (*ompF* mutants). The latter two mutational types confer resistance to various of quinolones and, with *ompF* mutants, to other antibiotics including tetracyclines, chloramphenicol, β-lactams and

aminoglycosides. Generally, chromosomal mutations are less common than plasmid mutations in clinical isolates and such mutants are frequently associated with loss of pathogenicity (Smith and Lewin, 1988).

Low MICs and pharmacokinetic properties of the quinolones make them attractive alternatives to drugs such as oxytetracycline. Fluoroquinolones are rapidly absorbed after oral dosing and achieve high intracellular and tissue concentrations. They are rapidly eliminated from serum by passive diffusion. Kidney and liver concentrations can be 5-10 times the serum concentration (Bergan, 1988).

Phase III was the determination of plasma concentrations of oxolinic acid and sarafloxacin in Atlantic salmon during and following the recommended oral treatment regime. Plasma concentrations were determined using a microbiological assay with *V. anguillarum* ATCC 19264 as the test organism. The assay was developed as part of this study. A pilot study was performed to determine the oral bioavailability of two quinolones and blood sampling times for the medicated feeding trial; however, due to problems with dosing procedures such information could not be calculated. An oral bioavailability of 25% for oxolinic acid in Atlantic salmon in seawater has been reported (Hustvedt *et al*, 1991).

In the medicated feeding trial herein, Atlantic salmon in seawater were treated at 10 mg/kg/d for 10 d with oxolinic acid and 10 mg/kg/d for 5 d with sarafloxacin. Tank biomasses

were determined one month prior to commencing the study; thus, actual dose rates were 7.3-8.0 mg/kg/d. Plasma concentrations reported at 6 h post-treatment were 0.93 μg/mL and 0.19 μg/mL for oxolinic acid and sarafloxacin, respectively. Minimum maintainable concentrations between 24 h feedings (trough concentrations) were 0.61-0.68 μg/mL and 0.14 μg/mL for oxolinic acid and sarafloxacin, respectively. Concentrations dropped below the limit of detection for the assay for both drugs at 72 h post-treatment (<0.125 μg.mL for oxolinic acid and 0.04 μg/mL for sarafloxacin). Blood concentrations of 3-5 times the MIC have been recommended for effective treatment (Washington, 1985). Based on these guidelines, plasma concentrations of oxolinic acid were sufficient for treatment of *A. salmonicida* and *Vibrio* spp. up to 72 h post-treatment. Sarafloxacin concentrations were within these recommendations for *Vibrio* spp. up to 48 h post-treatment, but concentrations of 3-5 times the MIC of *A. salmonicida* were obtained only at the 6 and 12 h samplings. However, the guidelines were established for homeotherms and that they do not take into consideration high intracellular and tissue concentrations achieved by fluoroquinolones. Thus, decline in blood concentration of sarafloxacin may be due to the passage into tissues and cells and not only to the elimination from the animal. *A. salmonicida* can be found within tissues and within macrophages (McCarthy and Roberts, 1980); therefore,

intracellular concentrations are likely important in effective therapy.

High intracellular concentrations of antibiotic raises another concern in the use of drugs to treat fish raised for human consumption: drug withdrawal time. Once the drug has reached the central compartment, namely the blood, it can then be metabolized by liver or kidney and excreted in bile, urine through skin or gills, or it can be stored. The antibiotic and its metabolites may be stored in fat, skin, liver, kidney or muscle. (Guarino and Lech, 1986). Withdrawal times for oxolinic acid of 20 d at a freshwater temperature of 18°C and ≤ 8 d at 12°C have been recommended for rainbow trout in freshwater (Jacobsen, 1989). The elimination of oxytetracycline is also complicated by the influence of water temperature. Withdrawal times of 90 d at < 6°C, 70 d at 6-12°C and 60 d at > 12°C have been suggested (Jacobsen, 1989).

The metabolism of ectotherms is greatly influenced by water temperature. Thus, the immune response of fish, the pharmacokinetics of antibiotics and the *in vivo* growth of the pathogen in fish are all influenced by water temperature. Although direct correlation of *in vitro* data with the *in vivo* situation is not possible, temperature effects on the MIC data are of interest from two viewpoints. First, farmed salmonids are subjected to seasonal variations in water temperature and second, incubation temperature may influence interpretation of MIC results (susceptible versus resistant). In this study the

MIC was influenced by temperature for some antibiotics. The most pronounced effect was seen with oxytetracycline against *Vibrio* spp, where the MIC increased by two-fold or greater with increasing incubation temperature.

Oxytetracycline and streptomycin resistance was not determined as plasmid-mediated by examination of resistant and susceptible isolates by agarose gel electrophoresis. Transferable antibiotic resistance may be under the control of plasmids or transposons, although resistance transfer was not examined in this study.

Plasma concentrations of oxolinic acid and sarafloxacin in Atlantic salmon in 10°C seawater were reported as sufficient for effective treatment of vibriosis and furunculosis, based upon a concentration 3-5 times the MIC.

In conclusion, effective antibiotic therapy involves knowledge of the *in vitro* and *in vivo* efficacy of the drug, as well as the pharmacokinetic properties of the antibiotic for that animal species and water temperature. The drug withdrawal time is important in treatment of fish raised for human consumption. Additional research is required in these areas as well as in the surveillance of the spread of antibiotic resistance and potential environmental pollution problems.

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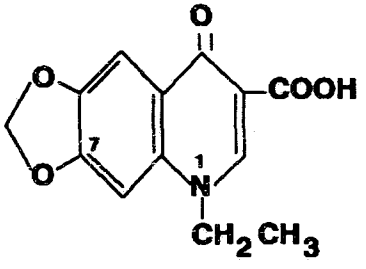
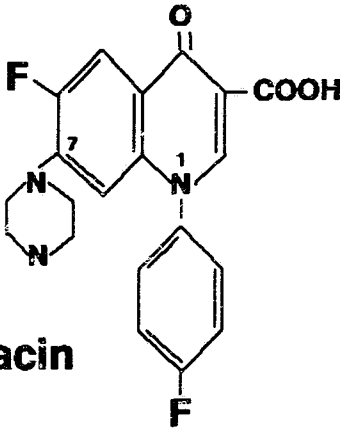
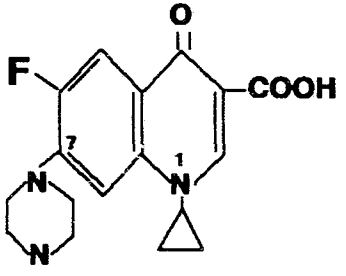
APPENDIX A



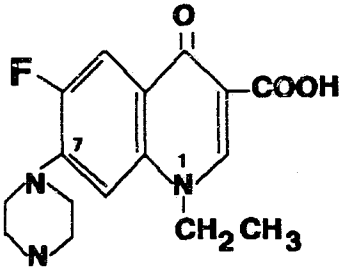
oxolinic acid



sarafloxacin



ciprofloxacin



norfloxacin

APPENDIX B

Lysis solutions

TELT solution

1M Tris-HCl, pH 8	5 mL
0.5M Na_2EDTA, dihydrate, pH 8	12.5 mL
250 mM lithium chloride	1 mL
Triton X-100	4 mL

sterile deionized water to 100 mL

SDS lysis solution

3% sodium dodecyl sulphate (SDS)

50 mM Tris

adjust pH to 12.6 with 5.7 mL 1N NaOH

sterile deionized water to 100 mL

APPENDIX C

TE buffer

1M Tris-HCl	5 mL
0.5M Na_2EDTA	2 mL

sterile deionized water to 100 mL

final concentrations of 0.05M Tris-HCl, 0.01M EDTA, pH 8

APPENDIX D

Ribonuclease A (RNase A)

RNase A (bovine pancreas) 4.3 mg

50 mM sodium acetate, pH 5 860 μL

heat at 80°C for 10 minutes

store at -20°C in 20μL aliquots

APPENDIX E

10X Tris-acetate/EDTA (TAE) electrophoresis buffer

Trizma base 48.4 g

Na_2EDTA, dihydrate 7.4 g

sodium acetate, anhydrous 16.4 g

glacial acetic acid 17.0 mL

sterile deionized water to 1 L

final pH 7.8

autoclave at 121°C for 15 minutes

Note: This is a 10X concentration solution. Dilute with sterile deionized water prior to use.

APPENDIX F

10X sample loading buffer

glycerol 5 mL

Na_2EDTA, dihydrate 0.37 g

sodium dodecyl sulphate (SDS) 0.1 g

bromophenol blue 0.01 g

sterile deionized water to 10 mL

APPENDIX G

1000X ethidium bromide solution

ethidium bromide 50 mg

sterile deionized water to 100 mL

wrap bottle in foil and store at 4°C

Note: This is a 1000X concentration solution (0.5 mg/mL). Dilute 1:1000 for working solution (0.5 μg/mL).

APPENDIX H

STANDARD OPERATING PROCEDURE

NUMBER: SOP 412/01

TITLE: Double bucket weighing technique

PURPOSE: To accurately determine biomass of a tank/aquarium without subjecting fish to anesthesia.

SCOPE: This SOP is a FHU management directive to Study Directors and all personnel engaged in GLP studies at the FHU.

EFFECTIVE DATE: _____

APPROVED BY:

POSITION	NAME	SIGNATURE	DATE
FHU MANAGEMENT	_____	_____	_____
CHAIRPERSON QAU	_____	_____	_____
PREPARED BY	_____	_____	_____
	_____	_____	_____

THIS SOP WILL BE POSTED, MAINTAINED AND REVISED AS DESCRIBED IN SOP 107/01. IN THE EVENT THAT THIS SOP CAN NOT BE FOLLOWED, THIS WILL BE REPORTED TO FHU MANAGEMENT. DEVIATION FROM THIS SOP MAY ONLY OCCUR UPON RECEIPT OF WRITTEN PERMISSION FROM FHU MANAGEMENT AND THE SPONSOR.

1.0 GENERAL CONSIDERATIONS

1.1 Fish to be weighed using the double bucket weighing technique will be fasted for 24 to 48 hours prior weighing.

1.2 The three buckets used in the technique will be disinfected for 30 minutes with water containing ≥ 5 ppm free chlorine. Buckets will be thoroughly rinsed with system water before use.

1.3 A sufficient number of disinfected holding containers (buckets or tubs), air tubing and air stones will be set up to accommodate the fish to be weighed.

1.4 The balance to be used will be levelled and calibrated according to the appropriate current SOP.

2.0 DOUBLE BUCKET WEIGHING TECHNIQUE

2.1 The double bucket weighing technique uses three white plastic buckets. The first bucket (**number 1**) has a solid bottom and a handle, the second bucket (**number 2**) has a solid bottom and no handle, the third bucket (**number 3**) is deeper than 1 and 2, has two handholds cut out of the side near the top and has a Vexar™ screen bottom (Appendix A).

2.2 Fill bucket 2 with system water. Bucket and water together will have a weight appropriate to the balance used to allow for the additional weight of the fish (for example, the Mettler PJ6000 balance has a total capacity of 6,000 gram, bucket and water together should weigh approximately 4,000 gram, allowing 2,000 gram for bucket 3 plus fish). Place the filled bucket 2 on the balance and tare.

2.3 Fill bucket 1 with sufficient system water to accommodate the number of fish to be weighed at any one time. Place bucket 3 inside bucket 1.

2.4 Capture fish to be weighed and put them into the bucket 1-3 combination. Fish will not be more than one layer deep when the screened bucket is lifted.

2.5 Lift bucket 3 slowly until clear from bucket 2. Shake bucket 3 three times firmly but gently to remove excess water.

2.6 Place bucket 3 into bucket 2 on the balance. Record the measured weight.

2.7 Remove bucket 3 gently out of bucket 2, tipping the bucket slightly to allow water to drain out. Replace bucket 3 into bucket 1. **Tare the balance.**

2.8 Transfer the fish in bucket 3 to a holding container containing aerated water. Repeat Sections 2.3 to 2.8 until all fish from the tank/aquarium have been weighed.

2.9 If the biomass of additional tanks/aquaria needs to be determined, disinfect all weighing and holding equipment in water containing free chlorine at ≥ 5 ppm. Rinse all such disinfected equipment with system water before use. Repeat steps 2.3 to 2.9 until all weighing is completed.

2.10 Determine the weight of the empty bucket 3 by repeating **all steps** of the weighing process, including the three shakes. Record this weight and repeat the procedure for a total of 5 times (make sure to tare balance between measurements). Take the average of the 5 measurements and subtract this weight from the recorded fish plus bucket weights.

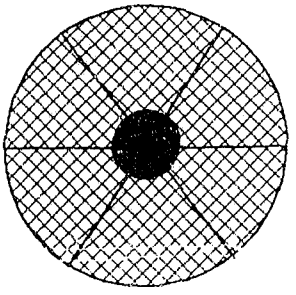
2.11 Upon completion of the weighing process, all equipment will be disinfected by soaking in water containing ≥ 5 ppm free chlorine or by spraying with 70% ethanol.

Attached: Appendix A - Equipment used in double bucket weighing technique.

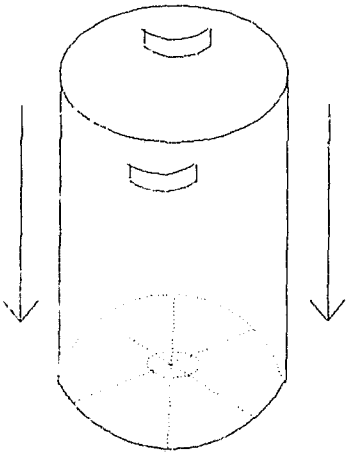
APPENDIX A

EQUIPMENT USED IN

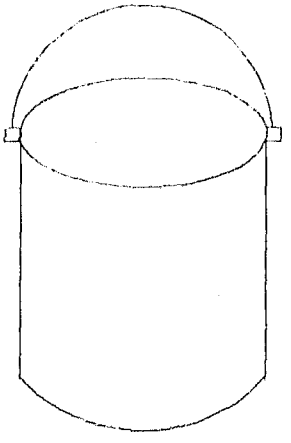
DOUBLE BUCKET WEIGHING TECHNIQUE



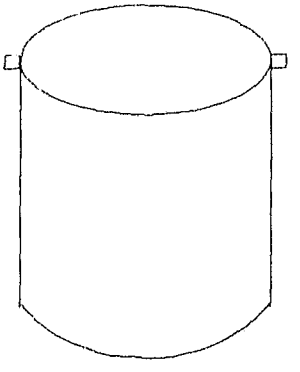
view of bottom
of bucket 3 with Vexar mesh in place



bucket 3



bucket 1



bucket 2