Control of Furunculosis by Antimicrobial Chemotherapy

A thesis submitted for the Degree of Doctor of Philosophy.

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ABSTRACT

In vitro activities of six fluoroquinolone antimicrobials, flumequine, sarafloxacin, PD127,391, PD117,596, enrofloxacin and Ro 09-1690, against *Aeromonas salmonicida*, isolated from outbreaks of furunculosis in farmed and wild Atlantic salmon (*salmo salar*), were evaluated in terms of minimum inhibitory concentration (MIC), bactericidal activity and the frequency at which mutation to resistance developed to the drugs compared to oxolinic acid, the 4-quinolone antibiotic currently licensed for furunculosis therapy in the UK. MICs were also determined for oxytetracycline and the ß-lactam amoxycillin.

In terms of MIC, the fluoroquinolones were more active than oxolinic acid with MICs reduced from 2- to 500-fold. Furthermore, the fluoroquinolones were bactericidal against both oxolinic acid-resistant and -susceptible isolates of *A.salmonicida* whereas oxolinic acid was not bactericidal against any bacteria tested. In addition to the increased antimicrobial activity, the frequency at which *A.salmonicida* mutated to resistance to the fluoroquinolones was lower than the frequency for resistance to oxolinic acid. Amoxycillin was active against all the *A.salmonicida* subsp. *salmonicida* isolates tested; however, the atypical *A.salmonicida* subsp. *achromogenes* isolates were resistant to amoxycillin with MICs in excess of 500mg/l. Ion effects were noted for the quinolones and oxytetracycline. For instance, the presence of 50mM $MgCl_2$ resulted in a 20- to 60-fold increase in the MICs of these drugs against the test strains. No ion effect was identified for amoxycillin nor the components of Romet. The quinolones were not affected by inoculum size but their efficacy, in terms of MIC, was reduced at the lower temperature of 10°C compared to 22°C.

Mechanisms of resistance to the fluoroquinolones, oxytetracycline and amoxycillin were investigated. Low levels of resistance to the quinolones and oxytetracycline could be induced with either oxolinic acid or oxytetracycline, with each drug inducing cross resistance to the other. This phenomenon was discovered to result from alterations in outer membrane proteins, specifically the increase in expression of a 42kDa and a decrease

in expression of a 37kDa protein. These two proteins were found to be non-covalently associated with peptidoglycan suggesting they may function as porins. It is suggested that high level resistance to quinolones in *A.salmonicida* may be due to an alteration in the target enzyme, DNA gyrase. The high level resistance to amoxycillin found in the *A.salmonicida* subsp. *achromogenes* isolates resulted from the production of a ß-lactamase enzyme. This enzyme was inducible and probably chromosomally expressed. Attempts to mobilise the resistance gene were unsuccessful and plasmid curing experiments had no effect on the amoxycillin MICs of the strains tested.

An experimental seawater infection challenge was developed that was capable of inducing significant furunculosis mortalities in Atlantic salmon smolts. The *in vivo* activity of the potentiated sulphonamide, Romet 30, was investigated. Romet (15mg/kg/body wt/day) was effective in the control of artificially induced outbreaks of furunculosis. Changes to the field unit at Aultbea necessitated an investigation into disinfection of undiluted seawater with iodine. Iodine was bactericidal in undiluted seawater, killing 90% of bacteria at normal pH (7.2), and 99% of bacteria at reduced pH (4.0). However, this was insufficient to proceed with further *in vivo* infection studies.

An alternative to efficacy studies requiring infection was developed. Fish were medicated with the quinolones oxolinic acid, flumequine, sarafloxacin, enrofloxacin, and Ro 09-1168. Serum levels were determined, and the bactericidal activities of these levels of antibiotic in serum were determined against resistant and susceptible isolates of *A.salmonicida.* Levels of antibiotic achieved in the fish were bacteriostatic against a resistant isolate of *A.salmonicida,* and, with the exception of oxolinic acid, were bactericidal against a susceptible isolate of *A.salmonicida.*

ACKNOWLEDGEMENTS

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Finally, Iwould like to thank Miss Allyson Bain of Microelectronics Education Development Centre, Paisley, Renfrewshire, for teaching me all there is to know about DTP on the Macintosh.

DECLARATION

The research described in this thesis is the sole work of the undersigned author unless otherwise stated.

Digitised images have not been altered in any way, apart from rescaling where necessary to fit in the document. Original photographs will be supplied on request.

CONTENTS

PUBLICATIONS & PRESENTATIONS

PUBLICATIONS

1. Barnes A.C., Lewin,C.S, Hastings,T.S. & Amyes,S.G.B. (1990). *In vitro* activity of fluoroquinolones against the fish pathogen *Aeromonas salmonicida. Antimicrob.Agents Chemother.* **34:**1819-1820.

2. Barnes, A.C., Lewin,C.S., Hastings,T.S. & Amyes,S.G.B. (1990). Cross resistance between oxytetracycline and oxolinic acid in *Aeromonas salmonicida* associated with alterations in outer membrane proteins. *FEMS Microbiol. Lett.* **72:**337-340.

3. Barnes, A.C., Lewin,C.S., Hastings,T.S. & Amyes,S.G.B. (1990). Susceptibility of *Aeromonas salmonicida* to enrofloxacin. *Bull.Eur.Assn.Fish Pathol.* **10:**138.

4. Barnes,A.C., Lewin,C.S., Amyes,S.G.B. & Hastings,T.S. (1990). *In vitro* activities of 4-quinolone antimicrobials against *Aeromonas salmonicida. ICES Mariculture Committee CM1990/F:49.*

5. Barnes, A.C., Lewin,C.S., Hastings,T.S. & Amyes,S.G.B. (1991) Susceptibility of the fish pathogen *Aeromonas salmonicida* to flumequine. *Antimicrob. Agents Chemother.* **35:**2634-2635.

6. Barnes, A.C., Amyes,S.G.B., Hastings,T.S. & Lewin,C.S. (1991) Fluoroquinolones display rapid bactericidal activity and low mutation frequencies against *Aeromonas salmonicida. J.Fish Dis.* **14:**661-667.

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9. Barnes, A.C., Lewin,C.S., Hastings,T.S. & Amyes,S.G.B. (1992). Bactericidal activity of 4-quinolones, including flumequine, against *Aeromonas salmonicida.* In: *Chemotherapy in Aquaculture: From Theory to Reality.* Michel,C. & Alderman, D.J. (eds.). OIE, Paris, France.

PRESENTATIONS

1. Lewin,C.S., Barnes, A.C., Hastings,T.S. & Amyes,S.G.B. (1990). Activity of fluoroquinolones against *Aeromonas salmonicida.* (Poster) *Bacterial Diseases of Fish. Institute of Aquaculture, Stirling.* 26th-29th June 1990.

2. Barnes, A.C., Lewin,C.S., Hastings,T.S. & Amyes,S.G.B. (1990) 4-quinolones in Aquaculture. (Oral). *Scottish Salmon Growers Association Furunculosis Seminar. Aviemore.* 8th Nov 1990.

3. Barnes, A.C., Lewin,C.S., Hastings,T.S. & Amyes,S.G.B. (1991). Bactericidal activity of quinolones, including flumequine, against *Aeromonas salmonicida.* (Poster). *Problems of Chemotherapy in Aquaculture: From Theory to Reality. Office Internationale des Epizooties, Paris.* 12th-15th March 1991.

4. Barnes, A.C., Lewin,C.S., Hastings,T.S. & Amyes,S.G.B. (1991). Multiple low level antibiotic resistance in *Aeromonas salmonicida* associated with OMP alterations. (Oral). *EAFP Joint-Branch Meeting on Salmon Diseases. SOAFD Marine Laboratory, Aberdeen, Scotland.* 11th-12th April 1991.

5. Barnes, A.C., Lewin,C.S., Love,D.M., Hastings,T.S. & Amyes, S.G.B. (1991). Romet 30 is active against *Aeromonas salmonicida in vitro* and is effective in the control of furunculosis in Atlantic salmon in seawater. (Oral).*IVth EAFP International Conference, Budapest, Hungary.* 25th-29th Aug 1991.

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INTRODUCTION

1.1 FURUNCULOSIS IN SCOTLAND.

1.1.1. Atlantic Salmon Farming in Scotland. Commercial farming ofAtlantic salmon (*Salmo salar* L.) in Scotland commenced in the early 1970s. Growth of the industry has been rapid, with 10,000 tonnes being produced in 1985, increasing to 40,000 tonnes produced by around 360 registered sites in 1991 (SOAFD 1991).

The farming cycle of Atlantic salmon varies considerably, but a typical cycle may proceed as follows: Ova, from brood stock hen salmon, are collected late in the year and fertilized. The eggs hatch in to alevins and develop into

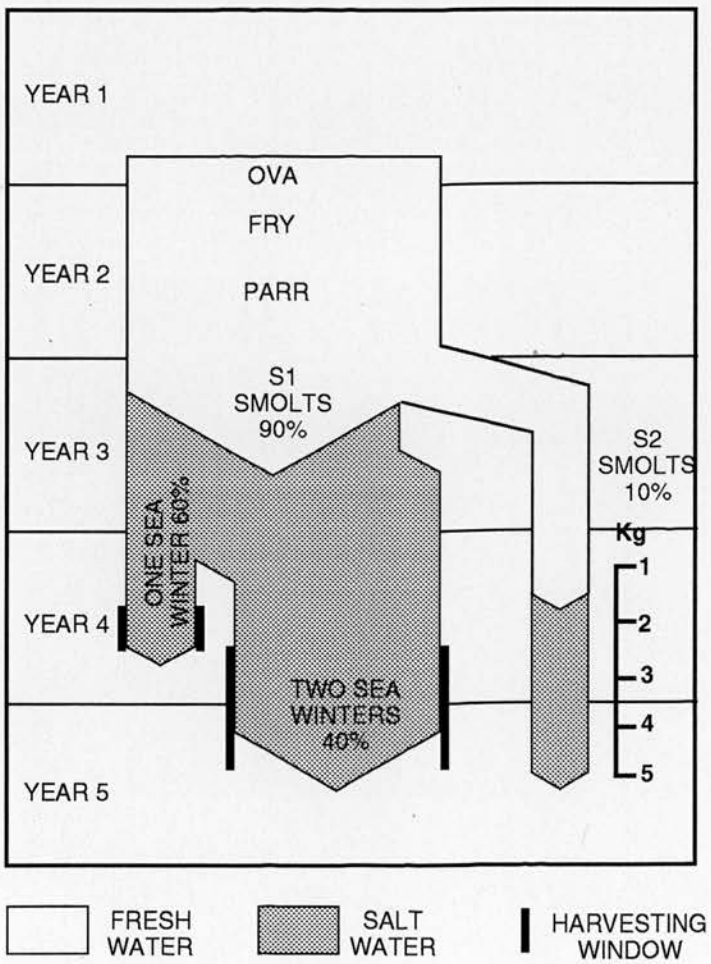


Figure 1. Farming Cycle of Atlantic Salmon in Scotland (source; SOAFD, Aberdeen.)

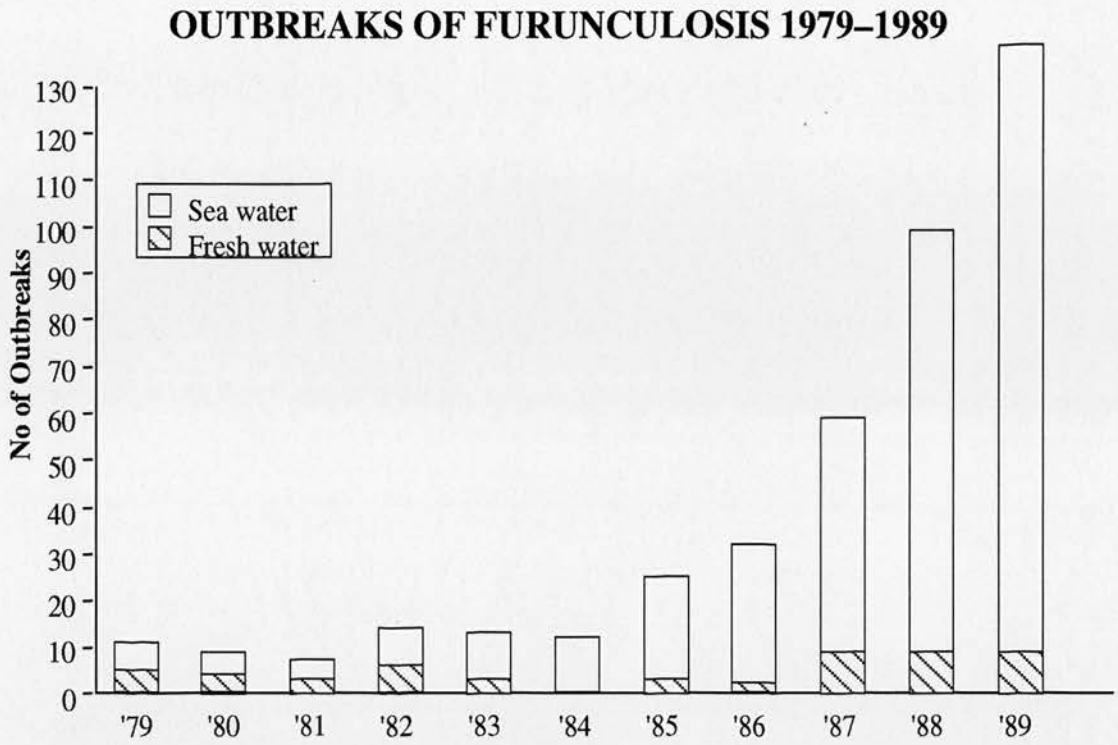


Figure 2. Outbreaks of Furunculosis in Farmed Salmonids Reported Between 1979 and 1989 (Source; SOAFD).

fry early the following spring. The parr are grown in fresh water tanks or cages until they are ready for smolting. In most cases, this occurs in the late spring of the following year when fish are one year of age (S1 smolts), although some fish may be ready for transfer to seawater within 6 months (S1/2 smolts), while others do not smolt until the following year (S2 smolts). During smoltification the fish undergo a series of physiological changes which enable them to adapt to life in salt water. Following transfer to sea, the fish are grown in pens or cages. Typically, up to 60% of these fish may be harvested and sold after one winter at sea. The remaining fish are kept at sea for a further winter before harvesting. By this stage the mature salmon may be 3-4 kilogrammes in weight (figure 1)

1.1.2. Scale of the Disease Problem. Farmed salmonids are susceptible to a range of viral, bacterial and parasitic infections (Hastings 1988). Bacterial diseases of fish have been reviewed in depth by Austin & Austin (1987). One of the most economically damaging diseases to the Scottish salmon farming industry in recent years, however, has been the bacterial disease, furunculosis.

Year	Number of Smolts	Years Harvested	% of Fish Recovered
1984	3.6m	1985,1986	86.5
1985	5.6m	1986,1987	70.4
1986	6.6m	1987,1988	75.6
1987	12.9m	1988,1989	65.5
1988	20.9m	1989,1990	NA

Table 1. Survival of Smolts to Harvest (Source; SOAFD)

Over the last decade the prevalence of the furunculosis has increased rapidly, with the number of cases reported in farmed salmonids in seawater escalating from 11 in 1979 to 137 in 1989 (fig. 2). Furthermore, the percentage of smolts recovered from production sites has decreased alarmingly over the past 7 years, the losses resulting largely from disease mortalities (table 1).

1.1.3. *Aeromonas salmonicida.* Furunculosis is caused by the Gram-negative, normally non-motile bacterium, *Aeromonas salmonicida* (Popoff, 1980) and has been reviewed by Austin & Austin, 1987. The species *A.salmonicida* has been divided into 3 subspecies, namely; subsp. *achromogenes,* subsp. *masoucida* and subsp. *salmonicida* (Popoff, 1980). In addition to these subspecies, which can be differentiated by means of biochemical and physiological characteristics, several unusual isolates have been described (Austin & Austin, 1987). Typical *A.salmonicida* produces a water soluble brown diffusible pigment when grown on Tryptone Soy Agar (TSA). Griffen *et al.* (1953) demonstrated that production of the pigment was dependent upon the presence tyrosine and phenylanaline in the growth medium, and this was confirmed by O'Leary (1956). Although the pigment is similar in appearence to melanin, produced by several species of *Pseudomonas,* Donlon *et al.* (1983) demonstrated that pigment biosynthesis from tyrosine differed substantially from from melanogenesis. Pigment production provides a useful tool when identifying the organism; however, non-pigmented strains of *A.salmonicida* occur. Furthermore, other organisms produce similar brown pigments on TSA, including *A.hydrophila,*

A.media (Austin & Austin, 1987) and certain strains of *Pseudomonas fluorescens* (Frerichs & Holliman, 1991). Interestingly, the brown pigmented *Ps.fluorescens* also react with some commercially available antisera for detection of *A.salmonicida*. The two species may, however, be distinguished by incubation at 37°C (personal observations). Furunculosis in farmed salmonids in Scotland is generally caused by typical *A.salmonicida* subsp. *salmonicida,* although *A.salmonicida* subsp. *achromogenes* does cause clinical outbreaks of the disease in wild fish in Scotland (T.Hastings pers.comm), and has been reported to cause outbreaks in farmed salmon in Finland (Rintamäki & Valtonen, 1991) and Canada (Harmon *et al.,* 1991)

A variety of pathogenicity and virulence factors have been associated with *A.salmonicida*. Munro (1984) divided these factors into two groups; cell-associated components and extracellular components.

The most thoroughly studied cell-associated factor is an additional proteinaceous layer surrounding the cell wall termed the A-layer (Udey & Fryer, 1978). Sakai (1986) determined that the A-layer was involved in auto-agglutination, and adhesion to fish tissue culture cells. Sakai (1986) proposed that the mechanism for auto-agglutination and adhesion was based on electrical charge associated with the cell surface. Trust *et al.* (1980) determined that the A-layer comprised a protein of molecular weight 50kDa and Ishiguro *et al.* (1981) demonstrated that loss of A-layer was associated with reduced virulence. Ishigura *et al.* (1981) also provided evidence that the A-layer may protect *A.salmonicida* from the action of bacteriophages by shielding their binding sites. Further evidence for the role of the A-layer was provided by Munn & Trust (1984) who showed that virulent *A.salmonicida* with A-layer was resistant to the bactericidal activity of both normal and immune complement in rainbow trout (*Oncorhynchus mykiss* L.) serum. Trust *et al.* (1983) reported that the A-layer also conferred hydrophobicity on the organism, providing cells with an enhanced ability to associate with rainbow trout macrophages in the absence of opsonizing antibody. This increased association with phagocytic monocytes may be advantageous to the cell as Olivier *et al.*, (1992) reported that *A.salmonicida* is cytotoxic to salmon macrophages in ratios as low as <10:1. Although the authors were unable to

correlate this with virulence, these observations coupled with resistance to both immune and normal complement (Munn & Trust, 1984) leave the salmon with few effective defences against furunculosis.

Extracellular virulence factors of *A.salmonicida* have recently been reviewed by Ellis (1991). Many extracellular enzymes are produced by *A.salmonicida*, two of which are known to be of prime importance in the pathogenesis of the organism. The first, a 70KDa protease causes tissue liquefaction and blood clotting, and has been demonstrated to have an LD_{50} of 2.4μg/g fish (Lee & Ellis, 1989). The second, a 25kDa phospholipase (GCAT), is mainly present in a high molecular weight complex with lipopolysaccharide (LPS), and the complex is haemolytic for fish erythrocytes (Lee & Ellis, 1990). A further extracellular product (ECP) demonstrating proteolytic activity was identified by Sheeran & Smith (1981). This protease conferred casein hydrolysing activity, and activity against gelatin and collagen.

1.1.5. Clinical Furunculosis. The disease is named after the raised liquefactive muscle lesions (furuncles) which sometimes occur in chronically infected fish, though these lesions are rarely seen in acute infections which are characterised by a rapid fatal septicaemia (McCarthy & Roberts, 1980). Some devastating epizootics have been recorded in wild fish populations (Mackie *et al.*, 1930, 1933, 1935) but the major impact in recent years has been on farmed salmon (Hastings, 1988).

Most if not all species of salmonid fish may be affected by furunculosis, though some species seem to be more susceptible than others (Cipriano,1983). For example, Atlantic salmon and brown trout (*Salmo trutta* L.)are highly susceptible whereas some strains of rainbow trout seem to be remarkably resistant to the disease (Cipriano, 1983).

The clinical signs of furunculosis are somewhat variable depending on the form of the disease, but affected fish often show lethargy, loss of appetite and darkening of the skin (Austin & Austin, 1987). Haemorrhaging may occur at the base of the fins and in the abdominal walls, heart and liver. Enlargement

of the spleen and inflammation of the lower intestine are common features of chronic infections, but in acute cases mortalities occur rapidly with few external symptoms (McCarthy & Roberts, 1980; Austin & Austin, 1987).

Furunculosis is primarily a disease of rising and elevated water temperatures, nevertheless, mortalities can occur at temperatures as low as 4-6°C. The risk of disease is especially high if fish are overcrowded, but outbreaks can precipitate by the stressful nature of intensive fish rearing, particularly during handling, grading, transporting or following transfer of smolts to salt water (Hastings, 1988).

Transmission of furunculosis is normally lateral, via infected fish or contaminated water (Austin & Austin, 1987). Although *A. salmonicida* has been isolated from the gonads of infected fish, attempts to demonstrate vertical transmission have generally been unsuccessful (McCarthy, 1977). Whether *A. salmonicida* is able to survive in the surface waters has been argued for many years. Indeed, by definition, the organism is an obligate fish pathogen, not surviving in the surface waters (Popoff, 1980). However, alternative studies have revealed the contrary (Michel & Dubois-Darnaudpeys, 1980; Allen-Austin *et. al.*,1984; Austin & Austin,1987). The length of time *A.salmonicida* resides in the water is likely to depend on factors such as water temperature, nutrient levels and the presence of other bacteria (Allen-Austin *et al.*,1984). Some studies have shown that the organism may remain viable for several weeks in both fresh and salt water (Austin & Austin, 1987), and for many months in river sediments (Michel & Dubois-Darnaudpeys, 1980). The normal routes of infection into the fish are also uncertain, infection through the gastrointestinal tract, skin or gills being possible (Hodgkinson, Bucke & Austin, 1987).

1.1.5. Asymptomatic Furunculosis. *A. salmonicida* may be harboured in the fish with no symptoms of disease being evident. These asymptomatic carriers were first recognized by Plehn (1911) who demonstrated that artificially infected carrier fish could be infective for healthy fish. Mackie *et al* (1930) considered the carrier state to be incubatory and that the disease would subsequently develop.

Carrier fish, which may be present in both farmed and wild populations, can transmit infection to other fish or succumb themselves to the disease when stressed (Bullock & Stuckey, 1975; McCarthy, 1977). How the bacterium is harboured in the fish during the carrier state is poorly understood. Bacteriological sampling of various organs revealed that the organism was most frequently isolated from the kidney of carrier fish (McDermott & Berst, 1968). McCarthy (1977) investigated the site of carriage of furunculosis and concluded that the kidney was the primary site, but that the organism could also be detected in the gut. However, a recent report (Cipriano, 1992) has demonstrated that *A.salmonicida* is more frequently isolated from the mucus on the external surface of the fish than from the kidney. This is supported by the observations of the SOAFD staff (T. Hastings, pers.comm.).

Detection of carrier fish is problematic as bacterial numbers can be very low and their presence is rarely revealed by conventional bacteriological techniques (Hastings, 1988). It was not until 1975 that Bullock & Stuckey reported more sensitive method of detecting carriers. This technique was based on intramuscular (IM) injection of a corticosteroid and thermal stress. Fish were injected with triamcinolone acetonide and the water temperature was raised form 12.5 to 18°C. McCarthy (1977), in a comparative study of corticosteroids, found that prednisolone acetate was most effective at activating asymptomatic furunculosis.

Further methods of detecting *A.salmonicida* have been investigated. Austin *et al.* (1986) developed a monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) for the detection of *A.salmonicida*. However, this group found that their system could only detect $>10^4$ cfu. Furthermore, this technique still required killing the fish. Further ELISA systems have been investigated with similar observations (Bernoth, 1990). More sensitive means of detection involve amplification of fragments of *A.salmonicida* DNA using specific probes and polymerase chain reaction (PCR) technology. Hiney *et al.* (1992) reported that their system could detect two *A.salmonicida* cells. Although such technology may be difficult to apply to carrier detection, it has ben used to characterize *A.salmonicida* isolates (Hennigan *et al.*, 1989), and may prove useful in identifying habitats and routes of transmission/infection of *A.salmonicida* (Hiney *et al.*, 1992).

The only non-lethal mode of carrier detection would appear to be by bacteriological sampling of external mucosa, based on the observations of Cipriano *et al.* (1992). However, the detection rate of this method has yet to be determined.

1.1.6. Control of Furunculosis. Furunculosis control has recently been reviewed by Munro & Hastings (1992). Potential means of control may include vaccination, improved husbandry, selection of resistant fish, chemotherapy, management and, perhaps more realistically, a combination of the above.

Vaccination against furunculosis has been reviewed by Munro (1984) and Hastings (1988). Vaccines have been based on whole killed cells (bacterins) (Olivier *et al,* 1985; McCarthy *et al.,* 1983) of undefined antigenic composition: Extracellular products (ECP) (Olivier *et al.,* 1985; Cipriano, 1982; Shieh,1985) or ECP toxoids (Rodgers & Austin, 1985). Mixtures of bacterins and ECP have also been tried (Cipriano *et al,* 1983). Live vaccines have received little attention, perhaps through concern over safety in the field. However, one live attenuated vaccine has been tested (Cipriano & Starliper, 1982). Many of these vaccines have only been modestly successful (Hastings, 1988). This may continue to be a problem when one considers the success of *A.salmonicida* in dealing with the fish immune system (Munn & Trust , 1984; Olivier *et al.*, 1992).

There is little doubt that *A.salmonicida* is an opportunistic fish pathogen awaiting periods of stress or trauma in the fish farming cycle; indeed, stress forms the basis of asymptomatic carrier detection (Bullock & Stuckey, 1975). Thus, management of the fishes' environment throughout the farming cycle to minimise stress would appear to be important. Management may also play a role in avoidance of the pathogen through fallowing and single year class policies. It has been suggested that lice infestations may provide a route of infection for furunculosis, and may spread the disease through transfer from fish to fish (Munro & Hastings, 1992). Indeed, McHenery *et al.* (1991) have already highlighted the importance of controlling lice infestations.

Species of salmonid fish, namely rainbow trout, resistant to furunculosis have been reported (Cipriano, 1983). Olivier *et al.* (1988) determined a genetic basis for furunculosis resistance in Atlantic salmon. Breeding programmes which select for more resistant strains may be a practical way of reducing the prevalence of furunculosis in future.

Chemotherapy can be an effective means of furunculosis control and this will be reviewed at the end of this introduction. However, there is concern over the use of antibiotics in the environment (Lunestad, 1992). Investigations have demonstrated that oxolinic acid may be found in wild fauna (Samuelsen *et al.*, 1992), and sediments (Husevåg *et al.*, 1991; Samuelsen *et al.*, 1991; Hansen *et al.*, 1992; Lunestad *et al.*, 1992) associated with aquaculture sites in Norway. Furthermore, fish pathogens and antibiotic resistant bacteria have been recovered from sediments at abandoned fish farms (Husevåg *et al.*, 1991), thus the removal of the site may not necessarily lead to recovery of the natural flora. There is also concern that use of antibiotics may affect bacterial diversity in marine sediments (Lunestad *et al.*, 1992).

Currently, oxytetracycline, oxolinic acid, potentiated sulphonamides and amoxycillin are licensed for use in Scottish aquaculture. The remaining part of this Introduction will review three classes of antimicrobial studied in this project: the quinolones, the potentiated sulphonamides, and the ß-lactams.

1.2 THE QUINOLONE ANTIMICROBIAL AGENTS

1.2.1 The Development of the Quinolones. The quinolones, also called fluoroquinolones, 4-quinolones and quinolone carboxylic acids, are analogues of the earlier developed agent nalidixic acid. Nalidixic acid was originally isolated by Lesher and associates (1962) from a distillate during chloroquine synthesis and thus was a by-product of antimalarial research. Additional older analogues include pipemidic acid, cinoxacin and oxolinic acid which is currently licensed for use in aquaculture in Scotland.

The newer quinolones are substantially more potent *in vitro* and broader in antibacterial spectrum than nalidixic acid (Andriole, 1988). Furthermore, they have relatively long half lives in serum, excellent penetration into many tissues and permeation into mammalian cells, resulting in antimicrobial activity against intracellular pathogens.

Nalidixic acid and the newer quinolones have been reviewed (Crumplin, 1990; Andriole, 1988; Wolfson & Hooper, 1989). They are wholly synthetic and structurally related compounds have not been identified as products of living organisms. More than a thousand quinolones and analogues have been synthesised and evaluated for antimicrobial activity, including compounds with substitutions and additions to many parts of the molecule. Moieties attached to N-1, a fluorine attached to C-6, and moieties such as a piperazinyl or methylpiperazinyl group attached to C-7, seem to be particularly important to increased antibacterial activity.

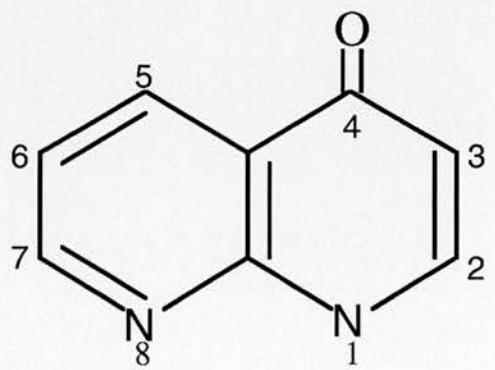


Figure 3. The Basic 4-Quinolone Skeleton.

The favourable potency *in vitro* and the pharmacological properties of the quinolones predicted potential for treatment of a variety of bacterial infections, and both clinical studies and medicinal use of these compounds have borne out this promise (Norris & Mandell, 1988). However, oxolinic acid is currently the only quinolone antimicrobial agent licensed for use in veterinary medicine in the United Kingdom.

1.2.2 DNA Gyrase: Bacterial Topoisomerase II. The primary target of the quinolone agents is DNA gyrase (bacterial topoisomerase II: EC number 5.99.1.3) (Gellert *et al.* 1976) and it is central to their mechanism of action.

Deoxyribonucleic acid (DNA) is a linear, double helical structure comprising a sequence of the four nucleotide bases, adenine, thymine, cytosine and guanine. This molecule encodes genetic information, allows mutation and recombination, and serves as a template for semiconservative replication, DNA repair and transcription.

The configuration of the DNA molecule leads to certain potential conformational difficulties. The first problem occurs because the DNA must be condensed within the cell. For the bacterium *Escherichia coli,* the chromosome is a circular DNA molecule 1,100μm in length (Cairns, 1963) present in a cell of only 1 to 2 μm in length. This DNA molecule, despite its 1000-fold condensed state, must be able to replicate, segregate into daughter chromosomes, and allow transcription of individual genes, without becoming lethally entangled.

A second problem is a result of the helical nature of the DNA duplex. For the *E.coli* chromosome, which contains 4×10^6 base pairs, the strands are intertwined about 400,000 times generating a linking number for *E.coli* of 400,000. To allow semiconservative replication to occur, therefore, the two strands must unwind 400,000 times. Cairns (1963) recognised that, to permit the unwinding of the DNA double helix during strand separation, a 'swivel' mechanism would be required to prevent the molecule from becoming tangled in front of the replication fork as it advanced.

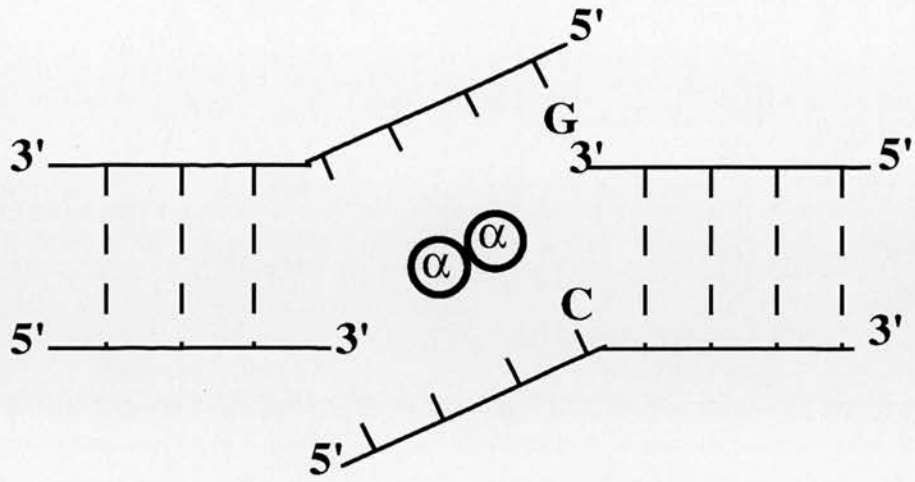


Figure 4. The α-subunits of DNAgyrase introduce nicks into the DNA strands at specific sites 4 base pairs apart (Source; Bayer, UK).

A third problematic situation arises in prokaryotes. Their DNA double helices contain negative supercoils which reduce the number of helical turns thus facilitating strand separation (Wang, 1974). However, these supertwists are energetically unfavourable thus an energy requiring process is needed within the cell for their generation.

For prokaryotic DNA, the problems of entanglement, strand unwinding, and negative supercoiling are solved, at least in part, by topoisomerases (Gellert, 1981; Wang, 1985). A topoisomerase is an enzyme that alters the number of times one single strand of a DNA duplex winds around its complementary strand; that is, a topoisomerase alters the linking number of a double stranded DNA molecule. Such DNA molecules which differ in linking number *only* are called topological isomers. Topoisomerases may be categorised into three groups: type II topoisomerases (prokaryotic DNA gyrase), type I topoisomerases, and special topoisomerases (Wang, 1985). In 1976, Gellert and associates isolated the first topoisomerase II, DNA gyrase, from *E.coli.* DNA gyrase is a tetramer containing two α subunits, 100 kilodaltons in mass and encoded by the *gyrA* gene located at 48 min. on the *E.coli* map, and two β subunits, 90 kilodaltons in mass and encoded by the *gyrB* gene located at 83 min. on the *E.coli* map. The α subunits introduce

nicks into each strand of the DNA helix at intervals of four base pairs. The action of the β subunits of the gyrase enzyme is the ATP-dependent introduction of extra twists into both strands of the helix. Once this has been affected, the α subunits reseal the 'nicked' strands, and stability and tension in the DNA helix are restored (fig. 4)(Wang, 1985).

Reactions catalysed by purified DNA gyrase include introduction of negative supercoils into DNA, catenation and decatenation of interlocked covalently closed circular DNA molecules, and formation and resolution of knots within the DNA duplex. These reactions require ATP and a divalent cation, optimally magnesium, for activity (Wang, 1985).

DNA gyrase and topoisomerase I within the living bacterial cell set the net level of negative supercoiling of DNA, with the introduction of negative supercoils by DNA gyrase and their removal by topoisomerase I. The amount of intracellular DNA gyrase and topoisomerase I is regulated at transcription level in response to the superhelicity of DNA: decreasing the negative supercoiled state of intracellular DNA stimulates transcription of the *gyrA* and *gyrB* genes and suppresses the transcription of the *topA* gene; increasing the superhelicity of intracellular DNA represses *gyrA* and *gyrB* transcription whilst inducing transcription of *topA* (Wang, 1985).

1.2.3 Inhibition of Bacterial Topoisomerase II by the Quinolone Antimicrobial Agents. Quinolone antimicrobial agents have marked effects on DNA gyrase and the bacterial cell. Many important consequences of exposure of bacteria to the quinolones nalidixic acid and oxolinic acid were determined prior to the discovery of DNA gyrase. In the 1960s, Goss (1964, 1965) and his co-workers (Deitz *et al.*,1966; Cook *et al.*,1966) reported that nalidixic acid selectively antagonised DNA synthesis, caused DNA degradation and induced filamentation of bacteria.

In 1977 Gellert and associates and Sugino *et al.* reported experiments that defined the α subunit of DNA gyrase as the primary target for oxolinic acid and nalidixic acid. Quinolone resistance mutations affecting DNA gyrase occur most frequently in the *gyrA* gene for *E.coli*, however two mutations have also been identified in the *gyrB* gene (Inoue *et al.*,1978; Yamagishi *et al.* 1981, 1986). Thus the β subunit as well as the α subunit, is a target for

quinolones, perhaps by direct interaction of the drug with the subunit or alternatively by indirect action via the α subunit. As a result of this interaction, nalidixic acid, oxolinic acid and other quinolones antagonise almost all activities of purified DNA gyrase, including supercoiling, catenation and decatenation, and unknotting (Crumplin *et al.* 1984; Gellert, 1981).

1.2.4. Inhibition of Bacterial Growth in the Presence of the Quinolones. Antagonism of bacteria by quinolones results in decreased introduction of negative supercoils into DNA, decreased decatenation of interlocked DNA circles, and damage to DNA (Cook *et al.*, 1966; Crumplin *et al.*, 1984; Deitz *et al.*, 1966; Drlica, 1984; Gellert, 1981; Goss *et al.*, 1964;1965; Inoue *et al.*, 1978; Smith, 1984; Wang, 1985;1987; Yamagishi, 1981). Induction of these changes requires higher drug concentrations in *gyrA* mutants indicating that gyrase is involved in these phenomena. The result of drug exposure is interference with DNA synthesis, demonstrated by an immediate halt in the uptake of DNA precursors. At high drug concentration, RNA and protein synthesis are also impaired and filamentation occurs as a result of induction of the SOS DNA repair system (Piddock and Wise, 1987).

In a review, Drlica (1984) hypothesised that the quinolones may complex with both DNA and DNA gyrase, blocking the replication fork thereby inducing the SOS DNA repair response. However, Lewin *et al.* (1991) demonstrated that SOS deficient mutants are still susceptible to the action of the quinolones. They suggested that recombination repair played a greater role in quinolone activity, as *rec* mutants were hypersensitive to nalidixic acid. *lex* mutants, which are SOS deficient but have an operational recombination repair system, are no more sensitive to nalidixic acid.

1.2.5. Killing of Bacteria by Quinolones. Most of the quinolone antimicrobials, particularly the newer drugs, do not merely inhibit the growth and division of susceptible bacterial strains. Exposure of most susceptible bacterial species to these agents results in rapid cell death (Smith, 1984a; Lewin & Hastings, 1989).

At concentrations above the minimum inhibitory concentration (MIC), the lethality of the drugs increases until a concentration known as the optimum bactericidal concentration (OBC) is reached, beyond which the bactericidal activity then declines. It has been suggested that this biphasic response results from the inhibition of bacterial RNA synthesis by the 4-quinolones at concentrations above the OBC (Smith, 1984; Smith & Lewin, 1988). It is possible that this occurs because the inhibition of DNA gyrase may cause relaxation of chromosomal DNA to such an extent that it can no longer be efficiently transcribed into RNA. Bacterial protein and RNA synthesis are prerequisites for the full bactericidal activity of the 4-quinolones (Smith & Lewin, 1988). For example, rifampicin, an inhibitor of bacterial RNA synthesis, completely abolishes the bactericidal activity of nalidixic acid against *E.coli* (Smith & Lewin, 1988).

Studying the bactericidal activity profiles of the quinolones has led to an improved understanding of the killing mechanisms of these drugs. As mentioned above, bacterial protein and RNA synthesis are essential for full lethality of the 4-quinolones. However, while the addition of chloramphenicol, an inhibitor of protein synthesis, or rifampicin, an inhibitor of RNA synthesis, completely inhibited the bactericidal activity of nalidixic acid against *E.coli* in nutrient broth they merely reduced, but did not completely abolish, the bactericidal activity of ciprofloxacin or ofloxacin in nutrient broth (Smith, 1984; Smith & Lewin, 1988). Furthermore, nalidixic acid was not lethal in phosphate buffered saline where bacteria cannot divide, whilst both ciprofloxacin and ofloxacin were active in this medium (Smith, 1984). It was therefore proposed that nalidixic acid displayed a single bactericidal mechanism, termed mechanism A, which requires protein and RNA synthesis as well as cell division for its activity, and that this is the principal mechanism of action of all 4-quinolones. Furthermore, Smith (1984) suggested that ciprofloxacin and ofloxacin possessed a second bactericidal mechanism, mechanism B, which was not dependent upon protein or RNA synthesis, and was active against non-dividing bacteria (table 2). Other newer fluoroquinolones have also been shown to exert mechanism B in addition to mechanism A. Although norfloxacin is unable to kill bacteria in the absence of protein or RNA synthesis as rifampicin or chloramphenicol completely abolish its bactericidal activity (Lewin, Amyes & Smith, 1989). However, unlike nalidixic acid, norfloxacin is active against non-dividing

Mechanism A	Mechanism B	Mechanism C
All 4-quinolones (only mechanism displayed by older drugs)	Ciprofloxacin Ofloxacin DR-3355 Lomefloxacin Pefloxacin Fleroxacin	Enoxacin Norfloxacin

Table 2. Categorisation of 4-quinolones by bactericidal mechanism. All fluoroquinolones so far tested display an additional mechanism of action, in addition to mechanism A, against *Escherichia coli.*

cells. It has therefore been proposed that it displays a third mechanism, termed C, in addition to mechanism A (Ratcliffe & Smith, 1985). It has also been shown that enoxacin exerts mechanism C (Lewin *et al.,* 1989). Thus, many of the fluoroquinolones appear to exert a second mechanism of action in addition to mechanism A, the principal bactericidal mechanism of all 4-quinolones (table 2).

Although the precise molecular events leading to the death of bacteria exposed to the 4-quinolones are not clear, several proposals have been made. The SOS genes are a set of genes encoding DNA repair enzymes which are induced by the 4-quinolones (Piddock & Wise, 1987) and it has been proposed that one of the SOS genes may code for a lethal protein (Philips *et al.,* 1987). Indeed, Walters and co-workers (1989) have implicated the SOS gene, *sfiA,* in the lethality of the 4-quinolones. However, mutations that disable the SOS response either have no effect or increase the susceptibility of bacteria to nalidixic acid (Lewin *et al.,* 1989a). These results would not be expected if an SOS gene encodes the lethal protein, and it has been suggested that the hyper-susceptibility of some SOS deficient mutants is a result of deficient recombination repair rather than SOS repair (Lewin *et al.,* 1989a). Thus recombination repair appears to play a role in restoring DNA damaged by the 4-quinolones.

Cook *et al.* (1966) suggested that, since DNA breakdown occurred in bacteria treated with nalidixic acid, the lethal protein might be an exonuclease and that bacterial death may result from degradation of chromosomal DNA. However, recent studies have demonstrated that this is not the case as DNA breakdown was shown to occur under conditions where nalidixic acid was not bactericidal (Lewin & Smith, 1990). Furthermore there was no correlation between DNA breakdown and the rate of cell death caused by the quinolones. Lewin and Smith (1990) have suggested that DNA breakdown occurs as a result of exonuclease V involvement and may merely serve as a signal for the induction of the SOS response, since exonuclease V activity is required for the quinolones to induce this response.

Protein and RNA synthesis, as well as bacterial cell division, are not the only factors which can affect the bactericidal activity of the quinolones. Multivalent metal ions have also been shown to affect the lethality of these drugs (Smith & Lewin, 1988). Varying the inoculum size has little or no effect on the MICs of the quinolones (Smith, 1984a), however an inoculum size effect is exhibited at the OBC with *E.coli* and *Staphylococcus aureus* (Smith & Lewin, 1988). Bactericidal activities are normally determined with a starting inoculum of 10^6 colony forming units per millilitre (cfu/ml), but it was found that if the initial inoculum size was increased to 10^8 cfu/ml, a reduction in bactericidal activity of ciprofloxacin and ofloxacin occurred at their respective OBCs. Furthermore, when the initial inoculum was increased to 10^{10} cfu/ml, the activity of both quinolones was reduced to mere bacteriostasis. The inoculum size effect could not be attributed to pH or multivalent ion concentration, or indeed to any destruction of the antibiotics at the higher initial inoculum sizes (Smith & Lewin, 1988). Aeration of cultures of high initial inoculum did, however, restore some of the bactericidal activity, suggesting that lack of oxygen may be the cause of the inoculum size effect (Smith & Lewin, 1988; Morrissey *et al.* 1990). This explanation seems valid as both ofloxacin and ciprofloxacin are bacteriostatic against *E.coli* and *Staph. aureus* under anaerobic conditions (Smith & Lewin,1988; Lewin, Morrissey & Smith, 1989; Morrissey *et al.*, 1990).

As the 4-quinolones affect DNA gyrase, DNA supercoiling may account for the lack of bactericidal activity under anaerobic conditions. DNA superhelicity has been implicated in the control of expression of certain genes involved

in anaerobicity in both *E.coli* and *Salmonella typhimurium* (Yamamoto & Droffler, 1985; Dorman *et al,* 1988). Obligate aerobic mutants of *S. typhimurium* have been found to be DNA gyrase deficient, implying that gyrase activity is essential for anaerobic growth. Furthermore, there is evidence linking anaerobic conditions with DNA supercoiling and with 4-quinolone uptake. Anaerobicity can influence the supercoiling of cellular DNA (Dorman *et al.,* 1988), which is subsequently responsible for the regulation of a number of specific genes. The ompC porin genes are among those genes induced by anaerobicity via a supercoiling regulated system (Bhriain *et al.* 1989). As the quinolones are known to enter *E.coli* via ompF and ompC (Hirai *et al.,* 1986a, 1986b; Hooper *et al.,* 1986), it is interesting to postulate that some form of altered porin configuration under anaerobic conditions may decrease quinolone uptake and thus bactericidal activity.

In summary, the bactericidal activity of all 4-quinolones is biphasic owing to a requirement for RNA and protein synthesis for full bactericidal activity. This biphasic response is less pronounced with the newer fluoroquinolones due to the possession of an additional mechanism of action, termed either B or C. All of the newer fluoroquinolones are active against non-dividing cells whereas the older drugs such as oxolinic, nalidixic, pipedimic acids require cell division for their activity. Bacterial cell division, protein and RNA synthesis do not appear to be the sole requisites for full activity of these drugs as oxygen has been shown to be essential for the drugs currently available to kill bacteria.

1.2.6. Mechanisms of Resistance to the Quinolone Antibacterials. Resistance to quinolones has been reviewed by Wolfson *et al.* (1989). A number of genes that can mutate to cause bacterial resistance to the quinolones have been identified. These genes in *E.coli* include *gyrA* (*nalA, nfxA, norA, cfxA* and *ofxA*) and *gyrB* (Yamagishi *et al.* 1981, 1986) encoding DNA gyrase (table 3). Other mutant loci (table 4), include *nfxB, cfxB, norB, norC* and *marA,* which appear to result in altered drug permeation.

The majority of DNA gyrase alterations so far identified occur in the *gyrA* gene encoding the A subunits of the enzyme. Recently, nucleotide sequences have been determined for *gyrA* genes isolated from two strains of *E.coli* selected for spontaneous resistance to nalidixic acid and two strains selected

DNA gyrase gene	Resistance mutations
gyrA	*nalA, nfxA, ofxA, norA, cfxA;* cause resistance to quinolones only.
gyrB	*nalC*; resistance to nalidixic acid and hypersusceptibility to certain fluoroquinolones; *nalD;* resistance to nalidixic acid and fluoroquinolones.

Table 3. Quinolone resistance mutations in genes encoding DNA gyrase in *Escherichia coli.*

for resistance to pipemidic acid (Yoshida *et al.,* 1988). In each mutant gene a single base pair was changed. The four mutations were located in close proximity in a sequence near the N-terminus of the *gyrA* polypeptide. The four sites were located in hydrophilic regions of the peptide, suggesting they reside near the surface of the α subunit protein. The mutations were also found at ser 83, close to the codon for tyrosine 122, which has been shown to be the site of covalent attachment of the α subunit to DNA when the enzyme cleaves DNA in the presence of quinolones (Horowitz & Wang, 1987). Nucleotide sequences have also been determined for the two *gyrB* mutations, *nalC* and *nalD* (Yamagishi *et al.,* 1981,1986). Mutations at the *nalD* locus cause increased resistance to the newer quinolones, particularly those with piperazinyl substitutions, while *nalC* mutants exhibit increased susceptibility to new quinolones. Each mutation results in a single nucleotide change in the midportion of the coding sequence. These alterations encode single amino acid changes which alter the protein charge in opposite directions, suggesting that charge is in some way related to drug-enzyme interaction (Yamagishi *et al,* 1986).

The second class of mutations is characterised by low level resistance to quinolones and structurally unrelated drugs. The pleiotropic nature of these mutations suggest that they are a result of alterations in drug uptake or permeability. This hypothesis is supported by the observations of Cohen and co-workers (1989). In their study, it was found that resistance to fluoroquinolones could be selected with tetracycline or chloramphenicol.

Selected with:	Resistance mutation	Characteristics
Nalidixic acid	*nalB, nalD, crp, cya, icd, purB, ctr.*	
Norfloxacin Ciprofloxacin	*nfxB, norB, norC cfxB*	Likely permeation mutants; resistant to quinolones and structurally unrelated drugs. Decreased OmpF porin production; decreased norfloxacin accumulation; altered LPS.
Tetracycline or Chloramphenicol	*marA*	Decreased accumulation of tetracycline; nalidixic acid resistance.

Table 4. Quinolone resistance in *Escherichia coli* as a result of mutations in genes other than *gyrA* and *gyrB*.

This cross resistance was a result of two changes in outer membrane proteins (OMPs), namely the porins OmpC and OmpF. The alterations resulted in a change in outer membrane permeability as a result of increased expression of the narrow channelled porin OmpC, coupled with decreased expression of the wider channelled porin OmpF. *norB* and *norC,* two additional mutants selected with norfloxacin in *E.coli*, also appear to result from decreased drug permeation (Hirai *et al.,* 1987). *norC* causes hypersusceptibility to hydrophobic quinolones such as nalidixic and oxolinic acids, detergents and dyes, and exhibits both decreased OmpF expression and altered lipopolysaccharides in the outer membrane (Hirai *et al.,* 1987).

For most classes of antibiotic, resistance genes may be carried chromosomally or may be encoded on extrachromosomal portions of DNA, plasmids. With a single, as yet unconfirmed, apparent exception (Munshi *et al.* 1987), mutations causing quinolone resistance are located exclusively on the bacterial chromosome, despite an extensive search (Courvalin, 1990). This apparent absence of plasmid mediated quinolone resistance has an important consequence. Resistance determinants carried on the bacterial chromosome

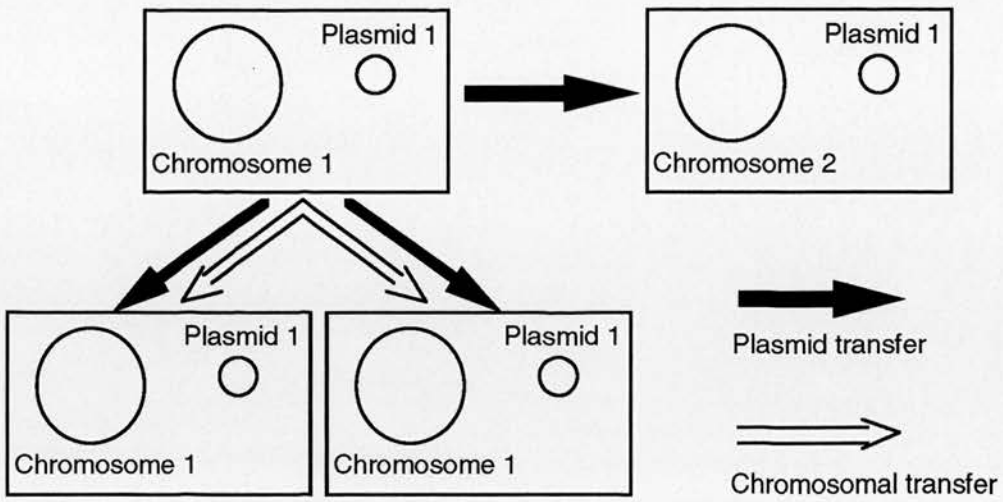


Figure 5. Transfer of genetic material encoding antibiotic resistance.

are generally restricted to vertical transmission, i.e. from mother to daughter cells at cell division (fig. 5). On the other hand, plasmids are frequently transferred laterally between bacteria of the same, or even different, species or genera (fig. 5). The result of this is that resistance carried on plasmids tends to spread significantly more rapidly than chromosomally determined resistance. Four attributes of quinolones and quinolone resistance may contribute to the scarcity of plasmid mediated resistance (Wolfson *et al.* 1989):

1). Wild type susceptible genes are dominant to many *gyrA* mutations encoding quinolone resistance (Hane & Wood, 1969; Lampe & Bott, 1984; Robillard, 1990; Swanberg & Wang, 1987; Yoshida *et al.,* 1988). This dominance pattern suggests that transfer of a mutant *gyrA* gene into a susceptible cell would not be likely to generate a resistant phenotype in the absence of gene conversion by recombination with a wild-type allele.
2). Expression of mutations which decrease outer membrane permeability is delayed when introduced into wild-type cells (Foulds, 1976). It seems fair to assume, therefore, that quinolone resistance determined by Omp alterations may also be delayed.
3). Plasmid conjugation is inhibited by quinolones (Burman, 1977a, 1977b; Nakamura *et al.,* 1976).

4). Some plasmids are eliminated from bacteria by sub-inhibitory concentrations of quinolones (Platt & Black, 1987; Weisser & Wiedemann, 1985).

For these reasons, the spread of quinolone resistance by plasmid mediated transfer seems unlikely, particularly in the presence of a quinolone agent. It must be stressed, however, that, in spite of the absence of plasmid mediated resistance to this class of compounds, there are therapeutic difficulties in certain bacterial species, notably *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The difficulties in *Ps.aeruginosa* can be explained by the poor uptake of the antibiotics by these bacteria, thus therapeutically effective levels of antibiotic are difficult to achieve, and are maintained for shorter periods. *Staph.aureus*, although occasionally treated with quinolones, is hardly an appropriate target as these drugs generally have poor anti Gram-positive activity (Norris & Mandell, 1988).

1.3. THE POTENTIATED SULPHONAMIDES.

The sulphonamides were the earliest wholly synthetic antibacterials, Prontosil being discovered by Domagk in 1935 for which he received the Nobel prize. The selective antimicrobial activity of Prontosil was attributed to the release of sulphonilamide which inhibits dihydropteroate synthetase. This enzyme exists only in bacteria but not in animals hence the drug is selective. (Tréfouel *et al.,* 1935; Colebrook *et al.,* 1936).

In all areas of antibacterial chemotherapy, resistance to the sulphonamides is widespread (Lacey *et al.,* 1972). Thus the efficacy of these compounds has been greatly enhanced by active potentiation with 2,4-diaminopyrimidines such as trimethoprim, and potentiated sulphonamides have now almost entirely replaced the sulphonamides in clinical use.

1.3.1. Folic Acid Biosynthesis. Trimethoprim and sulphonamides inhibit successive steps in the bacterial synthesis of tetrahydrofolic acid, a co-factor essential in many of the metabolic processes of the living cell (Bushby & Hitchings, 1968)(fig 6.). Folic acid or folate, is usually synthesised *de novo* as it may not be supplied exogenously. Brown (1959) found enzymes in *Escherichia coli* that used substituted pteridines for the formation of pteroic acid in the presence of *p*-amino benzoate (PABA). Brown *et al.* (1959) found that pteroic acid could only be formed from certain substituted pteridines and that PABA was a more efficient substrate for pteroate synthesis than *p*-aminobenzyl glutamate was for folate synthesis. This suggested that pteroate was an intermediate of folate synthesis. In a subsequent study, Brown *et al.* (1961) found that that dihydropteroate was a more effective precursor than the unreduced compound and proposed a pathway whereby PABA combined with dihydropteridine to form dihydropteroate (fig 6). This reaction had a requirement for ATP and magnesium ions, suggesting a phosphorylated pteridine intermediate may be involved (Jaenicke and Chan, 1960). Brown *et al.* (1961) proposed that condensing enzymes formed dihydrofolate from dihydropteroate and glutamic acid. Dihydrofolate was then reduced to tetrahydrofolate by a dihydrofolate reductase (Blakely & McDougall, 1961).

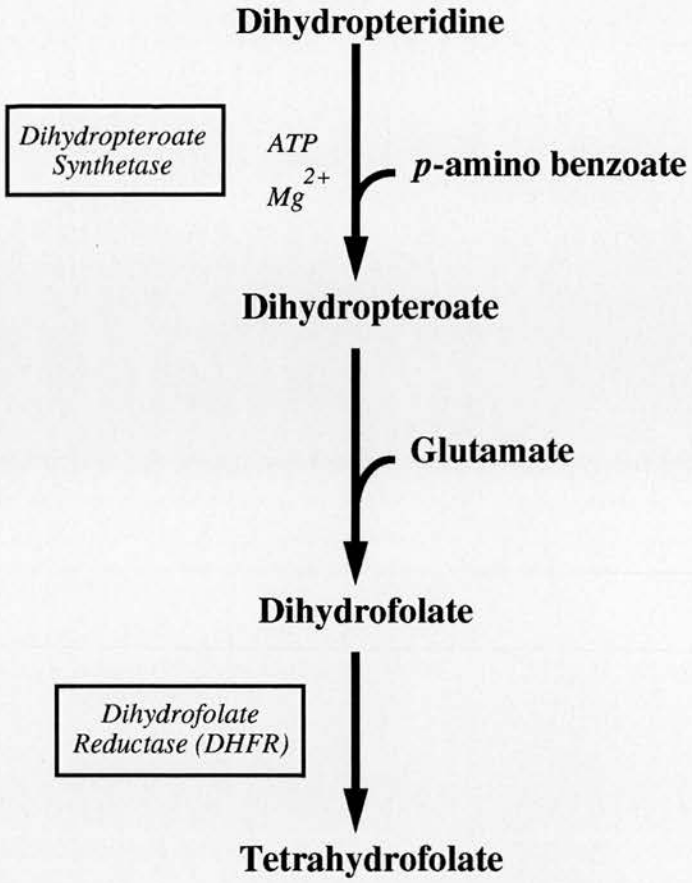


Figure 6. Pathway for the biosynthesis of reduced folate compounds in bacteria.

1.3.2. Mechanism of Action of Folate Inhibitors. Sulphonamides were noted to inhibit the production of folic acid (Nimmo-Smith *et al.,* 1948). Bacteria which were able to utilise exogenous folic acid were not affected by sulphonamide antimicrobials (Woods, 1954). However it was only when the pathway describing the synthesis of folic acid was deduced that their mechanism of action was determined (Brown, 1962). Brown (1962) deduced that sulphonamides inhibited the incorporation of PABA into folates by competition with the PABA condensing enzyme, dihydropteroate synthetase.

The 2,4-diaminopyrimidines, such as trimethoprim and ormetoprim, inhibit bacterial dihydrofolate reductase, an enzyme that converts dihydrofolate into an active reduced form, tetrahydrofolate (Hitchings & Burchall, 1965). 2,4-diaminopyrimidines are folate analogues which bind competetively

with dihydrofolate reductase (DHFR)(Hitchings & Burchall, 1965). Hitchings and Burchall (1965) also demonstrated that these folate analogues were more effective at binding bacterial DHFR than the mammalian counterpart. Burchall (1971) suggested that the mammalian enzyme had evolved further than the bacterial DHFR, enabling it to distinguish between the pteridine substrate and the diaminopyrimidine analogue. Thus a basis for chemotherapy was established.

As sulphonamides and 2,4-diamino pyrimidines act in the same biochemical pathway, their combined effect can sometimes be greater than the expected additive interaction. This is known as synergy (Bushby & Hitchings,1968).

Synergy between 2,4-diaminopyrimidines and sulphonamides is generally measured by the effect of each drug on the level of inhibition of the other. That is, the effect each drug has on the MIC of the other. Therefore, it is bacteriostatic synergy that is measured and this can be quantified by a 'checker-board' titration (Bushby, 1973). This synergystic effect is exploited in the potentiated sulphonamides.

1.3.3. Resistance to Potentiated Sulphonamides. Resistance to the potentiated sulphonamides is frequently plasmid-mediated. The biochemical mechanism of plasmid-mediated resistance to both sulphonamides and the dihydrofolate reductase inhibitors involves target enzymes resistant to the action of the antimicrobial. In the case of the sulphonamides, this is a drug resistant dihydropteroate synthetase, while for trimethoprim and ormetoprim it is a drug-resistant dihydrofolate reductase (Amyes & Towner, 1990; Skold, 1976; Wise & Abou-Donia, 1975). There are two known genes coding for plasmid-borne sulphonamide-resistant dihydropteroate synthetase and these have been termed *sul-I* and *sul-II* (Radstrom & Swedberg, 1988). There are over ten different plasmid-borne trimethoprim-resistant dihydrofolate reductases which can be differentiated by DNA:DNA hybridisation and biochemical characteristics (Amyes & Towner, 1990).

1.4. THE β-LACTAM ANTIMICROBIAL AGENTS.

1.4.1. The Development of the β-Lactams. The early success of penicillin prompted large scale research programmes in the pharmaceutical industry resulting in the development of the semisynthetic antimicrobials penicilllins G and V. Penicillin G was unstable at low pH and had no activity against Gram negative pathogens. Penicillin V, whilst being more stable at low pH, and thus suitable for oral administraion, was still poorly active against the Gram negatives.

The first semisynthetic penicillin to show significant activity against Gram-negative as well as Gram-positive pathogens was ampicillin. Ampicillin, however, was found to be poorly taken up into the serum of the patient resulting in high residues in the gut and thus potential for the development of a reservoir of resistant commensal flora. A slight modification to the side chain of ampicillin, however, gave rise to a molecule with similar antibacterial properties but with greatly improved pharmacokinetics. This molecule was named amoxycillin (fig. 7).

1.4.2. Mechanism of Action of Amoxycillin. β-lactam antimicrobials act by inhibiting the synthesis of peptidoglycan. Peptidoglycan is a complex, regular matrix which gives rigidity to the bacterial cell envelope, enabling the cell to withstand the relatively high internal osmotic pressure. Peptidoglycan is a highly ordered structure consisting of amino sugars and amino acids. The repeating units, N-acetyl muramic acid and N-acetyl glucosamine, coupled to their pentapeptide side chains, are synthesised within the cytoplasm while bound to the nucleotide UDP; they are then transferred to lipid carrier molecules that facilitate their movement across the hydrophobic membrane. Finally they are polymerised into peptidoglycan on the outside of the membrane by enzymes located on the membranes outer surface (Rogers *et al.*, 1980). It is during this cross linking, or transpeptidation reaction that the synthesis pathway is susceptible to attack by penicillins (Fig. 8). The β-lactam antibiotics appear to be able to bind to the cross-linking enzyme, alanine transpeptidase resulting in its inactivation. Penicillins are thought to be structural analogues of D-alanyl-D-alanine, the dipeptide in the amino acid side chains involved in cross-linking, and the

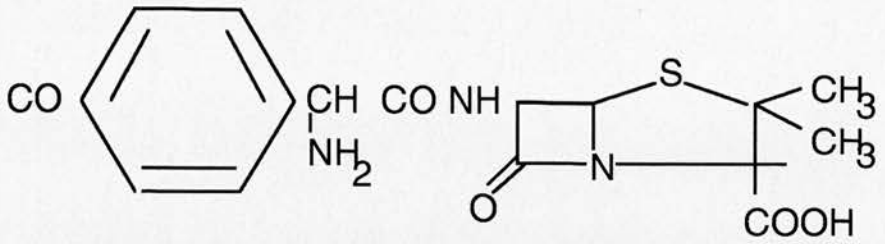


Figure 7. Amoxycillin, a derivative of ampicillin with improved pharmacokinetics and now licensed for aquaculture.

normal substrate of alanine transpeptidase (Waxman & Strominger, 1983). Thus the β-lactam is drawn to the active site of the transpeptidase normally occupied by the D-alanyl-D-alanine part of the pentapeptide side chain attached to the N-acetylmuramic acid. It seems that the antibiotic forms an irreversible bond with the enzyme preventing it from catalysing the cross-linking reaction by permanently occupying the active site. This appears to be the primary mechanism of action of the β-lactam antibiotics. However, the cross-linking of peptidoglycan occurs at multiple sites in the cell each catalysed by distinct enzymes.

Furthermore, the β-lactams have been shown to inhibit biochemical reactions which are not transpeptidations, specifically the actions of endopeptidases (Waxman & Strominger, 1983). These enzymes catalyse the hydrolysis of the peptidoglycan structure into different subunits. Thus it appears that the β-lactams have several biochemical reactions as their targets in the bacterial cell.

1.4.3. Resistance to the β-Lactams. There are several biochemical mechanisms by which an organism may resist β-lactam antibiotics: 1) decreased intracellular drug level, i.e modification of the cell envelope reducing the permeability to the drug; 2) destruction of the drug by β-lactamase enzymes; 3) an increase in target enzyme concentration; 4) repair or by-passing of the drug sensitive reaction and 5) decreased affinity of the receptor for the drug (Weidemann & Tolxdorff-Neutzling, 1985).

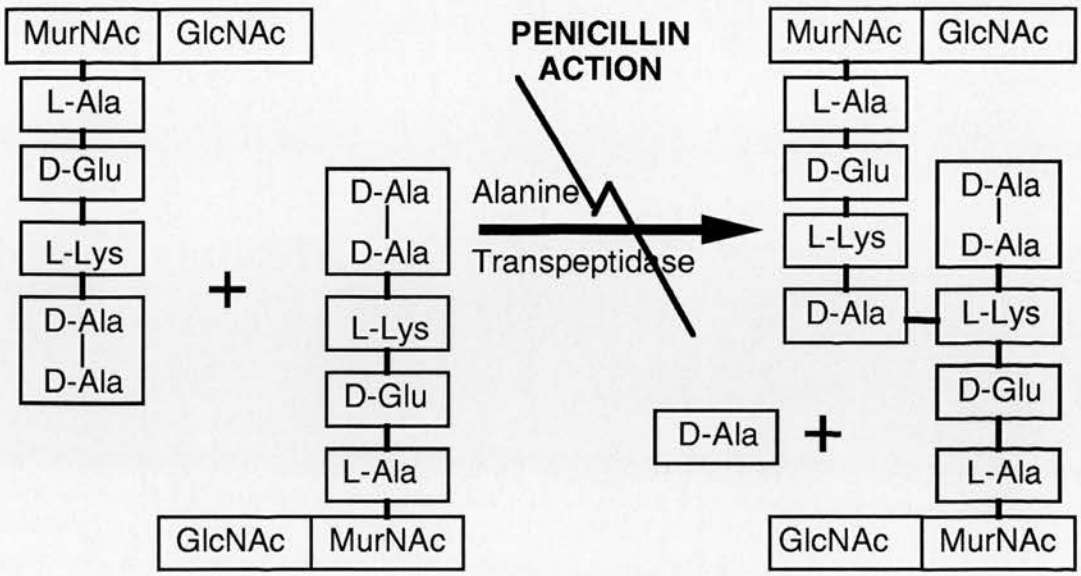


Figure 8. Action of penicillin on cross-linking of peptidoglycan.

Reduced permeability of the outer membrane of Gram negative pathogens can lead to resistance to most classes of β-lactam antibiotic, and cross resistance to unrelated classes of drug (Rella & Haas, 1982; Goldstein *et al.*, 1983; Bush *et al.*, 1988; Cohen *et al.*, 1989). The targets of the β-lactam antibiotics are a series of enzymes involved in cell wall synthesis in bacteria and are known collectively as penicillin binding proteins (PBPs). Clinical strains of pathogens with reduced or modified affinity for the penicillin binding proteins (PBPs), are being isolated with increasing frequency (Bryan, 1988).

β-lactamases, enzymes that bind and destroy β-lactam antibiotics, are the main cause of resistance to this class of antibiotics. They act by cleaving the lactam ring between C6 and the adjacent nitrogen moiety (fig. 9). It has been suggested that β-lactamase enzymes may have evolved from the penicillin sensitive enzymes of cell wall synthesis (Tipper & Strominger, 1965). Indeed, several PBPs have been found to exhibit β-lactamase activity and, furthermore, some homology has been demonstrated between the low molecular weight PBPs and β-lactamases (Waxman & Strominger, 1983).

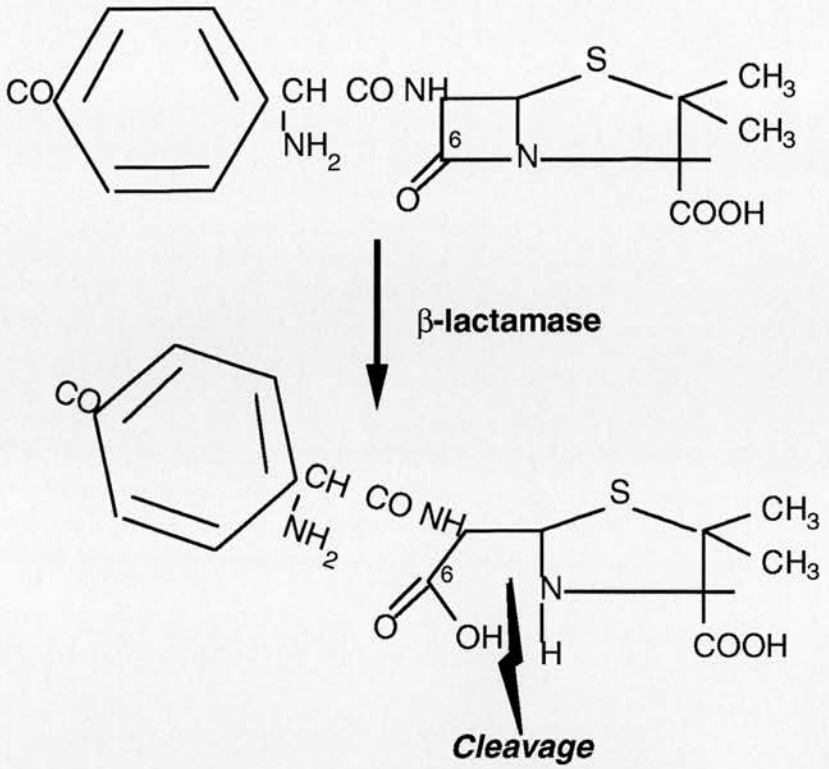


Figure 9. Action of β-lactamase on amoxycillin.

β-lactamases are identified on the basis of their subtrate hydrolysis profiles, molecular weight, and the point at which they focus on polyacrylamide gels containing carrier ampholines of various pH ranges, i.e. their isoelectric point (pI) (Matthew, 1976).

In Gram negative pathogens, β-lactamase production may be either plasmid mediated, or encoded on the chromosome. More than sixty plasmid encoded β-lactamases have been identified in Gram negative bacteria (Weidemann, 1989). The most prevalent of these enzymes is TEM-1. For example in *E.coli* of which almost 25% of isolates are ampicillin resistant (Kresken & Wiedemann, 1987), 70-100% of the resistance is due to TEM-1 or TEM-2. Furthermore, TEM-1 is responsible for most of the ampicillin resistance in *Salmonella* (Roy *et al.,* 1983, 1985; Simpson *et al.,* 1986). Indeed, TEM-1 has been found in all Enterobacteria so far examined along with strains of *Vibrio, Acinetobacter, Haemophilus,* and *Neisseria* (Roberts *et al.,* 1977;

Matthew, 1979; Dillon *et al.,* 1983; Joly-Guillon *et al.,* 1988).

Chromosomally determined β-lactamases are readily detected in Gram-negative bacteria such as the Enterobacteriaceae, *Ps.aeruginosa* (Sykes & Matthew, 1976), *Campylobacter* (Fleming *et al.,* 1982) and *Aeromonas* spp. (Bakken *et al.,* 1988; Hedges *et al.,* 1989; Iaconis & Sanders, 1990). Chromosomally mediated β-lactamases are generally species-specific enzymes (Matthew *et al.,* 1975). However, it has been demonstrated that some chromosomally determined enzymes can be mobilised and thus transferred to other bacterial species (Hedges *et al.,* 1985). A further characteristic of chromosomal β-lactamases is their inducibility. That is, different β-lactam antibiotics can stimulate the production of β-lactamases to varying extents (Minami *et al.,* 1980; Sanders *et al.,* 1982; Gootz & Sanders, 1983). It has been reported that new forms of β-lactamase occasionally arise after induction, though it is unclear whether these are new enzymes or merely a modification of the original β-lactamase (Sanders *et al.,* 1982).

β-lactamases of *Aeromonas* spp. have been widely identified and characterized (Hedges *et al.,* 1985; Bakken *et al.,* 1988; Iaconis & Sanders, 1990). These enzymes are mostly chromosomal, inducible, and active against penicillins, cephalosporins and carbapenems (Hedges *et al.,* 1985; Bakken *et al.,* 1988; Iaconis & Sanders, 1990). When analysed by isoelectric focusing (IEF) the enzymes so far identified in strains of *A.hydrophila, A.caviae* and *A.sobria* have pIs ranging between 5.9 (AER-1, Hedges *et al.,* 1985) and 8.4 (A2s, Iaconis & Sanders, 1990). Interestingly, AER-1 resembles plasmid determined carbenicillinases in substrate profile but differs in pI (5.9) and molecular weight (22,000) (Hedges *et al.,* 1989). No evidence for a plasmid locus has been found in *A.hydrophila*, but the AER-1 gene and resistance to chloramphenicol, streptomycin, and sulphonamide can be transferred to *E.coli* with *IncP* plasmids (Hedges *et al.,* 1985). The linked resistances are similar to those found on multi-resistant β-lactamase transposons. However the insertion of the *A.hydrophila* gene is site specific and *recA* dependent, suggesting that this is not a functioning transposon (Hedges *et al.,* 1985). In spite of the plethora of papers puplished on

Aeromonas β-lactamases, none of these studies have included *Aeromonas salmonicida.* Thus, the investigation of β-lactam resistance in this species will be included in this study.

1.5. CONTROL OF FURUNCULOSIS BY ANTIMICROBIAL CHEMOTHERAPY.

The use of chemicals in fish culture for the control of fungal, bacterial and parasitic infections has a long history and has been reviewed extensively (Meyer & Schnick, 1978; Meyer, 1989; Alderman, 1988; Alderman & Michel, 1991).

Antibiotics from the three classes described in this introduction are licensed for use in aquaculture in Scotland.

1.5.1. Folate Inhibitors in Aquaculture. Use of the sulphonamides in aquaculture has been reviewed by Alderman (1988). The sulphonamides were some of the first antibiotics made available for aquaculture use. The first sulphonamide, sulphanilamide, was tested on fish in 1937 (Tunison & McCay, 1937), and Gutsell (1948) demonstrated the efficacy of sulfamerazine and sulfathiazole in controlling furunculosis in brook trout *(Salvelinus fontinalis)*. In his study, Gutsell demonstrated that oral administration of 8g per 100lb fish per day of sulfamerazine was sufficient to control an outbreak of furunculosis in brook trout. In a similar study, Snieszko and Bullock (1957) found that 200mg/kg fish/day sulfamerazine could significantly reduce furunculosis mortalities in brook trout. Allison (1956) demonstrated that combinations of sulphaguanidine and sulphadiazine were equally effective in brook trout. Studies in Atlantic salmon demonstrated that intra-peritoneal (IP) injection of sulphisoxazole and sulphadimethoxine could effectively control furunculosis. However, sulphadimethoxine was found to cause abscesses at the injection site (Amend & Fryer, 1968). Amend *et al.* (1969) also found that sulphisoxazole was better absorbed from the feed by chinook salmon than sulphametazine and was more active against *A.salmonicida in vitro.*

More recent studies found that *A.salmonicida* was becoming resistant to sulphonamide antimicrobials when used alone (McCarthy *et al.*, 1974a). Indeed Aoki *et al.* (1983) found that 54 out of 129 *A.salmonicida* isolates recovered from culture ponds had MICs of 100mg/l or greater, and only 12 out of 46 isolates recovered from rivers had MICs below 25 mg/l. Aoki

(1988) reported that between 70 and 100 per cent of *Vibrio anguillarum* strains isolated in Japan between 1973 and 1977 were resistant to sulphonamides. However, the percentage of resistance decreased between 1978 and 1983, ranging between 6 and 20%, depending on year.

Although trimethoprim has been marketed for use on its own in human medicine since its patent expired in 1979, it tends to be used in aquaculture in combination with a sulphonamide. Nevertheless, several authors have examined the *in vitro* activity of ormetoprim and trimethoprim against *A.salmonicida.* Aoki *et al.* (1983) found that trimethoprim MICs ranged between 1.6 and >1000mg/l against *A.salmonicida* isolated from culture ponds in Japan, although the highest MIC found in *A.salmonicida* isolated from rivers was 3.1mg/l. In the same study, Aoki reported that ormetoprim MICs ranged between 0.1 and 400 mg/l in culture pond isolates, and between 1.6 and 12.5 mg/l in river isolates.

In human medicine, the effective life of sulphonamides has been increased by potentiation with a 2,4-diaminopyrimidine, trimethoprim. Potentiated sulphonamides are available for aquaculture use. In the US, Romet 30, a 1:5 combination of ormetoprim and sulphadimethoxine was licensed in 1984 (Maestrone, 1984; Meyer, 1988), and Tribrissen (1:4 combination of trimethoprim and sulphadiazine) was licensed in the UK in 1987 (K. Treeves-Brown, Pers.Comm). There are numerous reports of *in vitro* activity of potentiated sulphonamides against *A.salmonicida* (McCarthy *et al.*, 1974a,b,c; Inglis *et al.*, 1991a,b; Toranzo *et al.* 1991; Giles *et al.*, 1991). With the exception of the studies by McCarthy *et al,.* (1974a,b,c) most of the studies may be questioned due to the experimental techniques employed. Maestrone (1984), Inglis *et al.,* (1991a,b), Toranzo *et al.,* (1991) and Giles *et al.,* (1991), evaluated Romet 30, Co-trimoxazole, Tribrissen, or combinations of Sulphadiazine and trimethoprim in the ratios in which the drugs are supplied by the manufacturers. The ratios which are achieved *in vivo* rarely reflect the ratios in which the drugs are supplied due to the varying pharmacokinetic properties of the antibiotics (McCarthy, 1977b; D.Love, Pers.Comm.). Pharmacokinetics are of vital importance to the activity of potentiated sulphonamides as active potentiation may only occur in a very narrow range of ratios. Indeed, the optimum ratio of trimethoprim

to sulphadiazine is 1:20 (McCarthy *et al* (1974b), however the two components are presented in a 1:4 ratio in Tribrissen (1:4). In the case of Romet 30, which is supplied as 1:5 ormetoprim to sulphadimethoxine, the optimum ratio appears to be 1:20 (D.Love, Pers.Comm). The ratio of the components of Romet achieved *in vivo* has not yet been reported. Toranzo *et al.,* (1991) determined MICs for Romet and Tribrissen on Brain Heart Infusion Agar (BHIA) containing 1% NaCl. Use of BHIA may lead to erroneous results as the presence of thymine in the medium antagonises folate inhibitors and can, in the presence of amino acids and purines, reverse trimethoprim induced death (Amyes and Smith, 1974). McCarthy *et al.* (1974a,b,c) conducted a series of studies into the potentiation of a variety of sulphonamides with trimethoprim in fish. In these studies, seven sulphonamides were tested. Little difference between them was observed *in vitro,* however sulphamethylphenazole (SMP) had the most favourable pharmacokinetic properties. McCarthy *et al.* (1974b) found that the ratio of SMP to trimethoprim achieved in rainbow trout by admixture with feed (200mg sulphonamide:20mg trimethoprim:1kg fish) was approximately 20:0.75, close to the optimum ratio for maximum antibacterial effect of these two drugs against *A.salmonicida in vitro.* However, these favourable pharmacokinetics may not necessarily apply to Atlantic salmon. Indeed there are few published data on the pharmacokinetics of potentiated sulphonamides in Atlantic salmon. Since the ratio of the levels of the two components of these drugs achieved *in vivo* is essential for active potentiation, this dearth of information may be a serious failing.

In the present study, the optimum concentrations of the components of Romet 30 *in vitro* against *A.salmonicida* will be investigated. The efficacy of the combination as supplied by the manufacturer will be evaluated for the control of furunculosis in Atlantic salmon.

1.5.2. Quinolones in Aquaculture. The earliest application of quinolone antibiotics to fish pathogens was reported by Endo *et al.,* (1973) who tested nalidixic acid and oxolinic acid against *A.salmonicida,* both *in vitro* and *in vivo.* In 1983, Aoki *et al.,* also reported favourable results with oxolinic acid *in vitro,* demonstrating that over 90% of isolates from a culture pond were susceptible to oxolinic acid at 0.8mg/l. The results for *A.salmonicida*

isolated from rivers were more impressive, with all but one isolate susceptible to 0.4 mg/l. The first reports of successful control of furunculosis by a quinolone in Britain were made by Austin *et al.* (1983), who found that oxolinic acid administered orally at 10mg/kg/day could significantly reduce mortalities in brown and rainbow trout. Oxolinic acid was subsequently licensed for aquaculture use in the UK (K. Treeves-Brown, Pers.Comm.), however resistance to the drug was soon reported (Hastings & McKay, 1987; O,Grady *et al.,* 1987). In human medicine, the quinolones such as nalidixic and oxolinic acid have been superceded by the fluoroquinolones (Andriole, 1988). Several fluoroquinolones have been investigated *in vitro* and *in vivo* for use in aquaculture.

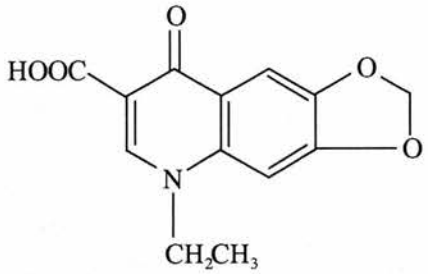
Flumequine, one of the earliest fluorinated 4-quinolones, has been investigated as a potential fish chemotherapeutant. In 1980, Michel *et al.* reported that oral administration of flumequine was successful in the control of furunculosis in salmonids. Chevalier *et al.* (1981) reported favourable pharmacokinetics and acceptable withdrawal periods (ie rapid absorption and elimination in rainbow trout at 12°C). Chevalier *et al.* (1981) employed two dose rates, 6mg/kg and 12mg/kg, and found that both dosing regimens resulted in elimination to the limit of detection within 72hours. O'Grady *et al.* (1988) reported that elimination of flumequine following bath administration of the antibiotic (50ppm in distilled water) was slower, with 1mg/l still present 14days following treatment. However, the levels of flumequine achieved in serum by bathing were very much higher than those determined by the oral route of Chevalier *et al* (1980). Elimination of flumequine following IP injection was slower than following bath administration, with 3mg/l being recorded 14 days after administration (O'Grady *et al.,* 1988). Scallan & Smith (1985) demonstrated that the carrier state of *A.salmonicida* infection could be successfully eliminated by bathing in a flumequine bath.

The fluoroquinolone enrofloxacin has been investigated for both *in vitro* and *in vivo* activity against *A.salmonicida* (Bragg & Todd, 1988; Tsoumas *et al.,*1989; Bowser & House, 1990; Bowser *et al.* 1990; Inglis *et al.,* 1991; Bowser & Babish, 1991; Bowser *et al.,* 1992). Favourable MIC data were obtained; Tsoumas *et al.* (1989) reported MICs ranging between 0.00024 and 0.5 mg/l. Similar results were reported by Bowser & House (1990), and

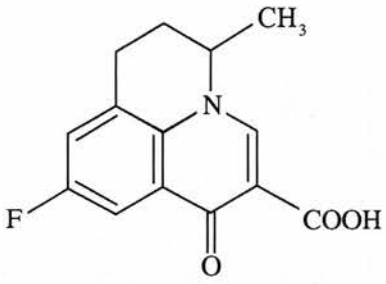
Inglis *et al.* (1991). In spite of these favourable *in vitro* results, Bowser *et al.* (1990) reported a failure to control furunculosis in Atlantic salmon with enrofloxacin. This was attributed to low temperature and poor feeding leading to low drug levels in the treated fish. It is interesting to speculate that adequate levels of antibiotic may have been achievable by ultra-fine preparation of the drug prior to administration, as increased bioavailability has been reported with ultra-fine size reduction of oxolinic acid in yellowtail (Takahashi & Endo, 1987; Endo *et al.,* 1987a) and in sea bream (Endo *et al.,* 1987b). In a subsequent study Bowser & Babish (1992) concluded that treatment of furunculosis in brook trout (*Salvelinus fontalis,* L.) x lake trout *(Salvelinus namaycush,* L.) hybrids with enrofloxacin was successful. However, the mortality data presented in this paper were ambiguous and inconclusive. The pharmacokinetic data, however, were clearer, and levels in excess of 8mg/l antimicrobial activity were detected in liver and skin, and 3.37 mg/l in muscle (Bowser & Babish, 1992).

Stamm *et al.* (1986) reported favourable *in vitro* activity of sarafloxacin against a range of human pathogens, and a low potential to induce resistance (Fernandes *et al.,* 1987). Markwardt & Klontz (1989) claimed that sarafloxacin was capable of eliminating an artificially induced "carrier state" of furunculosis in salmonids. The first report of *in vitro* activity of sarafloxacin against *A.salmonicida* was published by Stamm (1989) who reported an MIC of 0.06mg/l. However, only one isolate was used in this study, thus the significance of the results is limited. In a more recent survey, Stamm (1991) investigated the *in vitro* activity of sarafloxacin against 47 isolates of *A.salmonicida.* In this study, he reported that MICs ranged between 0.008 and 2.00mg/l. The only pharmacokinetic data available on sarafloxacin have been provided by Markwardt & Klontz (1989) who found that the maximum levels were attained in serum when Tween 80 was added to a 50mg/l bath treatment of sarafloxacin. However the data presented in this study were for a 10 minute treatment only and the levels attained were low (0.09mg/l).

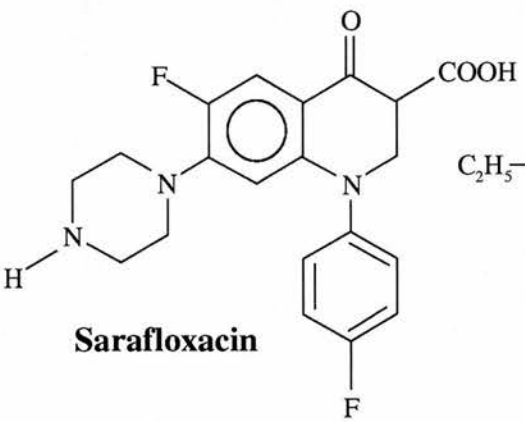
A search of the literature has failed to provide any published information on the other fluoroquinolones investigated in this study (PD127,391, PD117,596, Ro-09 1168 and CI934), in terms of fish pathogens.



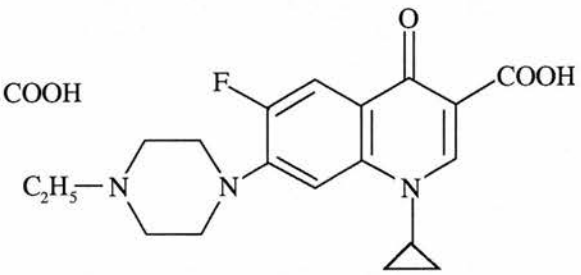
Oxolinic Acid



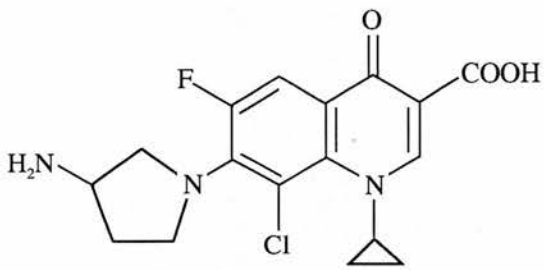
Flumequine



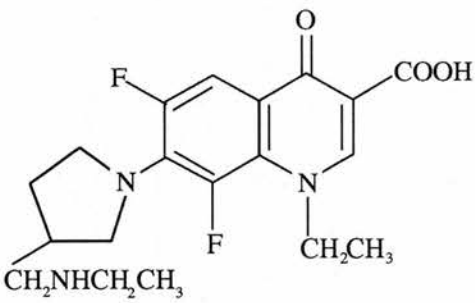
Sarafloxacin



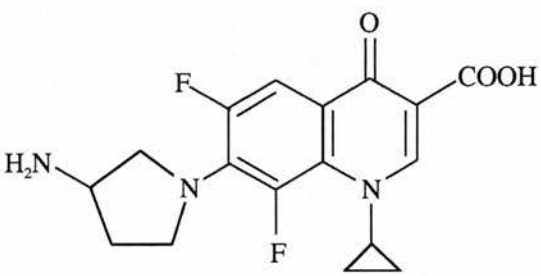
Enrofloxacin



PD127,391



CI934



PD117,596

Figure 10. Fluoroquinolones investigated in this study (NB. Structure for Ro 09 1168 not available).

1.5.3. Amoxycillin in Aquaculture. Amoxycillin is a recent addition to the antibiotics licensed for use in aquaculture in Scotland. Inglis & Richards (1991) found MICs ranging between 0.16 and 0.3 mg/l, however their study only included seven isolates of *A.salmonicida.* They also reported a limited temperature effect with 7% of isolates requiring a higher concentration of amoxycillin to inhibit their growth at 10 °C compared to 22°C. Inglis and Richards (1991) found that amoxycillin was poorly bactericidal, with 30% of bacteria surviving 3h exposure to 5mg/l of the antibiotic, a concentration of 9 x MIC. There appear to be no published pharmacokinetic data relating to the use of amoxycillin in fish.

1.5.4. Bacterial Resistance in Aquaculture. Despite the long history of the use of medicines in aquaculture, it was only in the late 1960s, when the rapid expansion of fish farming coincided with increasingly widespread use of antibiotics, that some observers remarked on the potential hazards of drug use in the aquatic environment. As in the case of other farmed animals, attention was drawn to the increasing occurrence of drug resistance.

Drug resistance problems have become increasingly evident over the past ten years (Hastings & Mackay, 1987; O'Grady *et al.,* 1987; Richards *et al.*, 1990) possibly due to the availability of only a limited number of compounds really suited to the control of aquatic pathogens, and their repeated therapeutic use (Aoki *et al.,* 1981; Aoki *et al.,* 1983; Tsoumas *et al.,* 1989).

One of the most serious examples of this problem has occurred recently in marine salmon farms in Scotland. Only two antimicrobials were available for use on these sites in the mid 1980s, oxytetracycline, for which transmissible plasmid-mediated resistance has been well documented (Aoki, 1988), and oxolinic acid. Both were used extensively during the rapid expansion of the salmon farming industry at this time and, as a result, resistance to these compounds became well established (Hastings & Mackay, 1987; O'Grady *et al.,* 1987; Tsoumas *et al.*, 1989; SOAFD, 1990).

With the exception of amoxycillin, which has only been available for use for a limited period of time, resistance to all aquaculture antibacterials is widespread in Scotland (Richards *et al.,* 1991; Inglis *et al.,* 1991). This observation, coupled with the prevalence of furunculosis in Scottish aquaculture emphasises the need for new antibiotics. Against this background, the fluoroquinolone antibacterials, amoxycillin and the potentiated sulphonamide Romet 30, have been examined.

1.6. AIMS OF THIS THESIS.

1. Fluoroquinolones which have potential for use in aquaculture will be investigated *in vitro* against *A.salmonicida,* and the bactericidal activity of the fluoroquinolones under study will be investigated.

2. Inhibitory and bactericidal concentrations of the quinolones *in vitro* will be compared with the levels which may be achieved in Atlantic salmon *in vivo.*

3. Potential mechanisms of resistance to the fluoroquinolone antibacterial agents in *Aeromonas salmonicida* will be studied.

4. The *in vitro* activity of the two components, individually and in combination, against *A.salmonicida* will be examined. The efficacy of Romet 30 in controlling a laboratory induced outbreak of furunculosis in Atlantic salmon will also be investigated.

5. The prevalence and mechanisms of resistance to the ß-lactam antibiotic amoxycillin in Scottish islolates of *A.salmonicida* will be investigated.

6. A sea water infection challenge model will be developed and methods of disinfecting effluent seawater so that clinical studies can be undertaken without risk of releasing *A.salmonicida* into the aquatic environment.

MATERIALS & METHODS

2.1. BACTERIAL STRAINS.

One hundred and thirty-seven isolates of *Aeromonas salmonicida* subsp. *salmonicida,* recovered predominantly from natural outbreaks of furunculosis in farmed salmonids in Scotland, between 1986 and 1989, were used in this study. A further nine isolates of *A.salmonicida* subsp. *achromogenes* isolated from wild salmonids over the same period were also obtained (Appendix A). All isolates were received from the Scottish Office Agriculture and Fisheries Department (SOAFD) Marine Laboratory, Aberdeen. The bacterial isolates were stored at -70°C in Tryptic Soy Broth (TSB, Oxoid, UK) containing glycerol (10%). Working cultures were stored on Tryptone Soya Agar (TSA, Oxoid, UK) plates at 4°C.

2.2. ANTIBIOTICS.

The fluorinated 4-quinolones enrofloxacin (Bayer, UK), PD127,391, PD117,596, CI934 (Parke-Davis, UK), Ro 09-1168 (F. Hoffman-la Roche, Switzerland),as well as amoxycillin (Amoxil®, Bencard, UK), Augmentin (SmithKline-Beecham, UK) and Oxytetracycline (Sigma, UK) were weighed aseptically and dissolved in sterile distilled water. Oxolinic acid (Sigma, UK), Sarafloxacin (A-56620, Abbott, Nth. Chic. Ill.) and flumequine (Apoflu®, Apothokernes Laboratorium A.S., Norway) were dissolved in 0.5N NaOH (0.02ml/mg) and diluted to the required concentration in sterile distilled water. Romet 30, ormetoprim and sulphadimethoxine were supplied by F. Hoffmann La-Roche, Basle, Switzerland. For *in vitro* studies, ormetoprim was dissolved in 1.0N lactic acid; sulphadimethoxine was dissolved in 0.5N NaOH. All drug solutions were prepared fresh on the day of use. Drugs were stored as dry powders in darkness at 4°C.

2.3. MEDIA.

2.3.1. Complex media. The following complex media were used; Tryptic soy broth (TSB), Tryptone Soya Agar (TSA) IsoSensitest Broth, Diagnostic

Sensitivity Test Agar (DSTA), Brain Heart Infusion Broth (BHIB), all supplied by Oxoid, Basingstoke, UK. Bacto Marine Agar (BMA) was supplied by Difco, UK.

Chemicals were supplied by Sigma Chemical Co Ltd., Poole, UK, unless otherwise stated.

2.3.2. DM Minimal Salts Medium. Double strength minimal salts medium was prepared as described by Davis & Mingioli (1950).

2.4. *IN VITRO* ACTIVITIES OF ANTIBACTERIAL AGENTS.

The *in vitro* activity of a range of antibacterials against the fish pathogen *A.salmonicida* was determined in terms of minimum inhibitory concentration, bactericidal activity, and the frequency at which mutation to resistance to the drugs occurred.

2.4.1 Determination of Minimum Inhibitory Concentrations (MICs). MICs were determined against 38 oxolinic acid-resistant and 45 oxolinic acid-susceptible isolates of *A.salmonicida.* Employing the criteria proposed by Tsoumas *et al.* (1989), strains for which the MIC of oxolinic acid was less than 1.0 mg/l were deemed oxolinic acid susceptible, whereas strains for which the MIC was equal to or greater than 1.0 mg/l were deemed oxolinic acid resistant. MICs were determined on TSA by an agar dilution technique. The dilution scheme followed either the arithmetic schedule 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 7.5; or a doubling dilution schedule where stated. The antibiotic plates were inoculated with a multipoint inoculator (Denley, UK) delivering approximately 10^5 cfu/spot and were incubated aerobically overnight at 22°C in a cooled incubator (Gallenkamp, UK) unless otherwise stated. The MIC was defined as the lowest concentration of antibiotic at which all visible growth was completely inhibited. The technique was validated using type strains of *Escherichia coli* and *Staphylococcus aureus.* Consistency of results was ensured by including *Aeromonas salmonicida* MT363 as a standard on every plate.

2.4.2. Effect of Inoculum Size on Antibacterial Activity of the Quinolones. Antibacterial activities, in terms of MIC, of oxolinic acid, sarafloxacin, enrofloxacin, PD117,596, PD127,391, and CI934 were determined against 20 isolates of *A.salmonicida* essentially as described above except that initial inocula of 10^2, 10^4 and 10^6 cfu per spot were employed.

2.4.3. Effects of Environmental Conditions on Antimicrobial Activity. The effects of temperature on the 4-quinolones was examined by determining MICs as described above (2.3.1) except that the plates were incubated at 10°C for 48hrs before calculating the inhibitory concentration. The antibacterial activities against *A.salmonicida,* of oxytetracycline, amoxycillin, oxolinic acid, sarafloxacin, and flumequine were determined on TSA, Bacto Marine Agar 2216 (Difco, UK) and TSA supplemented with 50mM $MgCl_2$ or 340mM NaCl, to elucidate the effect of these seawater cations on the antibacterials. Plates were incubated at 22°C for 24 hrs and MICs were calculated as described above.

2.4.4. Estimation of Bactericidal Activity of the Quinolone Antimicrobials. The bactericidal activities of the quinolones were estimated essentially as described by Lewin *et al.* (1989a), employing two oxolinic acid resistant isolates, MT364 and MT472, and two oxolinic acid susceptible isolates, MT363 and MT736 (Table 5). An overnight culture of the isolates containing approximately 10^8 cfu/ml was diluted 1 in 50 in fresh tryptic soy broth containing the various antibacterial agents. The antibacterial concentrations followed the arithmetic progression 1.5, 3, 5, and 9. One hundred microlitre samples were taken after 1h incubation at 22°C in the case of the newer fluoroquinolones, and after 1, 3 and 6h incubation at 22°C in the case of oxolinic acid and flumequine, and were serially diluted in TSB. Percentage survival was estimated by viable counting on TSA plates. Experiments were performed in triplicate and mean values calculated.

Isolate	Oxolinic acid	Oxyte-tracycline	Sarafloxacin	Enrofloxacin	CI934	PD117,596	PD127,391
MT363	0.03	0.2	0.05	0.015	0.4	0.015	0.0075
MT364	7.5	0.3	3.0	1.0	3.0	0.3	0.1
MT472	3.0	0.3	2.0	1.0	2.0	0.2	0.1
MT490	0.03	0.2	0.05	0.02	0.4	0.015	0.0075
MT494	0.03	0.2	0.04	0.02	0.4	0.015	0.0075
MT736	0.015	100.0	0.04	0.04	0.3	0.015	0.01
MT744	0.03	0.2	0.05	0.015	0.4	0.015	0.0075
MT747	0.03	0.2	0.03	0.02	0.4	0.015	0.0075

Table 5. MICs (mg/l) of strains used in mutation frequency and bactericidal activity experiments

2.4.5. Mechanisms of Bactericidal Action of the Veterinary Quinolones. The mechanisms of action of the fluoroquinolones enrofloxacin, sarafloxacin and Ro 09-1168 were investigated as described by Smith (1984a). Bactericidal activity was estimated, as described above, in PBS pH7.0, to assess the activity of the agents against non-dividing bacteria. To determine the action of these drugs when RNA or protein synthesis was inhibited, the bactericidal activities were determined in the presence of 4.0mg/l rifampicin and 2.0mg/l chloramphenicol respectively. Briefly, overnight cultures of *A.salmonicida* MT363 were diluted 1 in 50 into universals of fresh TSB containing the either rifampicin or chloramphenicol at the appropriate concentration, plus a series of dilutions of the quinolone under investigation as described above. After 1 hour of incubation at 22°C, samples (0.1ml) were taken and serially diluted into fresh TSB before estimation of survival by viable counting on TSA plates.

2.5. MECHANISMS OF QUINOLONE RESISTANCE.

2.5.1. Stability of Quinolone Resistance in *A.salmonicida*. MICs of oxolinic acid, sarafloxacin, enrofloxacin, PD127,391, PD117,596 and CI934 were determined for 20 oxolinic acid-resistant isolates of *A.salmonicida* after 10 successive passages of the strains on drug free TSA plates.

2.5.2. Frequency of Mutation to Resistance to the Quinolones and Oxytetracycline. Mutation frequencies were determined for five quinolone-

susceptible isolates of *Aeromonas salmonicida* MT363, MT490, MT494, MT744, MT747 (table 5) according to the method of Smith (1986).

Conical flasks containing 250 ml drug-free TSB were inoculated with 4.5ml overnight starter cultures of *A.salmonicida* and incubated at 22°C overnight with shaking. The resulting cultures were harvested by centrifugation at 11,500 x *g* and resuspended in 2 ml fresh TSB to give cell concentrations in excess of 10^{10} cfu/ml. Aliquots (0.1 ml) of undiluted, 10^{-1}, 10^{-2}, 10^{-3} & 10^{-4} dilutions were then spread onto TSA plates containing the various antibiotics at 5, 10, and 20 times their respective MICs. The plates were incubated at 22°C for 7 days and examined daily for the presence of resistant colonies. Resistant colonies were subcultured onto TSA plates containing antibiotic at the same concentration to verify resistance and the colonies were identified to species level with a latex bead agglutination kit (Aquaculture Vaccines Ltd., UK).

2.5.3. Preparation of Outer Membrane Proteins (OMPs). Quinolone and oxytetracycline resistant mutants isolated from mutation frequency experiments, along with wild type *A.salmonicida* isolates exhibiting low level resistance to the fluoroquinolones, were investigated for outer membrane alterations.

Outer membrane proteins were prepared from 1 litre TSB cultures shaken for 48 hours at 22°C. Cells were harvested by centrifugation at 10,000 x *g* for 15 minutes and lysed by sonication at 8μm for 30 second bursts totalling 3 minutes with an ultrasonic disrupter (MSE soniprep, MSE instruments, UK). The lysate was then centrifuged twice at 15,000 x *g* to remove whole cells and the inner membranes were solubilised by addition of lauryl sarcosinate (Sarkosyl) to a concentration of 0.7 %. The outer membranes were then precipitated by ultracentrifugation at 100,000 x *g* for 1h at 4°C. The membranes were then washed with approximately 7 ml pyrogen-free water (Milli-Q, Millipore, UK) and harvested by centrifugation at 100,000 x *g* for 1h at 4°C. The precipitated membranes were finally resuspended in 1 ml Milli-Q water.

2.5.4. Analysis of Outer Membranes by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Outer membrane proteins were resolubilised prior to electrophoresis by boiling in sodium

Isolate	MICs (mg/l)			
	Oxolinic acid	**Enro-floxacin**	**Sara-floxacin**	**Ro 09-1168**
MT438	1.50	0.20	1.00	0.20
MT362	5.00	0.75	2.00	0.40
MT487	5.00	1.00	2.00	0.10

Table 6. Quinolone resistant isolates of *A.salmonicida* employed in transformation experiments

dodecyl sulphate (SDS) for 5 min. The OMPs were analysed on gel gradient of 10-15% polyacrylamide using the PhastSystem® (Pharmacia LKB, Sweden). A coomassie brilliant blue R (Sigma) staining technique was employed to visualise the separated protein bands.

2.5.5. Examination of OMPs for Non-Covalent Association with Peptidoglycan. To determine whether the OMPs were non-covalently associated with peptidoglycan, the method of Darveau *et al.* (1983) was followed. Briefly, cell envelopes, isolated as described above, were solubilised in buffer containing 2% (w/v) SDS, 10% (w/v) glycerol, and 10mM Tris-HCl (pH 7.4) at 37°C for 30 min. The pellet obtained after centrifugation at 100,000 x *g* for 1 h was resuspended in the same buffer containing 0.1M NaCl. The resulting suspension was analysed by SDS-PAGE as described above.

2.5.6. Probing for DNA gyrase mutations. Genetic studies have shown that a quinolone sensitive *gyrA* gene is dominant over the quinolone resistant allele (Hane & Wood, 1969; Nakamura *et al.* 1989). Two gene probes have been made to screen bacteria for *gyrA* mediated quinolone resistance; a broad host range probe containing quinolone sensitive *gyrA* from *E.coli,* pNJR3-2, using a cosmid vector (Robilllard, 1990) and a probe, pBP513, on a high copy number plasmid (Heisig & Wiedemann, 1991).

pNJR 3.2 was supplied by Bayer, UK, and pBP513 was supplied by Dr Eddie Power, UMDS, London, UK with the kind permission of Dr Peter Heisig.

The two plasmids, carrying the wild type *E.coli gyrA* gene were used to probe high level quinolone resistant isolates of *A.salmonicida* (table 6).

L-F Base	L-F Broth	L-F Recovery Agar	SMM	SMMB
BHI (1%) Sucrose (10%) Yeast Extract (0.5%)	L-F Base + Horse serum (deactivated & added when cool) (1%)	L-F Base + Agar No. 3 (1%)	Sucrose 0.5M Maleate 0.02M MgCl 0.02M (pH6.5, adjusted with NaOH)	SMM mixed with an equal volume of 4x BHIB

Table 7. Media used in protoplast transformation experiments.

pUC19, a high copy number plasmid, was used as a negative control to ensure that transformation *per se* did not alter quinolone resistance.

Minipreparations of plasmid DNA were obtained by the alkaline lysis method of Takahashi & Nagano (1984).

Competent cells for transformation were prepared as follows; fifty millilitres of brain heart infusion broth (BHIB, Oxoid) at 22°C was inoculated with 0.5ml overnight culture of the required *A.salmonicida* isolate and incubated at 22°C to give a culture of OD_{660} = 0.15-0.2. Cultures were chilled on ice and cells harvested by centrifugation at 4°C. Pellets were resuspended in 12.5 ml ice-cold calcium chloride (10mM) and left on ice for 15min. The suspensions were centrifuged, resuspended in 75mM calcium chloride and kept at 4°C for 24 hours.

Transformations were carried out based on the methods of Saunders & Saunders (1988). Briefly, 0.2ml competent cells were mixed in Eppendorf tubes with plasmid DNA (30µl) and made up to 0.5ml with ice-cold 75mM calcium chloride. The transformation mixture was kept on ice for 30 minutes and then transferred to a 40°C water-bath for 2 min. BHIB (0.5ml) was then added and incubation continued at 22°C for 4h to allow expression of the plasmid. Dilutions of the transformation mixture were then plated onto DST

agar plates containing the appropriate antibiotics (20 or 40mg/l tetracycline for pNJR3-2; 20mg/l kanamycin for pBP513; 20 mg/l ampicillin for pUC19) and transformants selected after 48h at 22°C.

The presence of the relevant plasmid in the transformants was determined by agarose gel electrophoresis. Plasmids, prepared as described previously (Takahashi & Nagano, 1984) were electrophoresed in 0.7% agarose minigels in buffer A (40mM Tris-acetic acid, 2mM EDTA (pH8.0)) at 100V for 2h. Plasmid DNA was stained with ethidium bromide and visualized with ultraviolet light (330nm).

2.5.7. Transformation of Protoplasts. *A.salmonicida* protoplasts were prepared according to the method of Dr D I McIntosh (personal communication), based on the technique of Wyrick and Rodgers (1973). L-F broth (10ml)(McIntosh & Austin, 1988) (table 7) was inoculated with *A.salmonicida* and incubated overnight at 22°C. The resulting cultures were transferred to 50ml fresh L-F broth and incubated with shaking for 4 hours. Benzyl penicillin was added to a concentration of 50 mg/l and the incubation continued statically overnight. The cultures were then examined by phase contrast microscopy to determine whether protoplasts had been formed. The protoplasts were harvested by centrifugation at 3500 x *g* for 15min and washed in 10ml fresh L-F base (table 7). The washed protoplasts were reharvested and suspended in 10ml SMMB (table 7) before dilution to one tenth of the concentration of the starting culture (approx. 10^7 cfu/ml).

Protoplasts were transformed by a method adapted from that of Chang & Cohen (1979). Serial dilutions of plasmid pNJR 3,2 in TE buffer (10mM Tris.HCl, 1mM EDTA, pH8.0) were mixed with an equal volume of double strength SMM (table 7). To this mixture was added protoplast solution (0.5ml) followed by immediate addition of 40% w/v Polyethyleneglycol (PEG) (1.5ml). After 2min, 5ml L-F Base was added to dilute the PEG and the protoplasts were recovered by centrifugation at 3500 x *g* for 15min. The pellets were resuspended in 1ml L-F base and incubated for 6hours to allow expression of the plasmid. Transformants were selected on L-F recovery agar containing tetracycline (40mg/l) and incubated at 22°C for up to 5 days.

2.6. *IN VITRO* ASSESSMENT OF ROMET.

This work was carried out in collaboration with C.J.Thomson in our laboratory.

2.6.1. Minimum Inhibitory Concentrations (MICs). The minimum inhibitory concentrations of ormetoprim and sulphadimethoxine, the components of Romet, were determined. In addition to this, the 2,4-diaminopyrimidine trimethoprim was included in this study for comparison. MICs of the antifolate inhibitors were determined on Oxoid DSTA plates incorporating the various chemotherapeutants according to a doubling dilution schedule. Overnight cultures of the bacteria in nutrient broth were centrifuged and washed in DM minimal medium. They were then resuspended to their original cell density in fresh DM medium. The viable count of the cell densities was established such that they could be diluted sufficiently in order that 2µl would give approximately 10^5cfu. The antibiotic plates were inoculated with a multipoint inoculator (Denley, UK) delivering 2µl/spot (10^5 cfu) and incubated for 48h at 22°C. Results were read at both 24h and 48h, though the results at 48h were recorded as they were poorly defined after 24h.

2.6.2. Fractional Inhibitory Concentrations (FICs). FIC Indices were determined by the two dimensional serial dilution procedure devised by Bushby (1973). Twenty strains were selected for further study. The MICs of ormetoprim and sulphadimethoxine were repeated; however, on this occasion the determination was performed on agar plates containing the various antibiotics at concentrations following an arithmetic dilution schedule. This progression gives a closer determination of their true MIC values. To determine the fractional inhibitory concentrations, the inhibitory concentrations of ormetoprim and sulphadimethoxine were determined in the presence of sub-inhibitory concentrations of the other drug. An isobologram was constructed for each test organism and the FIC of each drug at the point of maximum interaction was determined. The sum of these two FICs, the FIC index, gives a quantifiable determination of the drugs' interaction. A value of <0.7 indicates synergy, 0.7-1.0 indicates an additive effect, >1.0-2.0 indicates indifference and >2.0 indicates antagonism.

2.7. RESISTANCE TO AMOXYCILLIN.

Amoxycillin resistant isolates of *A.salmonicida* were selected to determine the mechanisms of resistance to this class of antibiotics.

Substrate Antibiotic	λ_{max} (nm)	Final Concentration (M)
Benzyl-penicillin	238	10^{-3}
Carbenicillin	238	10^{-3}
Ampicillin	238	10^{-3}
Cefotaxime	265	10^{-4}

Table 8. β-lactam substrates used in hydrolysis assays.

2.7.1. Preparation of Crude β-Lactamase Enzyme Extracts. Resistant isolates were grown overnight in 10 ml TSB at 22°C with shaking. Cells were harvested by centrifugation at 3500 x *g* for 15 minutes. The pellet was resuspended in 3 ml 25mM sodium phosphate buffer (pH7.0) and cells were lysed by sonication at 8 μm for 4 bursts of 30 seconds each with an ultrasonic disruptor (MSE Soniprep, MSE Instruments, UK). Whole cells were then removed by centrifugation at 32,000 x *g* for 10 minutes. The supernatant containing the crude enzyme preparation was stored at -20°C until required.

2.7.2. Confirmation of β-Lactamase Activity. Crude cell extract (30μl) was mixed with 100μl of nitrocefin solution (500mg/l) in a microtitre well and the time taken for the colour of the mixture to change from yellow to red was measured.

2.7.3. Determination of Spectrum of Activity of the β-Lactamase by Hydrolysis Assay. Into a 3ml cuvette was added 2.6ml 25mM sodium phosphate buffer (pH7.0) and 0.3ml substrate antibiotic solution at the appropriate concentration (table 8) which were then allowed to equilibrate at 37°C. Enzyme (0.1ml) was added and the change in optical density (OD) at the λ_{max} for the particular substrate (table 8) was measured against a blank

Material	Supplier	Final Conc.
5% tetramethylethylenediamine in distilled water (TEMED)	Sigma, Poole, UK	0.25µg/ml
40% ampholines w/v (pH 3.5 - pH 10.0)	LKB, Uppsala, Sweden.	2% w/v
Acrylamide (100g) + methylene bisacrylamide (2.7g) in distilled water (300ml)	BDH, UK	Acrylamide 70 µg/ml, bis.2µg/ml
Distilled water (200ml)		
Riboflavin (20µg/ml in distilled water)	Sigma, Poole, UK.	2µg/ml

Table 9. Composition of IEF gels

containing 2.9ml 25mM sodium phosphate buffer and 0.1ml enzyme. Optical densities were determined with a Perkin-Elmer Lambda 2 dual beam UV/Vis spectrophotometer with a thermostatically controlled cuvette carriage.The rate of substrate hydrolysis was determined according to the equation;

$$R=\frac{\Delta OD \times C \times 10 \times d}{\text{Initial OD} \times t}$$

where;

ΔOD = Decrease in OD at λ_{max} over reaction time t.
C = Concentration of substrate at t0 (0.3 µmoles for penicillins, 0.03 µmoles for cephalosporins)
10 = Correction for enzyme (0.1 ml) to 1ml.
d = Dilution factor (if required).
t = Time (mins) during which reaction is linear.

The rates were then expressed in terms of initial rate of hydrolysis of substrate relative to that of hydrolysis of benzylpenicillin (V_{max}).

2.7.4. Analytical Isoelectric Focusing of β-Lactamase Enzymes. The method used for isoelectric focusing (IEF) was described by Matthew *et al.* (1975). The crude β-lactamase enzyme preparations were focused on a glass plate supporting a thin layer polyacrylamide gel containing carrier ampholines of pH range 3.5-10.0. The composition of the gel mixture is given in table 9. β-lactamase activity was detected with nitrocefin; a sheet of Whatman No. 54 paper dipped in nitrocefin solution (500mg/l) was laid over the gel surface. Focused bands of β–lactamase activity appeared red on a yellow background. Gels were photographed on Polaroid 655 film using transmitted light and a Wratten 58 green filter.

2.7.5. Plasmid Curing with Ethidium Bromide. Ethidium bromide (EtBr) is a known plasmid curing agent (Bouanchaud *et al.* 1969). MICs of EtBr were determined by serial doubling dilutions in TSB. The MIC was taken as the lowest concentration at which visible growth was completely inhibited after 24h incubation at 22°C. One hundred microlitre samples were taken from the broth containing the MIC of EtBr and from two dilutions on either side. These aliquots were washed in fresh TSB prior to plating onto TSA and replica plating onto TSA containing amoxycillin (100mg/l) to identify any cells which may have become sensitive. Plates were incubated at 22°C for 48 h.

2.7.6. Induction of β-Lactamase Production. Overnight cultures (10ml) of *A.salmonicida* were inoculated into 90 ml fresh TSB containing 10mg/l (1/4 of the MIC) of cefoxitin, a potent inducer of β-lactamase production (Minami *et al.* 1980).

The cultures were then incubated at 22°C with shaking for 4 h. The cells were harvested by centrifugation at 3500 x *g* and resuspended in 10ml 25mM sodium phosphate buffer (pH7.0) prior to lysing by sonication as described above. The protein concentration in the induced and uninduced enzyme

preparations was then assayed by the method of Waddell (1956) and the activity of the enzymes was determined in terms of rate of hydrolysis of nitrocefin.

2.7.7. Estimation of Rate of Hydrolysis of Nitrocefin by a Spectrophotometric Technique. The rate of hydrolysis of nitrocefin was determined per milligramme of protein for both induced and uninduced enzyme by the spectophotometric technique described above (2.7.3.).

2.7.8. Mobilisation of Resistance Genes from *A.salmonicida* to *E.coli* K12. In attempts to mobilise resistance genes from *A.salmonicida* subsp. *achromogenes* to *E.coli* K12 (KT106. K.Towner, University of Nottingham) containing the *Inc P* plasmid R702, equal volumes of overnight cultures of donor and recipient were mixed gently and incubated statically overnight at 25°C. The mixture was then filtered through a 0.45µm membrane filter (Millipore, UK), which was placed on a nutrient agar plate and incubated for a further 4h at 25°C. The cells were dislodged from the filter by gentle shaking in 10ml Davis and Mingioli (DM) minimal broth then harvested by centrifugation at 3500 x *g* prior to washing in a further 10ml DM and finally resuspended in 1 ml DM broth. One hundred microlitre samples were then spread onto TSA plates containing 50mg/l amoxycillin and incubated at 37°C for 24h. The incubation temperature of 37°C was sufficiently high to prevent any growth of *A.salmonicida* and thus acted to select in favour of amoxycillin resistant *E.coli* K12.

2.8. AN INFECTION CHALLENGE MODEL.

2.8.1. Experimental animals. Atlantic salmon S1 post smolts, mean weight 120g, were held in 1m circular tanks supplied with pumped sea water (15 l/ min) and were fed commercial pelleted diet (BP S2). These fish had no previous history of *A.salmonicida* infection nor of any antibiotic treatment. Effluent water, diluted 1:10 with fresh water, was disinfected with iodine (5ppm, holding time 30 minutes).

2.8.2. The challenge. The challenge model was developed with a virulent Scottish isolate of *A.salmonicida* (MT879). Seed cultures were transferred from liquid nitrogen to tryptone soya agar (TSA, Oxoid) plates and incubated at 15°C for 72 hours. The resulting cultures were used as inocula for further TSA plates which in turn were incubated at 15°C for 48 hours. Bacterial cells were removed from the plates and suspended in phosphate buffered saline (PBS) pH7.0 immediately prior to commencement of the challenge.

The preliminary infection challenges were performed on fish of mean weight 120g. Fish were starved for 24hours prior to commencement of infection challenge. Noon seawater temperatures were between 13°C and 15°C during the period of the experiment.

Suspensions of *A.salmonicida* were added to tanks containing 50 post-smolts in static aerated water to give bacterial densities of 1×10^4, 1×10^5 or 1×10^6 cfu/ml. After six hours, running sea water supplies were resumed to each tank and maintained, thereafter, at normal flow rates.

Mortalities were removed daily during the ensuing challenge period of 22 days. Bacteriological samples were taken from all dead fish: kidney samples were inoculated onto TSA plates and incubated at 22°C for up to seven days. Mortalities resulting from furunculosis were confirmed by isolation of pure cultures of *A.salmonicida.*

2.9. A PILOT *IN VIVO* EFFICACY STUDY ON THE POTENTIATED SULPHONAMIDE ROMET 30®.

Sea water temperatures were approximately 2°C lower during the period of the efficacy study than during the development of the infection challenge model. As lower sea water temperatures tend to reduce both the occurence and severity of furunculosis outbreaks in Atlantic salmon, it was decided to use the highest challenge dose of *A.salmonicida* tested in the infection challenge model (ie. 10^6cfu/ml) and subject both treated and control populations to handling stress 5 days and 16 days post infection.

Three replicate efficacy trials were performed with approximately 50 control and 50 treated fish in each trial. Each trial was performed in two phases: phase 1 examined efficacy of a single 5 day oral treatment with Romet in preventing mortalities due to furunculosis. In phase 2, the fish were administered a second five day treatment with Romet and subsequently examined for evidence of *A.salmonicida* infection.

2.9.1. Phase One. Two groups of fish (mean weight 177g), held in separate tanks in sea water, were exposed to water borne infection with *A.salmonicida* (10^6cfu/ml for six hours). Noon sea water temperatures were between 11°C and 13°C during the period of the experiment.

Commencing one day after exposure to infection, one group of fish (group 1) was fed a medicated diet (50 mg Romet 30/kg body weight/day; i.e. 15mg active drug/kg/day) for five days. The feeding rate to fish was 0.5% of body weight per day. The second group of fish (group 2) received a normal unmedicated diet at 0.5% of body weight per day.

Five days following exposure to infection, six fish from each tank were removed with a hand net and then immediately returned to the water. A second stress event was initiated 11 days later. The sea water supply to all six groups of fish was interrupted for one hour; during this time, six fish were briefly removed from each tank with a hand net.

Mortalities were removed from tanks daily. Bacteriological samples were taken from all fish, and mortalities caused by furunculosis confirmed as described above.

2.9.2. Phase Two. Commencing 24 days after initial infection, group 1 was administered a five day oral treatment with Romet, similar to the first treatment. Group 2 continued to receive an unmedicated diet.

Thirty days following initial infection, all surviving fish were sacrificed and examined bacteriologically for evidence of asymptomatic *A.salmonicida* infection: kidney and lower intestine samples were inoculated onto TSA plates and incubated at 22°C for up to 10 days. Asymptomatic infection was confirmed by isolation of one or more colonies of *A.salmonicida* either from kidney or lower intestine.

Relative percentage survival (RPS) values were calculated according to the equation:

$$RPS = 1 - \frac{\%\ \text{mortalities in treated fish}}{\%\ \text{mortalities in control fish}} \times 100$$

2.10. SUITABILITY OF IODINE AS A SEAWATER DISINFECTANT.

The suitability of iodine for disinfection of effluent water was determined in terms of bactericidal activity in seawater, seawater at reduced pH and artificial seawater (table 10, Dr B. Austin, personal communication). Seawater was obtained from the effluent sump of the Marine Laboratory Fish Cultivation Unit, Aultbea, Rosshire.

Compound	Concentration (mM)	g/500ml
NaCl	400	11.69
$MgSO_4.7H_2O$	100	12.32
$CaCl_2.2H_2O$	20	1.47
KCl	20	0.75

Table 10. Composition of artificial seawater.

Bactericidal activity was assayed essentially as described in section 2.4.4. Briefly, an overnight culture (10ml TSB) of *A.salmonicida* MT879 was harvested by centrifugation and resuspended in 10ml PBS pH7.0. The culture was then diluted 1 in 50 into seawater (pH7.2, unadjusted), seawater pH 4.0 (pH adjusted with HCl), or artificial seawater, containing iodine at various concentrations. The iodine concentrations followed a doubling dilution schedule from 0.25parts per million (ppm) to 32ppm. Aliquots (0.1ml) were taken immediately after, and 20 minutes following inoculation, and serially diluted in 0.5M thiosulphate to inactivate the iodine. Percentage survival was estimated by viable counting on TSA.

2.11. AN ALTERNATIVE TO INFECTION MODELS FOR STUDYING ANTIMICROBIAL EFFICACY IN FISH.

2.11.1. Selection and maintenance of fish. Eight circular sea water tanks, 1m in diameter, were each stocked with 25 Atlantic salmon post smolts, which were acclimatised for 2 weeks prior to commencing the study. Eight fish from each group were netted, anaesthetised and weighed to determine a mean weight for each tank (table 11). Having been maintained at a feeding rate of 2% body weight per day, the fish were starved for 48 hours prior to commencement of medication.

			Weight of Fish (grammes)	
Tank No.	**Medication**	**Dose (mg/kg/day)**	**Mean**	**Range**
93	Flumequine	10	417.7	319-525
94	Enrofloxacin	5	488.7	301-633
95	Sarafloxacin	10	392.4	235-498
96	Ro 09-1168	2	390.3	259-565
97	Oxolinic acid	10	460.4	232-618
98	Oxolinic acid	10	419.4	280-582
99	Controls	-	362.0	319-490
100	(Unmedicated)	-	374.8	173-492

Table 11. Mean fish weights and medication dose rates.

2.11.2. Preparation and Administration of Medicated Feed. Medicated feed was prepared as follows: the appropriate weight of BP S2 feed pellets was mixed with preweighed drug (table 11) by hand to give an even surface coating. Fish feeding oil (6ml/kg feed) was added to assist binding of the antibiotics and as an appetiser. The fish were fed to appetite, by hand, at a rate of 0.5% body wt / day, for three days.

	MIC (mg/l)				
Strain	**Oxolinic acid**	**Sarafloxacin**	**Enrofloxacin**	**Flumequine**	**Ro 09-1168**
MT363	0.03	0.05	0.02	0.10	0.01
MT477	1.50	0.75	0.20	1.50	0.10

Table 12. MICs of quinolones against *A.salmonicida* MT363 & MT477.

2.11.3. Sampling of Serum. Eight fish were netted from each tank and anaesthetised 3h, 8h and 24h following the administration of the final dose of medicated feed. The fish were bled from the caudal vein using 10ml Vacutainers® (Becton-Dickinson, France). The blood was allowed to clot at room temperature and then cooled. The samples were transported on ice and centrifuged at 4500rpm in a Heraeus Labofuge to remove blood cells. Aliquots (0.5ml) of serum were removed from each sample and pooled into two groups from four fish each, for each of the drugs and sampling times. Sera from the two tanks of unmedicated control fish (tanks 99 & 100, table 11) were pooled.

Antibiotic Standards (mg/l)				
Oxolinic Acid	**Flumequine**	**Sarafloxacin**	**Enrofloxacin**	**Ro 09-1168**
50	90	50	50	50
15	50	15	30	15
5	15	9	15	5
3	5	5	5	1.5
0	3	1.5	1.5	0.5
	0.9	0.5	0.5	0.15
	0	0	0	0

Table 13. Standard antibiotic concentrations used in bioassays.

2.11.4. Determination of Antibiotic Levels in Serum by Bioassay. Antibiotic levels in the serum samples were determined by microbiological assay using a quinolone sensitive isolate of *Aeromonas salmonicida* (MT363, table 12) as the detection organism. Bioassays were performed according to the method of Reeves and Wise (1978). Briefly, square bioassay plates (Nunc, UK) were levelled and poured with 100ml cooled DST agar (Oxoid, UK). The agar was allowed to set and the plates were dried at 37°C for 1 h. Plates were cooled to 20°C before inoculation.

	Well Number		
Standard 1	7	19	27
Standard 2	4	15	22
Standard 3	5	12	23
Standard 4	3	20	29
Standard 5	1	13	25
Standard 6	6	17	28
Standard 7	2	14	24
Test 1	8	11	30
Test 2	9	16	26
Test 3	10	18	21

Table 14. Codes for Latin square 7,3.

Standard inocula of *A.salmonicida* MT363 were prepared as follows. Overnight IsoSensitest broth cultures (10ml) of MT363 were diluted fourfold in fresh IsoSensitest broth. This culture (20ml) was then poured onto the surface dried bioassay plate. The culture was drained from each of the four corners of the plates and the plates were surface dried at 20°C for 2 h. Thirty sample wells (9mm) were then cut in the inoculated agar using a sterile No. 4 cork borer.

Antibiotic standards were prepared in fish serum isolated from the control fish according to an arithmetic dilution schedule following the progression 1.5, 3, 5, 9. With the exception of oxolinic acid, sample wells were

inoculated with 7 selected standards (table 13) and 3 test samples, each in triplicate, according to an encrypted latin square 7,3 technique (table 14). In the case of oxolinic acid, a 5,5 latin square pattern was employed.

2.11.5. Bactericidal activity of antibiotics in serum. To samples (0.9ml) of serum isolated from the various medicated fish, was added 0.1ml of a logarithmic phase culture of *A.salmonicida* MT477 or MT363 (MICs shown in table 12). Aliquots (0.1ml) were taken at 20, 40, 60, 120 and 180 minutes and diluted in fresh TSB before spreading onto TSA plates to estimate percentage survival by viable counting.

RESULTS

3.1 MINIMUM INHIBITORY CONCENTRATIONS

Minimum inhibitory concentrations (MICs) give a comparative estimation of the amounts of various antibiotics required to inhibit completely the growth of the target organism. With the quinolones, MICs must be determined by agar dilution as opposed to the broth dilution technique, as filamentation of bacteria caused by the quinolones can lead to erroneous MIC values determined by the latter method (Smith, 1984b).

3.1.1. Survey of MICs of Quinolones Against *Aeromonas salmonicida.* MICs of enrofloxacin, flumequine, sarafloxacin, oxolinic acid, PD127,391, PD117,596, CI934 and Ro 09-1168 were determined, employing an arithmetic dilution schedule, against 38 oxolinic acid-resistant and 45 oxolinic acid susceptible isolates of *Aeromonas salmonicida* (Appendix B).

The fluoroquinolones, PD117,596 and PD127,391 were, on average, at least 15 times more active, in terms of MIC, than oxolinic acid against oxolinic acid-resistant isolates and 10 times more active against oxolinic acid-susceptible isolates (table 15). Enrofloxacin was slightly more active than oxolinic acid against both oxolinic acid-susceptible and oxolinic acid-resistant isolates. Sarafloxacin was also slightly more active than oxolinic acid against both susceptible and resistant isolates (table 15). CI934 was as active as oxolinic acid against resistant isolates but less active against oxolinic acid susceptible isolates. Flumequine, on the other hand, was more active than oxolinic acid against both susceptible and resistant isolates. Interestingly, the range of flumequine MICs was broader than those recorded for oxolinic acid (table 15). In terms of MIC, the most active of the compounds tested was the Roche quinolone Ro 09-1168. This drug had very low MICs when tested against both oxolinic acid-susceptible and -resistant isolates, with MIC_{90}s of 0.015 and 0.2 respectively (table 15).

3.1.2. Effect of Inoculum Size on the Fluoroquinolones. MICs of five fluoroquinolones and oxolinic acid (figure 11) were determined, employing an arithmetic dilution schedule, against three oxolinic acid-susceptible and three oxolinic acid-resistant isolates of *A.salmonicida* by the agar dilution

Strain type (no. of isolates)	Test agent	MIC at 22°C (mg/l) 50%	90%	Range
Oxolinic acid resistant (38)	Oxolinic acid	3.00	7.50	1.00-15.00
	Sarafloxacin	1.50	4.00	0.20-4.00
	Enrofloxacin	0.50	1.00	0.05-5.00
	Flumequine	2.00	4.00	0.10-20.00
	CI934	3.00	7.50	0.30-10.00
	PD127,391	0.10	0.15	0.015-0.30
	PD117,596	0.15	0.50	0.075-0.75
	Ro 09-1168	0.20	0.20	0.075-0.50
Oxolinic acid susceptible (43)	Oxolinic acid	0.03	0.40	0.01-0.75
	Sarafloxacin	0.05	0.20	0.0075-1.00
	Enrofloxacin	0.02	0.15	0.004-0.75
	Flumequine	0.075	0.10	0.075-0.50
	CI934	0.40	1.00	0.04-3.00
	PD127,391	0.0075	0.03	0.002-0.10
	PD117,596	0.015	0.05	0.003-0.50
	Ro 09-1168	0.01	0.015	0.01-0.10

Table 15. *In vitro* activities of 4-quinolones against 83 isolates of *A.salmonicida*.

technique as described previously. Plates were inoculated with 10^2, 10^4 and 10^6 cfu/spot in place of the standard inoculum of 10^5 cfu/spot.

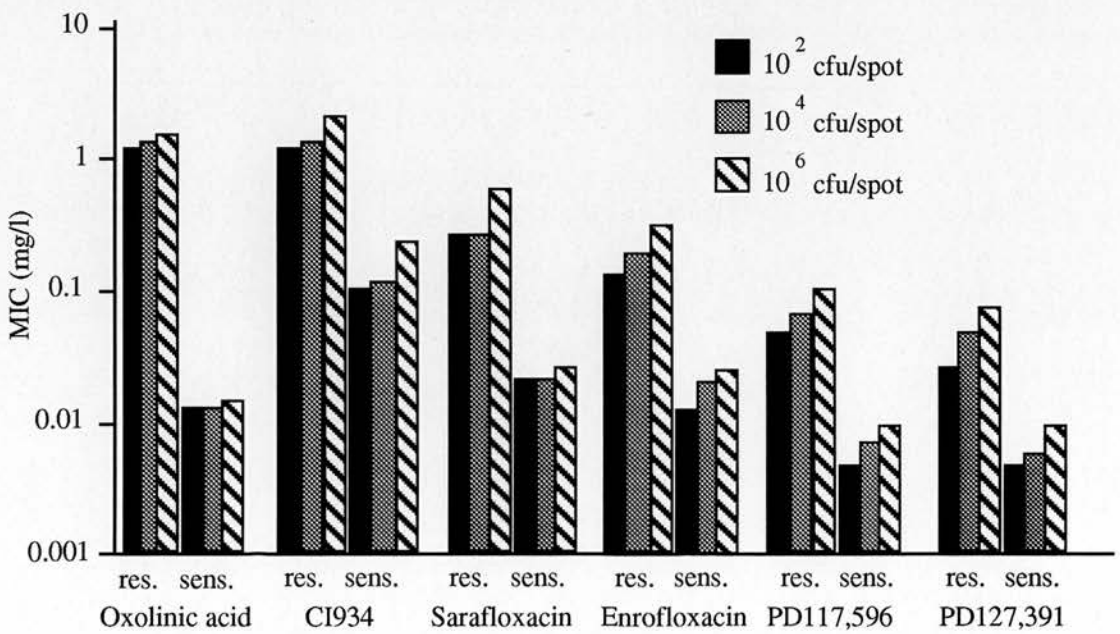


Figure 11. Inoculum size effect on MICs of 4-quinolones.

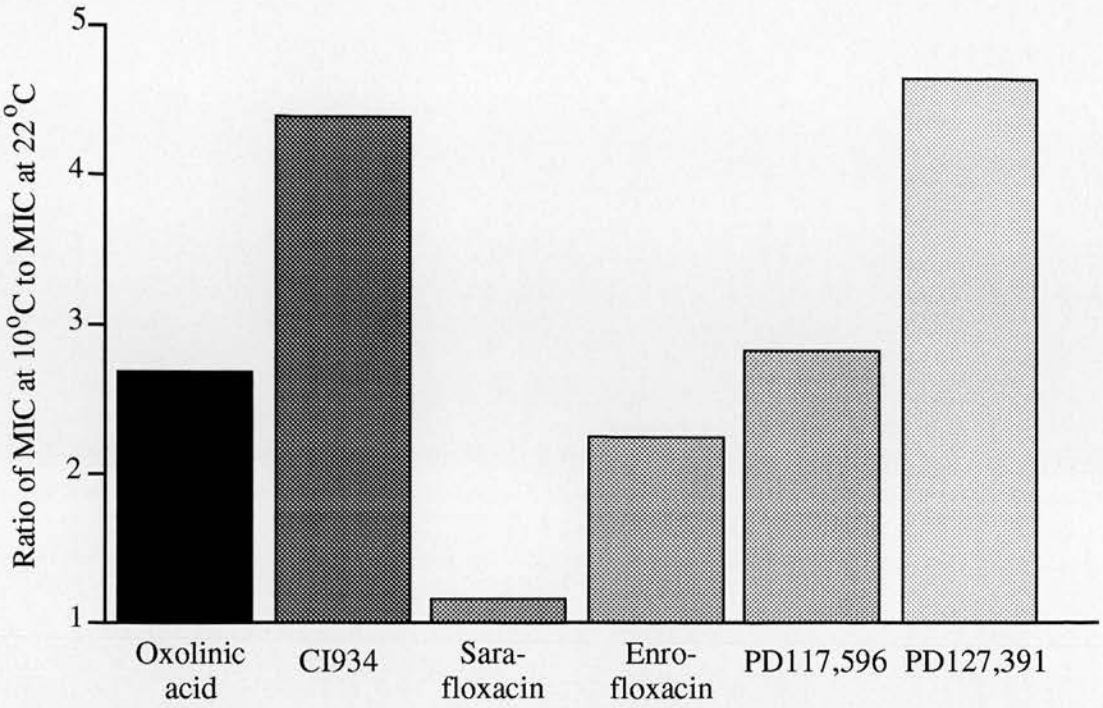


Figure 12. Effect of temperature on MICs of 4-quinolones. (Results shown recorded after 7 days incubation at 22°C).

The maximum change in MIC with inoculum size was found to be approximately threefold (1.5mg/l to 5.0 mg/l). However, considering the 10,000 fold increase in cell concentration, a threefold increase in MIC seems insignificant.

3.1.3 Temperature Effect and the 4-Quinolones. The MIC of each of the five fluoroquinolones (above) and oxolinic acid were determined against 18 oxolinic acid-susceptible isolates of *A.salmonicida* at 10°C, a temperature which more closely reflects those at which outbreaks of furunculosis may occur. Results were read after 5 and 7 days incubation at this temperature. With the exception of sarafloxacin, the quinolones exhibited a two- to five-fold increase after both 5 and 7 days incubation, compared with the MICs at 22°C (figure 12). Interestingly, sarafloxacin did not appear to be affected significantly by the reduction in temperature.

3.1.4. Effects of Seawater Cations on Antibacterial Agents. Treatment regimens for furunculosis are normally based on disc susceptibility testing on commercial media of *A.salmonicida*, isolated from fish mortalities.

Strain type (No. of isolates)	Test Agent	Range of MICs Determined on			
		TSA (IsoSensitest)	TSA (IsoSensitest) +340mM NaCl	TSA (IsoSensitest) + 50mM MgCl2	Bacto Marine Agar
Oxolinic Acid Susceptible (10)	Oxolinic Acid	0.03-0.50	0.03-0.50	0.50-32.0	0.25-16.0
	Flumequine	0.06	0.06-0.50	2.00-64.0	1.00-16.0
	Sarafloxacin	0.03-0.50	0.03-0.50	1.00-2.00	0.50-1.00
	Oxytetracycline	0.50	0.25	16.0-32.0	8.00
	Amoxycillin	0.50	0.50	0.50-1.00	0.50-1.00
	Ormetoprim	(0.50-1.00)	(0.50-1.00)	(0.50-1.00)	NT
	Sulfadimethoxine	(16.0->128)	(16.0->128)	(16.0->128)	NT
Oxolinic Acid Resistant (10)	Oxolinic Acid	1.00-4.00	1.00-2.00	32.0->128	16.0->128
	Flumequine	2.00	1.00-2.00	64.0->128	64.0->128
	Sarafloxacin	0.50-2.00	0.50-2.00	16.0-128	8.00-128
	Oxytetracycline	0.50	0.25	16.0-32.0	8.00
	Amoxycillin	0.50	0.50	0.50-1.00	0.50-1.00
	Ormetoprim	(0.50-1.00)	(0.50-1.00)	(0.50-1.00)	NT
	Sulfadimethoxine	(16.0->128)	(16.0->128)	(16.0->128)	NT

Table 16. Antagonism of aquaculture antibacterials by sea water cations. (Figures in parentheses indicate MICs determined on IsoSensitest Agar).

However, these media do not take into account the ion balance of the salt water environment in which the drugs are used. MICs of oxolinic acid, sarafloxacin, flumequine, oxytetracycline and amoxycillin were determined on Bacto marine agar (BMA) and TSA. A 40 to 60 fold increase in the MICs of the quinolones and oxytetracycline, but not amoxycillin, was observed on BMA. This antagonism appeared to be due to Mg^{2+} in the BMA, as addition of 50mM $MgCl_2$ but not 340mM NaCl (the ion concentrations in BMA) to TSA resulted in similar antagonism (table 16). Furthemore, the activities of the two components of the potentiated sulphonamide Romet 30, ormetoprim and sulphadimethoxine, against the same isolates of *A.salmonicida* were unaffected by the addition of 50mM $MgCl_2$ to IsoSensitest agar (table 16).

3.2. KILLING OF *AEROMONAS SALMONICIDA* BY THE QUINOLONES.

Minimum inhibitory concentrations of the 4-quinolones provide information about the inhibition of bacterial multiplication by these antibiotics. They do not, however, give any indication of the ability of these drugs to kill the target organism (Smith & Lewin, 1988). In order to determine the potency and mechanism of bactericidal activity of the quinolones, a further series of experiments was undertaken.

3.2.1. Bactericidal Activity of the Quinolones. Lewin & Hastings (1990) demonstrated that two fluoroquinolones in clinical use in humans, ciprofloxacin and norfloxacin, were bactericidal against both oxolinic acid-resistant and -susceptible isolates of *A.salmonicida* after 3h exposure. In contrast, oxolinic acid was merely bacteriostatic under these conditions. Therefore, the bactericidal activities of oxolinic acid and the seven fluoroquinolones, sarafloxacin, enrofloxacin, CI934, PD127,391, PD117,596, Ro 09-1168 and flumequine, were examined against oxolinic acid-susceptible and -resistant isolates of *A.salmonicida.*

Isolate	Oxolinic acid	Sarafloxacin	Enrofloxacin	CI934	PD117,596	PD127,391	Ro 09-1168
MT363	0.03	0.05	0.015	0.40	0.015	0.0075	0.01
MT736	0.015	0.04	0.04	0.40	0.015	0.010	0.01
MT364	7.50	3.00	1.00	3.00	0.30	0.10	0.10
MT472	3.00	2.00	1.00	2.00	0.20	0.10	0.20

Table 17. MICs of the test 4-quinolones against *A.salmonicida* isolates used in bactericidal activity experiments.

After 1h exposure, neither oxolinic acid nor flumequine were bactericidal with approximately 100% of the bacteria surviving at concentrations of up to 100 times the MIC for the isolate. Furthermore, oxolinic acid was merely bacteriostatic even after 6h exposure, with approximately 100% of the bacteria surviving (figs. 13, 14 & 15). In contrast, flumequine was bactericidal, killing 90% and >99% of the bacteria after 3h and 6h exposure respectively, at concentrations above five times the MIC at 22°C (figs. 14 & 15).

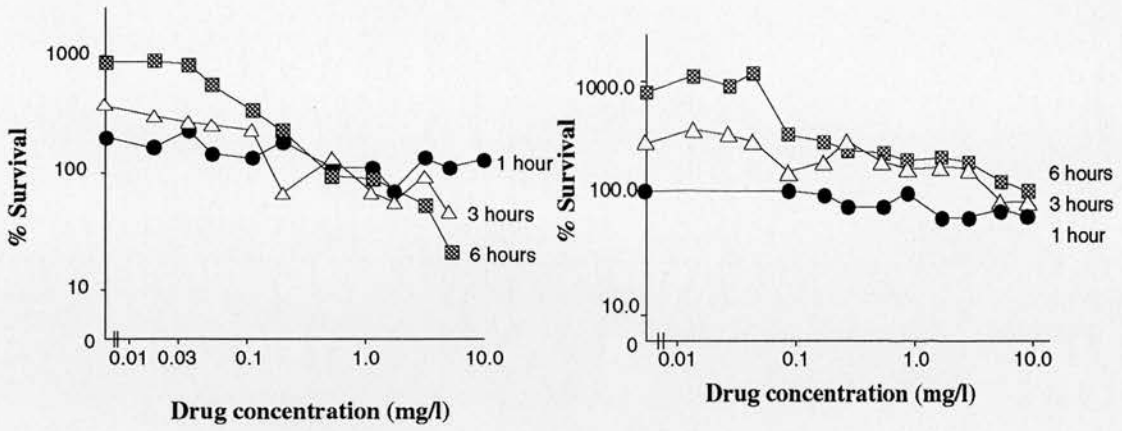


Figure 13. Bactericidal activity of oxolinic acid against *A.salmonicida* MT363 (left) and MT736 (right). Both isolates were oxolinic acid-susceptible.

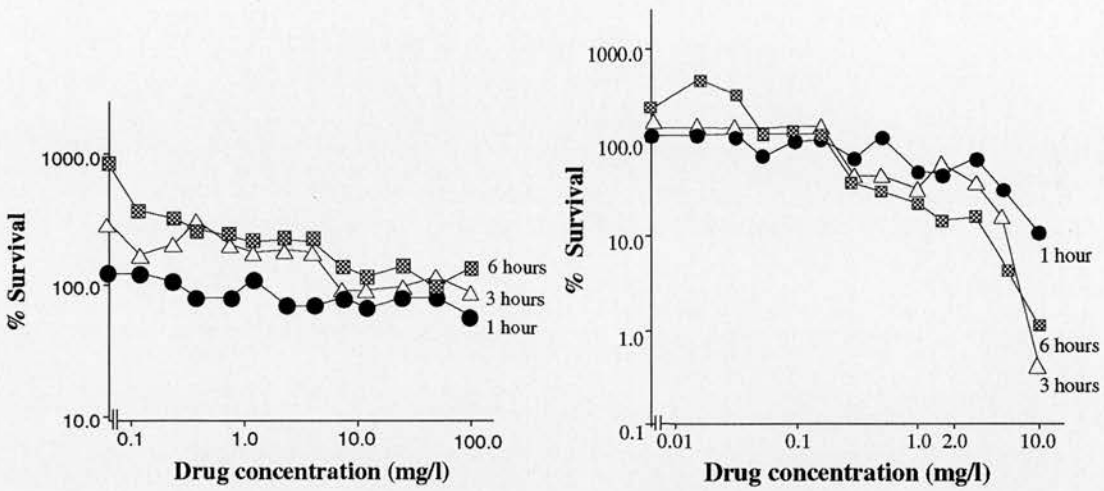


Figure 14. Bactericidal activity of oxolinic acid (left) and flumequine (right) against *A.almonicida* MT472 (oxolinic acid-resistant).

With the exception of CI934, the remaining fluoroquinolones were rapidly bactericidal against oxolinic acid-susceptible isolates of *A.salmonicida* MT363 (fig. 16) and MT736 (fig. 17). Less than 1% of the bacteria survived at concentrations just above the MIC after 1h exposure (table 17, figs. 16 & 17). CI934 was less bactericidal than the other fluoroquinolones as 90% of the bacteria survived 1h exposure to the drug. Sarafloxacin and enrofloxacin

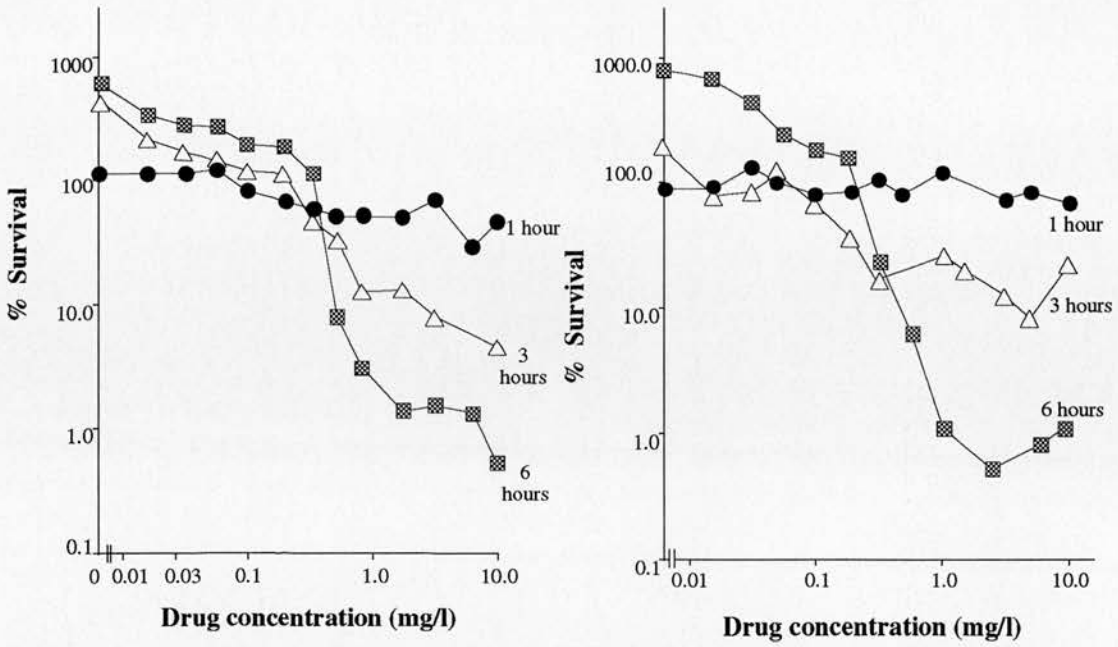


Figure 15. Bactericidal activity of flumequine against *A.salmonicida* MT363 (left) and MT736 (right). Both isolates are oxolinic acid-susceptible.

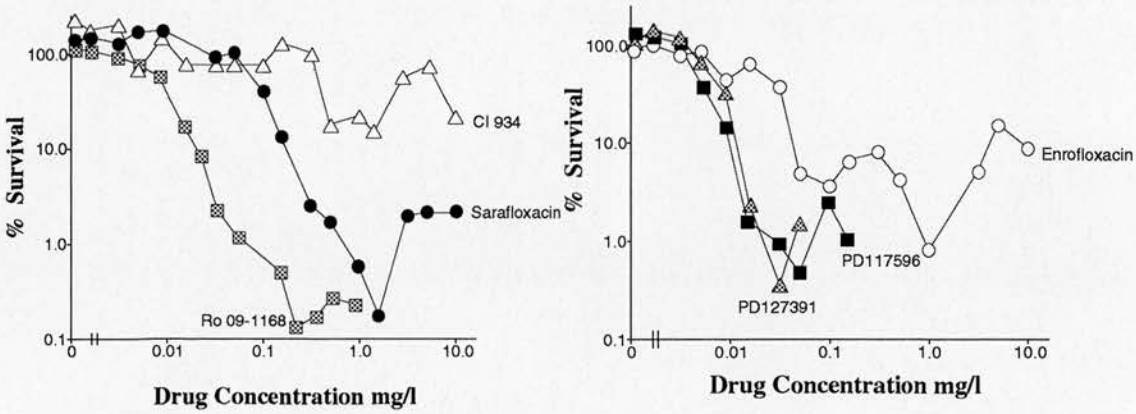


Figure 16. Bactericidal activity of fluoroquinolones against *A.salmonicida* MT363 (oxolinic acid-susceptible).

appeared to be less active against oxolinic acid-resistant isolates MT364 (fig. 19) and MT472 (fig. 18), with 10% of the bacteria surviving after 1h exposure. However, PD127,391, PD117,596 and Ro 09-1168 were as active against the resistant isolate MT472 as they were against the two susceptible isolates, killing > 99% of the bacteria (fig 18).

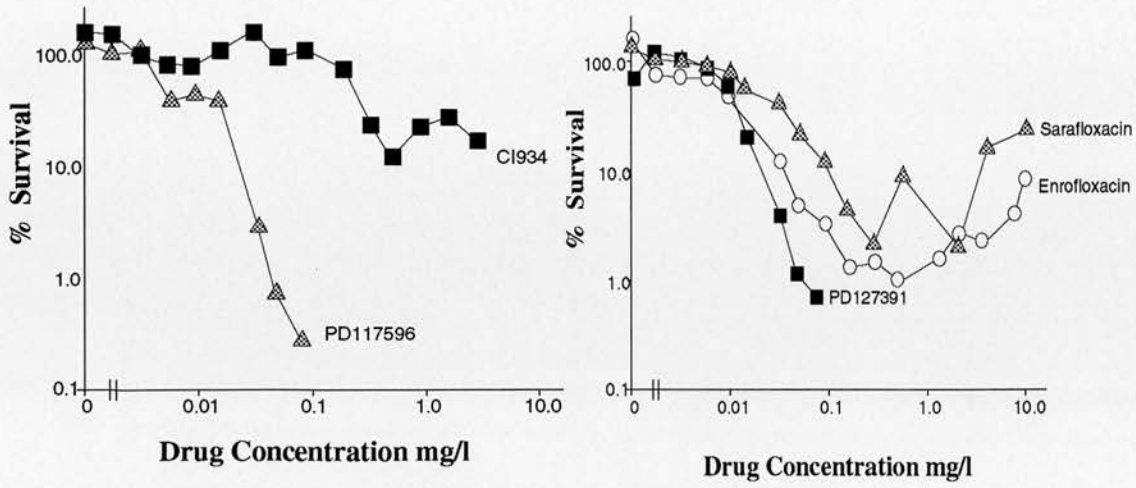


Figure 17. Bactericidal acivity of fluoroquinolones against *A.salmonicida* MT736 (oxolinic acid-susceptible).

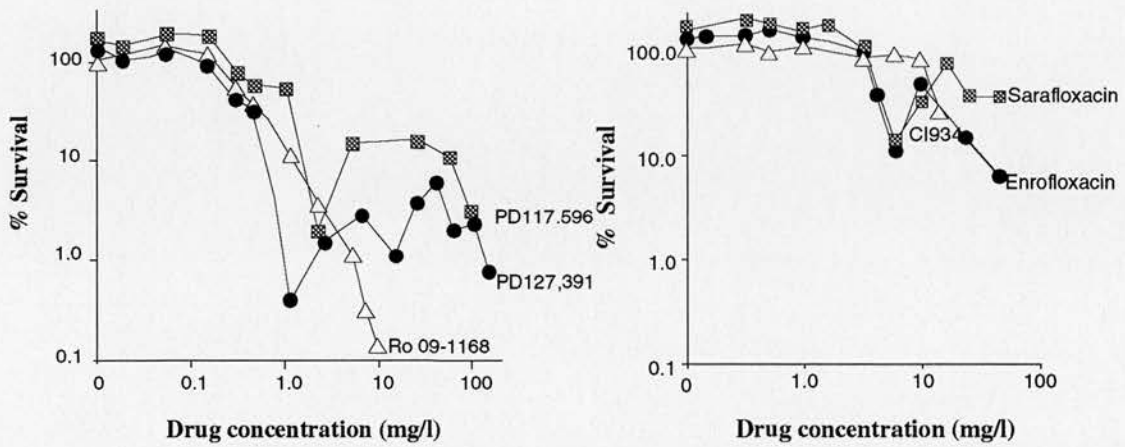


Figure 18. Bactericidal activity of fluoroquinolones against *A.salmonicida* MT472 (oxolinic acid-resistant).

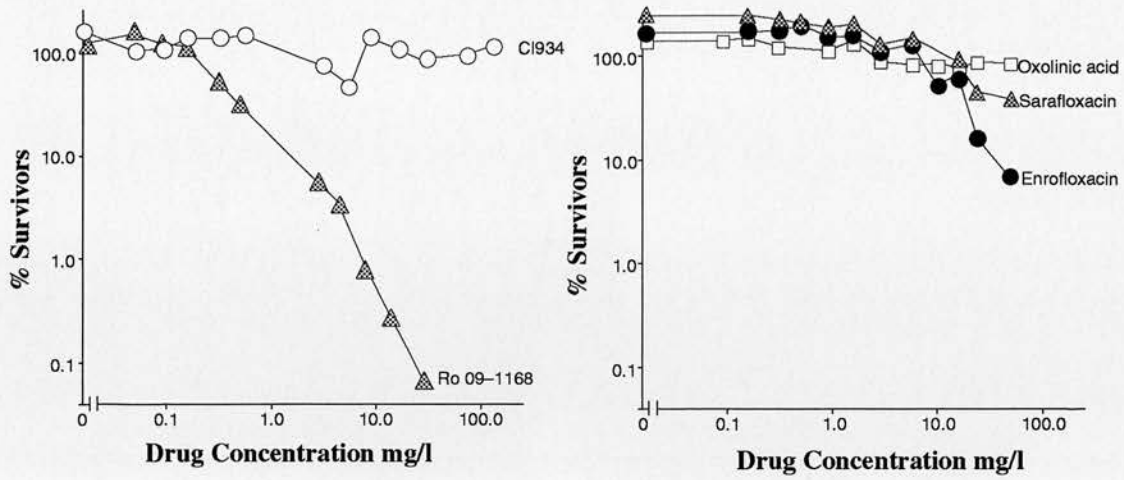


Figure 19. Bactericidal activity of fluoroquinolones against *A.salmonicida* MT364 (oxolinic acid-resistant).

3.2.2. Optimum Bactericidal Concentrations (OBCs). One of the characteristics of the fluoroquinolone antibiotics is their biphasic antibacterial activity. The killing efficacy increases to a point known as the OBC after which it decreases (refer to page 13).

	OBCs Determined after 1 hour			
	Oxolinic acid Susceptible		Oxolinic acid Resistant	
Drug	**MT363**	**MT736**	**MT472**	**MT364**
CI934	1.50	0.90	ND	ND
PD127,391	0.03	ND	1.00	ND
PD117,596	0.05	ND	1.50	ND
Enrofloxacin	0.90	0.90	5.00	ND
Sarafloxacin	1.50	1.50	5.00	ND
Ro 09-1168	0.30	ND	ND	ND

Table 18. Optimum Bactericidal Concentrations (OBCs, mg/l) of fluoroquinolones. (ND = not determined).

OBCs were determined, where possible, for the quinolones from their bactericidal curves (figures 13-19). The OBCs are presented in table 18. As flumequine and oxolinic acid were not bactericidal after 1h their OBCs were

not determined. The OBC of enrofloxacin against the quinolone susceptible isolates of *A.salmonicida,* MT363 and MT736, and the quinolone resistant isolate MT472, was approximately 5 times the respective MICs. On the other hand, sarafloxacin had an OBC 30 times its MIC against MT363, whereas the OBC of sarafloxacin against the resistant isolate MT472 was merely 2.5 times its MIC. The three Parke-Davis compounds, PD127,391, PD117,596 and CI934 had OBCs approximately 3 times their respective MICs against the oxolinic acid-susceptible isolates (tables 17 & 18), However, against the oxolinic acid-resistant isolate MT472, the OBCs of PD127,391 and PD117,596 were almost 10 times the MIC. The OBC of Ro 09-1168 was 30 times its MIC against the quinolone susceptible isolate MT363.

3.2.3. Mechanism of Bactericidal Action of Three Fluoroquinolones. All 4-quinolones, including the older examples oxolinic acid and nalidixic acid, possess one mechanism of bactericidal action, termed mechanism A. The fluoroquinolones, however, generally possess an additional mechanism of action termed B or C (refer to page 13).

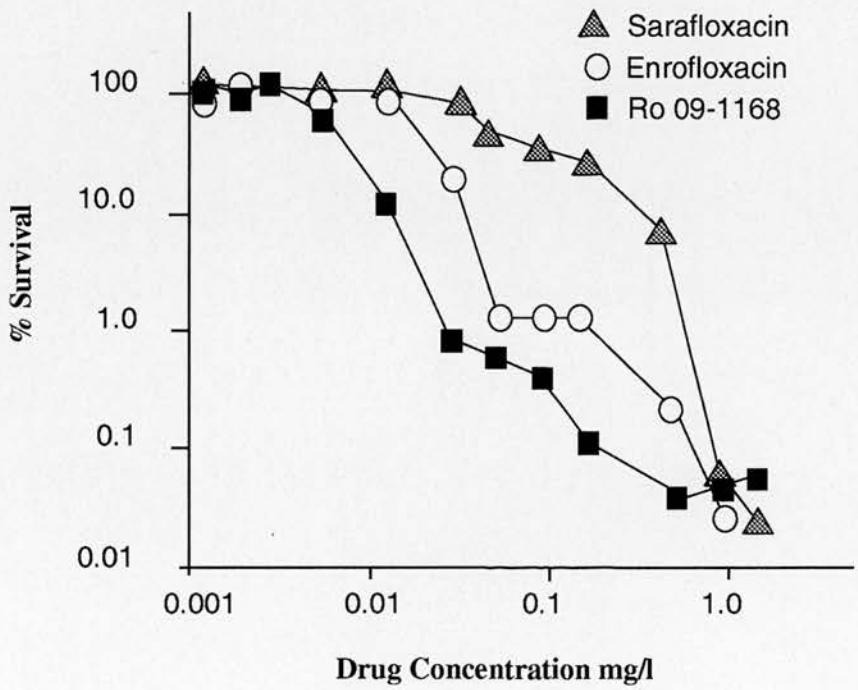


Figure 20. Bactericidal acivity of sarafloxacin, enrofloxacin and Ro 09-1168 against non-dividing *A.salmonicida* MT363 in PBS.

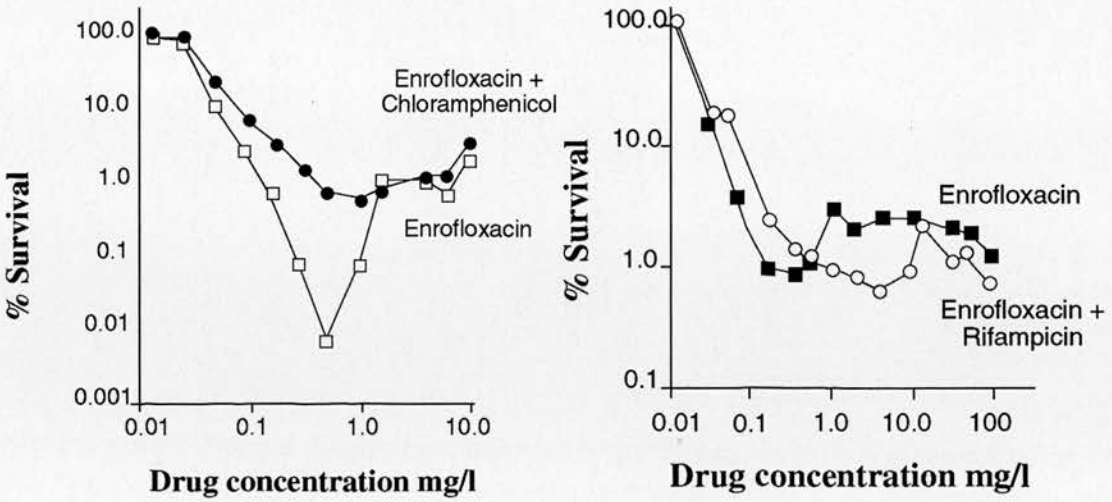


Figure 21. Bactericidal activity of enrofloxacin in the absence of protein synthesis (left) and RNA synthesis (right).

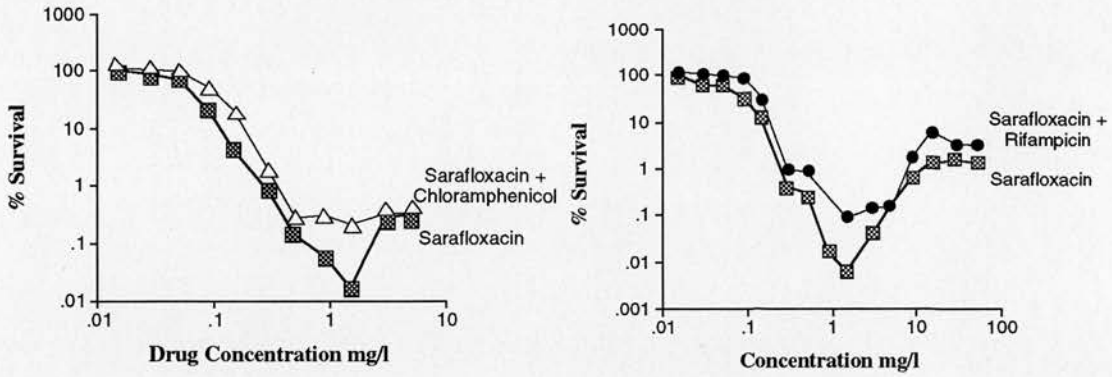


Figure 22. Bactericidal activity of sarafloxacin in the absence of protein synthesis (left) and RNA synthesis (right).

The previous experiments have demonstrated that the fluoroquinolones enrofloxacin, sarafloxacin and Ro 09-1168 are rapidly bactericidal, killing 90->99% of oxolinic acid sensitive bacteria after 1h exposure. In this study, the antibacterial action of these quinolones against a) non-dividing bacteria, b) bacteria where protein synthesis is prevented by chloramphenicol, and c) bacteria in which the RNA synthesis has been terminated with rifampicin, was investigated in order to elucidate the mechanisms of antibacterial action.

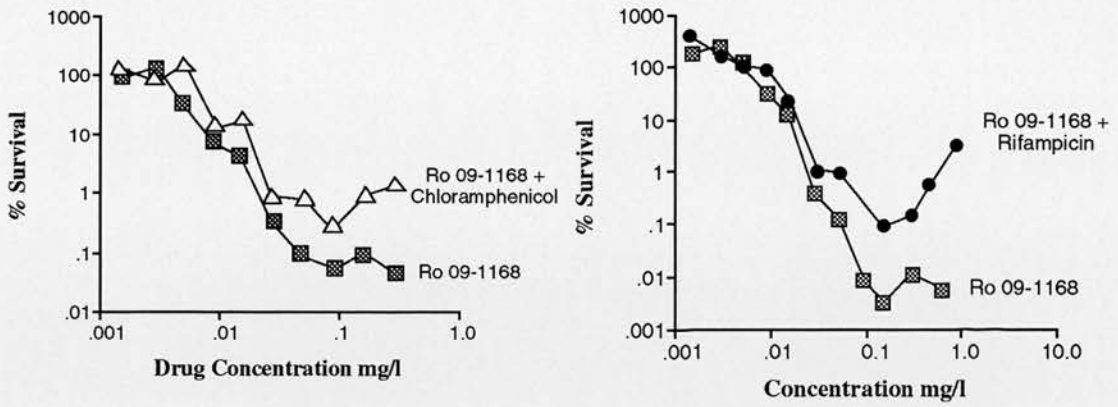


Figure 23. Bactericidal activity of Ro 09-1168 in the absence of protein synthesis (left) and RNA synthesis (right).

Sarafloxacin, enrofloxacin and Ro 09-1168 were all active against non dividing bacteria (fig. 20) demonstrating that these drugs possess a second mechanism of bactericidal action in addition to mechanism A. Furthermore, all three antibiotics showed only slightly reduced bactericidal activity in the presence of either rifampicin or chloramphenicol (figs. 21, 22 & 23). This suggests that the secondary mechanism of action utilised by these compounds is mechanism B. Thus sarafloxacin, enrofloxacin and Ro09-1168 possess both mechanisms A and B.

3.3 MECHANISMS OF QUINOLONE RESISTANCE.

Almost half of the *A.salmonicida* isolates tested in this study were resistant to oxolinic acid (table 15), according to the criteria proposed by Tsoumas *et al.* (1989). It was, therefore, considered important to investigate resistance to the 4-quinolones further in this species.

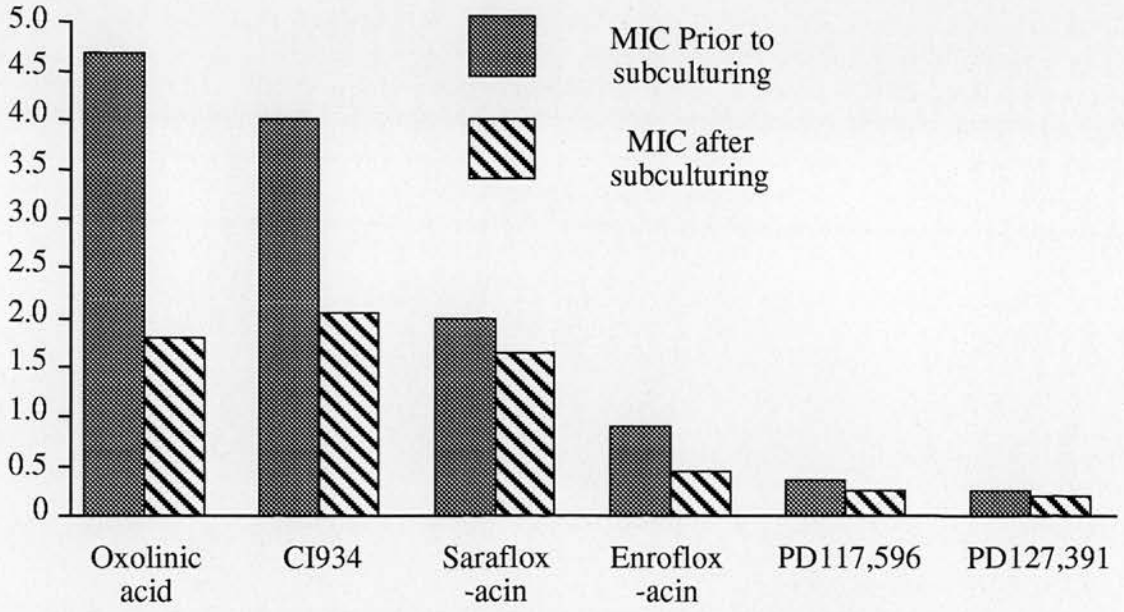


Figure 24. Stability of resistance to 4-quinolones.

3.3.1. Stability of quinolone resistance. It has been reported that resistance of *A.salmonicida* to oxolinic acid is reduced after repeated passage on tryptone soy agar medium (Tsoumas *et al.,* 1989). After 10 successive passages of 20 oxolinic acid resistant strains of *A.salmonicida* on drug-free TSA, there was a mean threefold decrease in the MIC of oxolinic acid. However, no such decrease was observed for PD117,596, PD127,391 or sarafloxacin, whereas a twofold decrease in MIC was noted for enrofloxacin and CI934 (fig. 24). Despite the threefold decrease in MIC observed for oxolinic acid, all strains are still classified as resistant by the criteria of Tsoumas *et al.*(1989), thus the significance of such a small change must be questioned.

Drug	Range of mutation frequencies at:		
	5 x MIC	10 x MIC	20 x MIC
Oxolinic acid	3.3×10^{-9} - 3.64×10^{-8}	1.7×10^{-9} - 6.5×10^{-8}	$<1.1 \times 10^{-9}$ - 3.2×10^{-8}
Sarafloxacin	$<8.3 \times 10^{-10}$ - 1.76×10^{-8}	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$
Enrofloxacin	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$
CI934	$<6.7 \times 10^{-10}$ - 8.5×10^{-9}	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$
PD117,596	$<5.0 \times 10^{-10}$ - 1.6×10^{-7}	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$
PD127,391	$<5.0 \times 10^{-10}$ - 2.9×10^{-7}	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$
Flumequine	1.5×10^{-9} - 2.0×10^{-8}	$<3.3 \times 10^{-10}$	$<3.3 \times 10^{-10}$
Oxytetracycline	1.27×10^{-8} - 6.82×10^{-7}	$<6.7 \times 10^{-10}$ - 7.8×10^{-8}	$<5.0 \times 10^{-10}$

Table 19. Frequency of chromosomal mutation to resistance to the 4-quinolones and oxytetracycline.

3.3.2. Frequency of Mutational Resistance to the 4-Quinolones and Oxytetracycline. Mutation frequencies give some idea of the rate at which resistance to a quinolone will develop during therapy as plasmid mediated resistance to these drugs has not yet been identified (Lewin, Allen & Amyes, 1990; Courvalin, 1990). The frequencies at which five oxolinic acid-susceptible strains of *A.salmonicida* mutated to develop resistance when exposed to oxolinic acid, oxytetracycline and 6 fluoroquinolones were determined at drug concentrations of 5, 10 and 20 times their respective MICs (table 19). Mutants resistant to oxolinic acid were isolated at 5, 10 and 20 times the MIC at frequencies ranging from 10^{-9} to 10^{-8} (table 19). Oxytetracycline resistant mutants were isolated at similar frequencies at 5 and 10 times the MIC. However, at 20 times the MIC, the mutation frequency was less than 10^{-10}, which was the limit of detection for this experiment. For the 6 fluoroquinolones tested, the frequency at which resistance developed at 10 and 20 times their respective MICs in all five test strains was less than 10^{-10} (table 19). When enrofloxacin was used as the test agent, no mutants were obtained at 5 times the MIC (table 19). Mutation frequencies for the remaining fluoroquinolones varied between isolates exposed to the drug at five times their MICs. Resistant mutants were detected for three of the five isolates tested with sarafloxacin, one of the five isolates with CI934, four of the five isolates with PD127,391, two of the five isolates with PD117,596 and two of the five isolates with flumequine (table

	MT494	MT363	MT744	MT747	MT490
Oxolinic acid	$<3.3 \times 10^{-8}$	3.64×10^{-8}	1.05×10^{-8}	3.33×10^{-9}	2.19×10^{-8}
Sarafloxacin	$<8.3 \times 10^{-10}$	1.76×10^{-8}	7.38×10^{-9}	2.20×10^{-9}	$<1.25 \times 10^{-9}$
PD117,596	$<8.3 \times 10^{-10}$	$<6.6 \times 10^{-10}$	$<5.0 \times 10^{-10}$	1.63×10^{-7}	3.75×10^{-7}
CI934	$<8.3 \times 10^{-10}$	$<6.67 \times 10^{-10}$	8.5×10^{-9}	$<1.1 \times 10^{-9}$	$<1.25 \times 10^{-9}$
PD127,391	$<8.3 \times 10^{-10}$	3.42×10^{-7}	1.83×10^{-8}	1.31×10^{-7}	2.93×10^{-7}
Enrofloxacin	$<8.3 \times 10^{-10}$	$<6.67 \times 10^{-10}$	$<5.0 \times 10^{-10}$	$<1.1 \times 10^{-9}$	$<1.25 \times 10^{-9}$
Flumequine	2.0×10^{-8}	3.75×10^{-9}	1.5×10^{-9}	NT	1.0×10^{-9}

Table 20. Mutation frequencies of *A.salmonicida* isolates to 4-quinolones at 5x their respective MICs

20). Resistant mutants were selected only with flumequine for *A.salmonicida* MT494 but not with any of the fluoroquinolones tested. With the other four strains tested, resistance was selected with one or more of the quinolones at 5 times their MIC (table 20).

The susceptibility of some of the quinolone resistant mutants isolated from the mutation frequency experiments was examined. All of the isolates tested were cross-resistant to the other quinolones, with an increase in MIC of approximately 10 fold (table 21). Two of the oxolinic acid resistant isolates selected from the mutation frequency experiments, AB174 & AB176 derived from MT363 and MT744 respectively, were also cross-resistant to oxytetracycline (table 21). These isolates were investigated further to determine the reason for this.

3.3.3. Alterations in Outer Membrane Proteins Associated with Cross Resistance Between Oxytetracycline and Oxolinic Acid. Previous work has shown that low level resistance to antibiotics in *A.salmonicida* may be associated with changes in outer membrane proteins (Wood *et al.*, 1986; Griffiths & Lynch, 1989). Wood *et al.* (1986) found that resistance to a wide range of antibiotics including 4-quinolones, β-lactams and tetracycline was associated with the appearance of a 37 kDa protein in the outer membrane. Cross-resistance between oxolinic acid and oxytetracycline could have serious implications for the aquaculture industry. Therefore, the cross resistance between these two compounds in *A.salmonicida* observed in the mutation frequency studies was investigated.

Parent Strain	Mutant	Selected with :	Oxolinic acid	Sara-floxacin	Enro-floxacin	CI934	PD117,596	PD127,391
MT494	AB173	Oxolinic acid	1.5(0.03)	1.0(0.04)	0.20(0.02)	4.0(0.4)	0.15(0.015)	0.015(0.0075)
MT744	AB177		1.0(0.03)	1.0(0.05)	0.15(0.015)	2.0(0.4)	0.15(0.015)	0.05(0.0075)
MT490	AB183		1.0(0.03)	1.0(0.05)	0.15(0.02)	3.0(0.4)	0.15(0.015)	0.05(0.0075)
MT747	AB180	PD117,596	0.1(0.03)	0.3(0.03)	0.15(0.02)	3.0(0.4)	0.15(0.015)	0.1(0.0075)
MT490	AB182		0.1(0.03)	0.3(0.05)	0.15(0.02)	2.0(0.4)	0.15(0.015)	0.1(0.0075)
MT747	AB181	PD127,391	0.1(0.03)	0.3(0.03)	0.15(0.02)	2.0(0.4)	0.15(0.015)	0.1(0.0075)
MT490	AB185		0.1(0.03)	0.3(0.05)	0.15(0.02)	3.0(0.4)	0.15(0.015)	0.1(0.0075)
MT363	AB174	Oxytetracycline	1.0(0.03)	0.4(0.05)	0.15(0.015)	2.0(0.4)	0.15(0.015)	0.1(0.0075)
MT744	AB176		0.1(0.03)	0.2(0.05)	0.1(0.015)	2.0(0.4)	0.15(0.015)	0.05(0.0075)

Table 21. MICs of resistant mutants isolated during mutation frequency studies. (Figures in parentheses indicate MICs of parent isolates).

Oxytetracycline resistant mutants were isolated on TSA containing oxytetracycline at 5 times the MIC. The MICs of oxytetracycline for both resistant mutants, AB174 and AB176, increased approximately 5-fold (table 21). Resistance was stable as ten repeated passages on drug free TSA had no effect on the MICs. When the susceptibility of these mutants to oxolinic acid was examined, both were less susceptible to the 4-quinolone than their respective parents (table 21). The MIC of AB174 was increased by 30-fold compared to its parent strain MT363, while the MIC of AB176 was increased only threefold compared to its parent MT744 (table 21). Both mutant strains were also less susceptible to enrofloxacin and sarafloxacin, and the other fluoroquinolones.

Outer membranes were prepared and the proteins were analysed by SDS-PAGE on the PhastSystem®. The PhastSystem® was calibrated for coomassie staining by running a series of dilutions of a preparation of the OMPs of *A.salmonicida* MT363 on a 12% polyacrylamide PhastGel®. It was found that the optimum protein concentration for giving clear bands on this system was 0.86 -1.30g/l (figure 25). Proteins were run against molecular mass standards, the sizes of which are given in table 22.

Prior to analysing the resistant mutants, it was necessary to investigate both quinolone sensitive isolates, and isolates bearing high level resistance to the quinolones, for comparison. OMPs of six quinolone sensitive isolates of

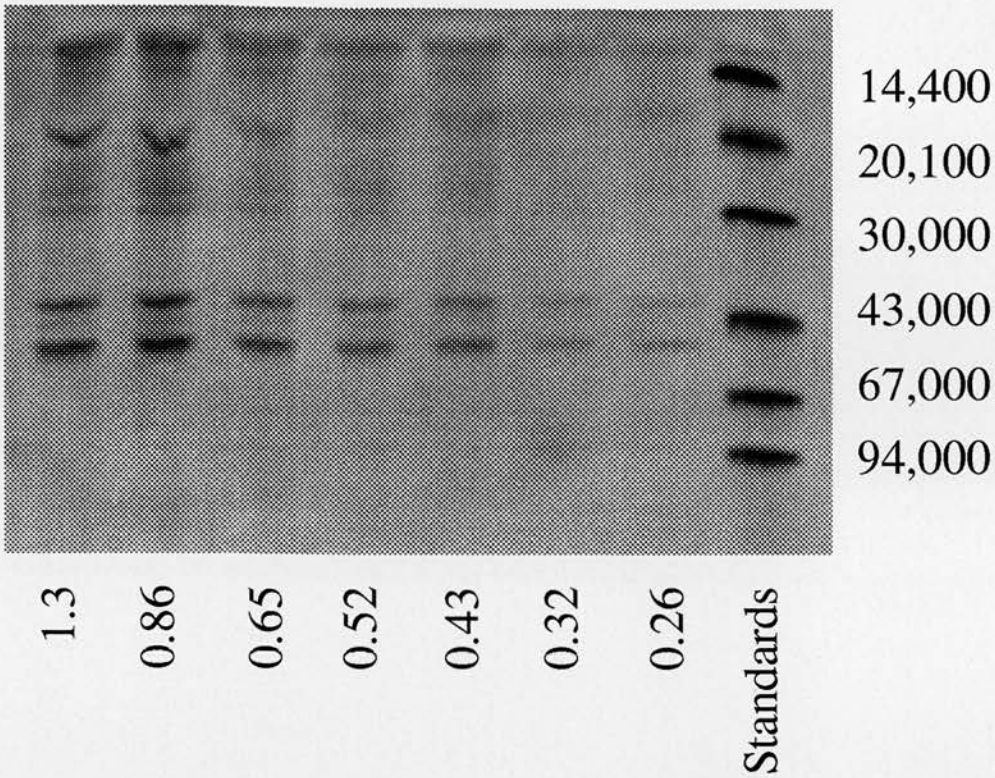


Figure 25. Effect of protein concentration (g/l) on detection of OMPs of *A.salmonicida* separated by SDS-PAGE using the PhastSystem®.

A.salmonicida (MICs given in table 23) were separated by the PhastSystem® and are shown in figure 26. No differences in major proteins were observed in the sensitive isolates examined. Furthermore, when the outer membrane protein profiles of four *A.salmonicida* isolates exhibiting high level resistance to the quinolones (MICs given in table 23) were analysed, no differences between the major proteins were observed (figure 27)

Molecular Mass Standards (Da)	
Phosphorylase B	94,000
Bovine Serum Albumin	67,000
Ovalbumin	43,000
Carbonic Anhydrase	30,000
Soybean Trypsin Inhibitor	20,100
α-Lactalbumin	14,400

Table 22. Molecular masses of standards run on PhastGels®.

Isolate	MICs (mg/l)			
	Oxolinic acid	**Sarafloxacin**	**Enrofloxacin**	**Oxytetracycline**
MT363	0.03	0.05	0.015	0.20
MT199	0.20	0.15	0.15	0.30
MT350	0.04	0.05	0.02	40.00
MT736	0.015	0.04	0.04	100.00
MT494	0.03	0.05	0.02	30.00
MT744	0.03	0.05	0.015	0.30
MT491	0.04	0.075	0.05	0.30
MT340	0.75	0.50	0.15	NT
MT458	1.00	0.75	0.30	50.00
MT438	1.50	1.00	0.20	0.30
MT464	1.50	1.00	0.20	40.00
MT740	1.50	1.00	0.20	NT
MT475	3.00	2.00	0.75	NT
MT472	3.00	2.00	1.00	0.30
MT335	5.00	4.00	1.50	50.00
MT364	7.50	3.00	1.00	0.30

*NT = not tested.

Table 23. MICs of quinolone sensitive and resistant isolates of *A.salmonicida* used in OMP studies.

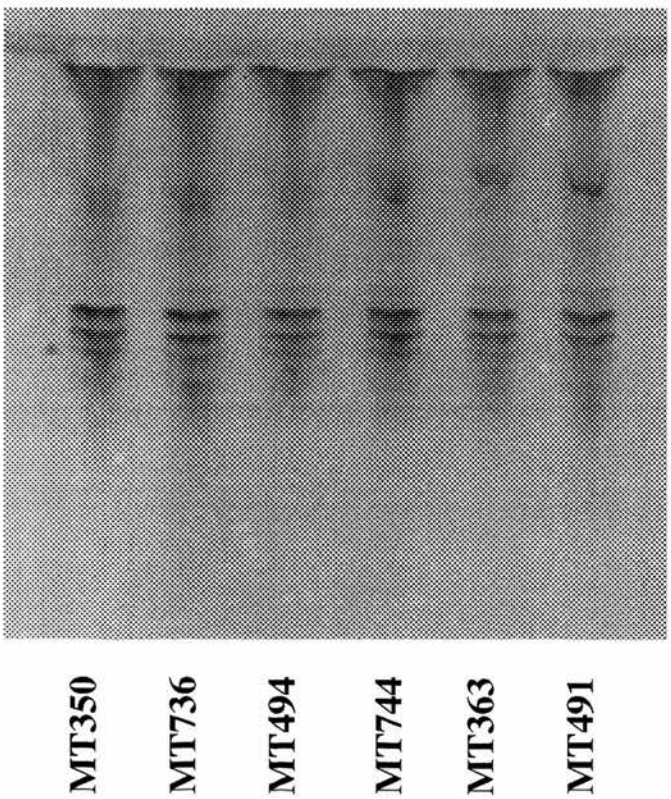


Figure 26. Outer membrane protein preparations of six quinolone sensitive isolates of *A.salmonicida*.

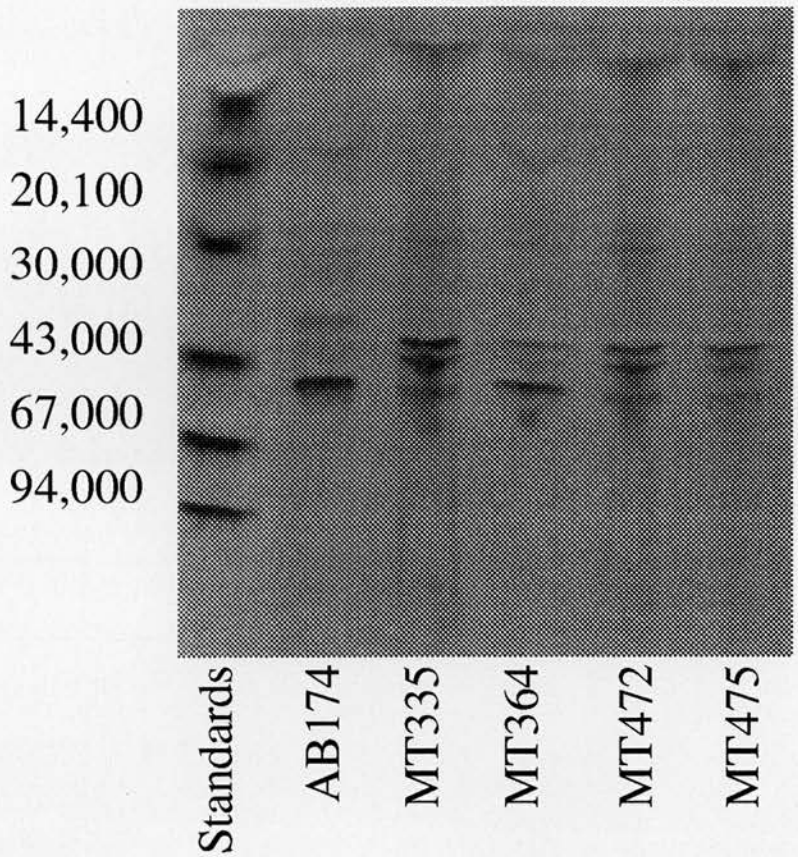
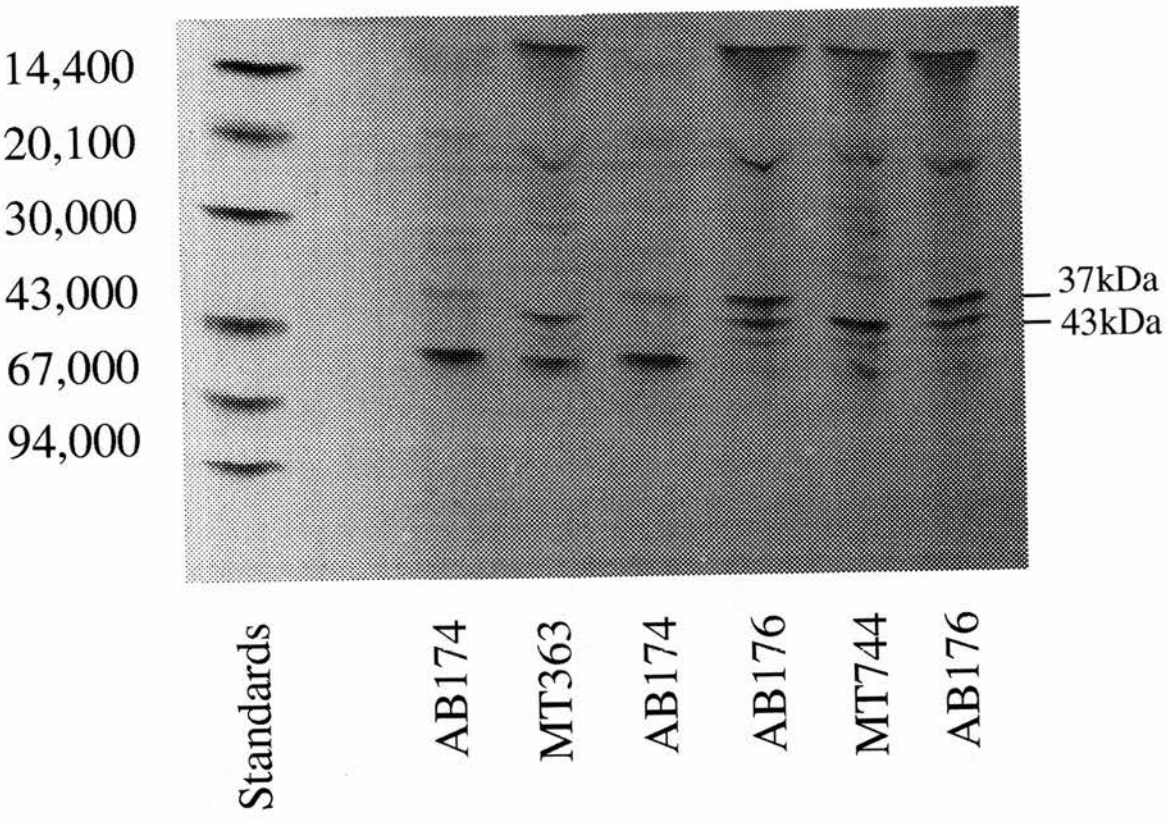


Figure 27. OMP profiles of *A.salmonicida* isolates with high level quinolone resistance.

The outer membrane proteins of the resistant mutants and their respective parents were then analysed by SDS-PAGE. Both mutants were found to express an additional protein, molecular mass approximately 37 kDa, which was not present in the parent strain (figure 28), or indeed, in any of the sensitive isolates examined (figure 28). However, differences were observed in the OMP profiles of mutants AB174 and AB176. A 43 kDa protein which was present in AB176 as well as both wild-type strains could not be detected in AB174. The complete absence of this protein may explain why resistance to oxytetracycline and oxolinic acid was higher in AB174 compared to AB176.

It may be that the single OMP change observed in AB176, namely the increased expression of the 37 kDa protein, is merely a single step towards the higher level resistance observed in AB174, where there were two distinct



Fugure 28. OMP changes in quinolone and oxytetracycline resistant mutants of *A.salmonicida*.

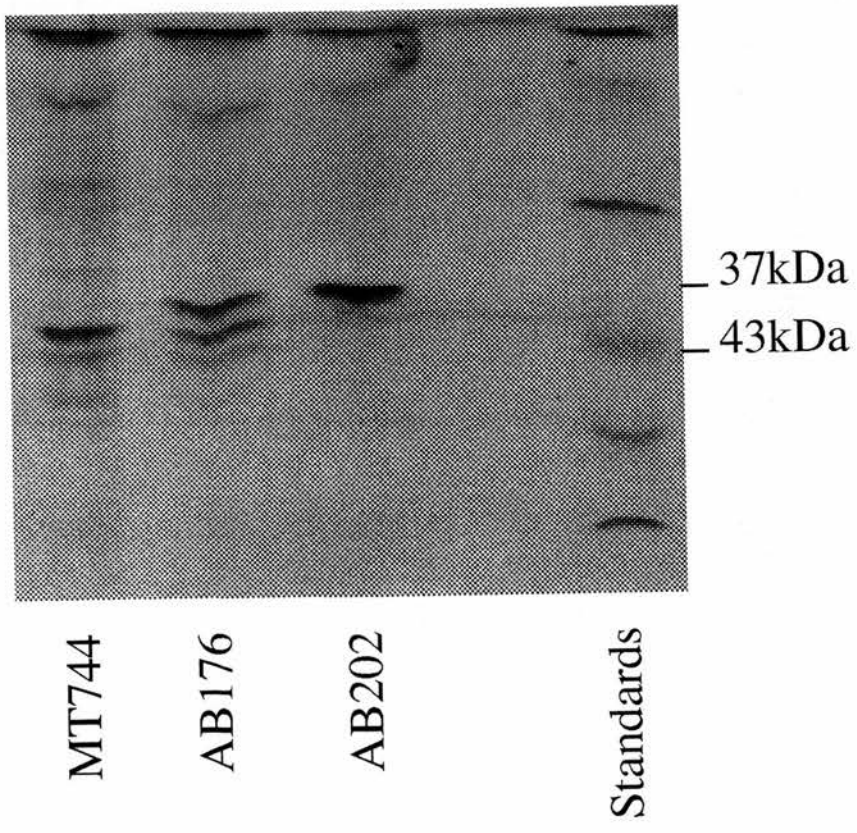


Figure 29. A two-stage mutation to high level quinolone resistance?

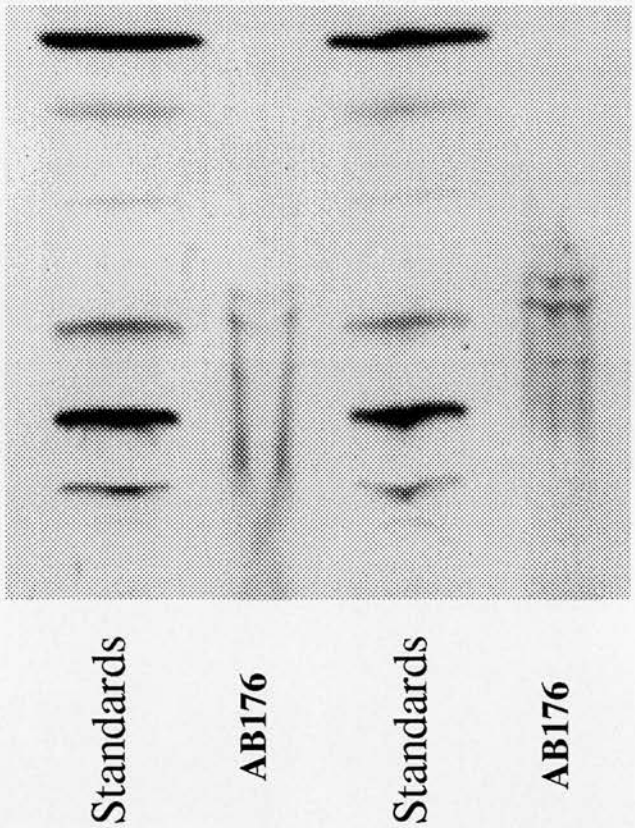


Figure 30. PhastGel® showing proteins from AB176 which are non-covalently associated with peptidoglycan (see text).

changes in the outer membrane profiles. In order to determine whether this was the case, AB176 (10^{10} cfu/ml) was lawned onto TSA plates containing oxolinic acid at 5 x the MIC. The plates were incubated for 7 days and examined daily for the presence of resistant colonies. A resistant mutant (AB202) was isolated and identified as described for the mutation frequency experiments (section 2.5.2). The mutant isolate (AB202) was found to have elevated MICs to the fluoroquinolones and oxytetracycline (table 24). Furthermore, on analysis of the outer membrane proteins of AB202, the 43 kDa protein, which was expressed in its parent, AB176, was not expressed in AB202 (figure 29). This supports the idea that there may be two stages to OMP associated fluoroquinolone resistance. A single change, the increase in expression of a 37 kDa protein, which results in low level resistance to quinolones and oxytetracycline. This change may be followed by a second change, the reduced expression of the 43 kDa protein, which leads to higher level antibiotic resistance.

Strain	MIC (mg/l)					
	Oxolinic acid	**Nalidixic acid**	**Sara-floxacin**	**Enro-floxacin**	**Amoxy-cillin**	**Oxytetra-cycline**
MT363	0.03	0.20	0.04	0.02	0.30	0.20
AB174	1.50	40.00	0.30	0.10	0.75	1.00
MT744	0.03	0.20	0.04	0.02	0.30	0.20
AB176	0.10	0.75	0.20	0.10	0.75	1.00
AB202	1.50	40.00	0.30	0.10	0.75	1.00

Table 24. MICs of quinolones and unrelated antibacterials against *A.salmonicida* OMP mutants.

Both the 37- and the 43-kDa proteins appeared to be non-covalently associated with peptidoglycan as they both remained with the insoluble peptidoglycan fraction after solubilisation with 2% (w/v) SDS at 37°C (fig 30). These proteins were, however, released from the peptidoglycan by heating in 2% SDS at 100°C for 5 minutes. This suggests that these two proteins may function as porins.

This proposal is supported by the observation that hydrophilic quinolones such as nalidixic acid and oxolinic acid are affected more significantly by these changes than the more hydrophobic fluoroquinolones such as sarafloxacin, enrofloxacin, PD127,391 and PD117,596 (table 24). This is a result of the reliance of hydrophilic antibiotics on the porins for uptake into the bacterial cell. Hydrophobic compounds can be taken up by other routes.

These phenomena are not solely laboratory artefacts. A number of *A.salmonicida* isolates exhibiting low level resistance to quinolones were screened for alterations associated with multiple antibiotic resistance. The outer membranes are shown in figure 31. *A.salmonicida* MT464 (fig.31, lane

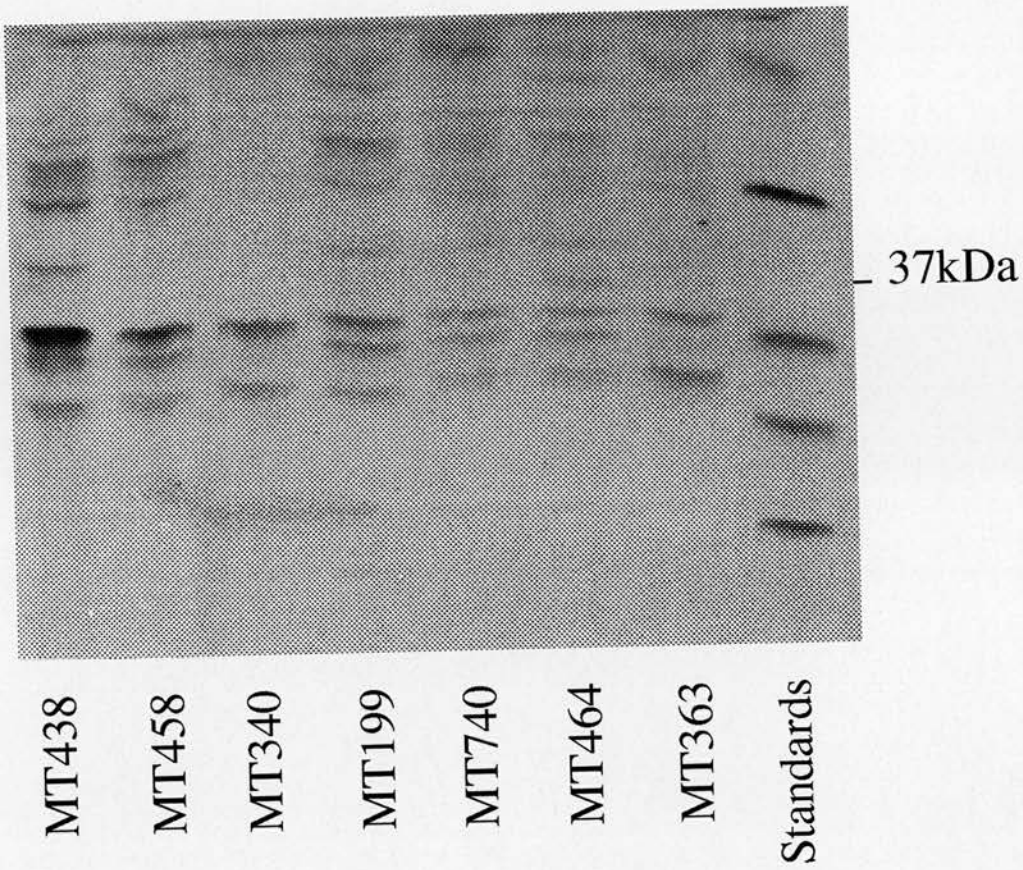


Figure 31. PhastGel® showing OMP changes in a wild-type isolate of *A.salmonicida* exhibiting low level quinolone resistance.

6), an isolate exhibiting low-level resistance to oxolinic acid (MIC 1.00 mg/l), isolated from an outbreak of furunculosis at a Scottish salmon farm in 1989, demonstrated increased expression of a 37kDa protein. As discussed previously, this protein is associated with decreased permeability to antibacterials.

3.3.4 Probing for *gyrA* Mutations. High level quinolone resistance in many pathogens is due predominantly to alterations in the α-subunit of the target enzyme DNA gyrase, encoded by the gene*gyrA* (Lewin *et al.* 1991). Two probes, pNJR 3,2 and pBP513 were used to determine whether *gyrA*

mutations are prevalent in *A.salmonicida* isolates exhibiting high level resistance to the quinolones.

Transformation was used as the method of introducing the probes into *A.salmonicida* as the difficulty in separating the recipient *A.salmonicida* isolates from a donor *E.coli* deemed conjugation unsuitable.

Parent Isolate	**Oxolinic Acid MIC (mg/l)**	**Kanamycin resistant isolate (MIC >20.0mg/l)**
MT438	1.50	265, 266, 267 268
MT362	5.00	269, 270, 271 272, 273
MT487	5.00	274, 275, 277 279, 280, 281

Table 25. Kanamycin resistant isolates recovered from transformation experiments.

In spite of numerous attempts, transformants were not obtained using plasmid pNJR 3,2, either by the ice-cold calcium chloride technique of Saunders & Saunders (1988), or by the protoplast transformation technique of Chang and Cohen (1979). Indeed, no colonies resistant to the tetracycline marker on the probe were isolated on the selective plates after 48h incubation.

Plasmid pNJR3,2 is quite large (23kb). Thus a smaller probe, pBP513, of only 10kb was obtained. Using the ice cold calcium chloride technique of Saunders & Saunders (1988), colonies resistant to kanamycin (the marker on pBP513) were isolated after 48h incubation. The colonies were speciated with a Bionor Mono AS latex bead agglutination kit and found to be *A.salmonicida.* Minipreparations of plasmid DNA from these resistant colonies was analysed on a 0.7% agarose gel. However, pBP513 was not present in any of the resistant isolates (fig. 32 & table 25).

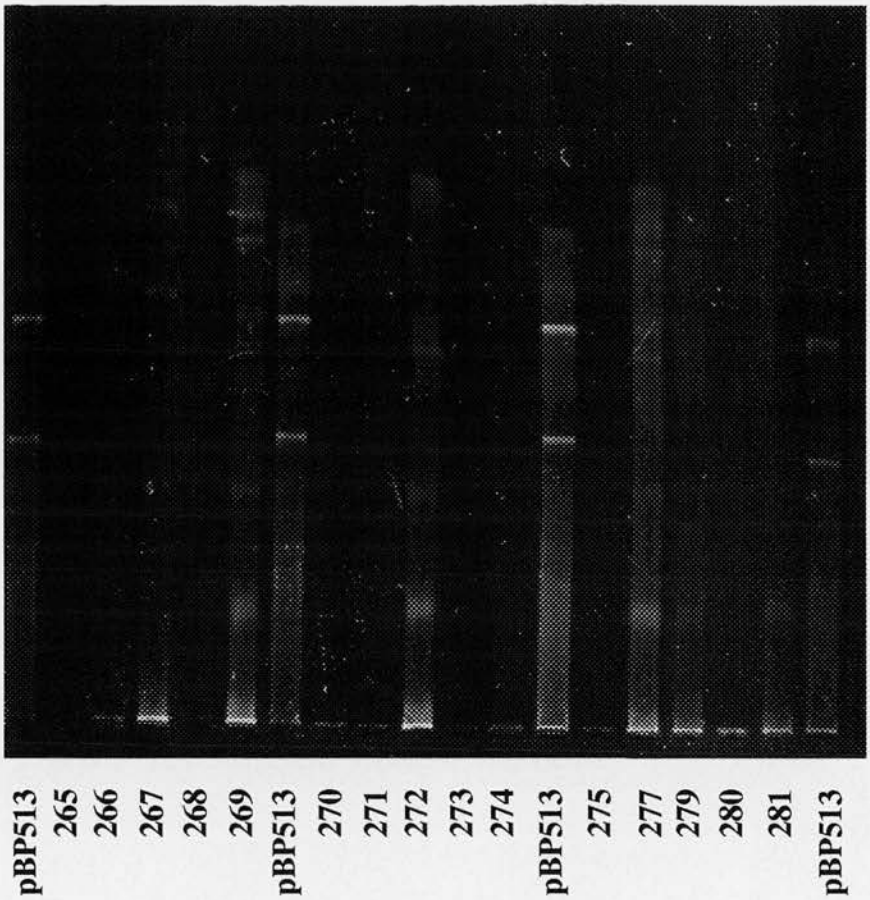


Figure 32. Agarose gel electrophoresis of plasmid DNA from transformants. Also shown is purified pBP513.

Furthermore, transformation of protoplasts by the method of Chang and Cohen (1979) with pBP513 was also unsuccessful, as no kanamycin resistant colonies were recovered.

3.4. *IN VITRO* ACTIVITY OF THE POTENTIATED SULPHONAMIDE ROMET.

Romet 30 is a potentiated sulphonamide comprising two antibiotics, ormetoprim and sulphadimethoxine. Romet has been used for a number of years in aquaculture and agriculture in the USA. It is now being considered for use in aquaculture in the UK, thus the efficacy of Romet was investigated *in vitro* against Scottish isolates of *A.salmonicida*.

3.4.1. Minimum Inhibitory Concentrations (MICs). The MICs of ormetoprim and sulphadimethoxine were estimated in parallel with trimethoprim, the prototype 2,4-diaminopyrimidine considered to be the standard dihydrofolate reductase inhibitor. The results show that all of the isolates were susceptible to ormetoprim at 1mg/l (table 26). Approximately 75% of the Scottish isolates of *A.salmonicida* were susceptible to sulphadimethoxine at 16mg/l. Generally speaking, the trimethoprim concentrations required to inhibit the bacteria were half that required by ormetoprim. Examination of the MICs suggested that two main phenotypes were prevalent, based on their resistance levels to ormethoprim and sulphadimethoxine; a) Sensitivity to ormetoprim and sulphadimethoxine; and b) Sensitivity to ormetoprim but resistance to sulphadimethoxine.

	MICs (mg/l)		
Antibiotic	**MIC_{50}**	**MIC_{90}**	**Range**
Ormetoprim	0.5	1.0	0.5 - 1.0
Sulphadimethoxine	16.0	>64.0	8.0 - >64.0
Trimethoprim	0.25	0.5	0.0625 - 0.5

Table 26. Range of MICs (mg/l) of dihydrofolate reductase inhibitors

3.4.2. Fractional Inhibitory Concentrations of Ormetoprim and Sulphadimethoxine. Isolates of both phenotypes were taken for further examination of the fractional inhibitory concentrations (FIC), 10 from phenotype a, and 10 from phenotype b. The isolates within each group were

distinct, based on their resistance profiles of oxytetracyline and oxolinic acid. The FICs were determined at their points of maximum interaction (for examples see figures 33 & 34) and the FIC index determined (table 27).

	MIC (mg/l)		FIC		FIC
Isolate	**Orm**	**Su**	**Orm**	**Su**	**Index**
MT491	0.75	12	0.083	0.166	0.25
MT199	1.00	12	0.125	0.166	0.29
MT328	0.75	6	0.083	0.33	0.41
MT495	0.50	6	0.125	0.33	0.46
MT489	0.75	12	0.083	0.166	0.25
MT736	1.00	12	0.062	0.166	0.23
MT494	0.75	8	0.083	0.25	0.33
MT321	1.00	16	0.125	0.125	0.25
MT496	0.75	6	0.083	0.33	0.41
MT350	1.00	8	0.062	0.25	0.31
MT320	0.75	>2048	0.5	<0.125	<0.625
MT427	0.75	2048	0.5	0.125	0.625
MT674	1.0	2048	0.5	0.187	0.687
MT448	0.75	2048	0.33	0.125	0.45
MT464	0.75	2048	0.5	0.187	0.687
MT757	0.75	2048	0.5	0.187	0.687
MT740	0.75	2048	0.33	0.125	0.45
MT741	1.00	768	0.187	0.33	0.52
MT350	1.00	2048	0.375	0.125	0.5
MT487	1.00	2048	0.25	0.125	0.375

Table 27. Fractional inhibitory concentrations of the component antibiotics of Romet 30.

Amongst the 12 isolates which were sensitive to both ormetoprim and sulphadimethoxine, the highest FIC index was 0.46. This value is considerably lower than the value of 0.7, which is the accepted value associated with the upper limit of synergy. In other words, the two drugs are actively interacting with one another to produce potentiation. The highest FIC index for the eight sulphadimethoxine resistant strains was 0.687. Although close to the upper limit, this value indicates that active potentiation of the drugs is occurring.

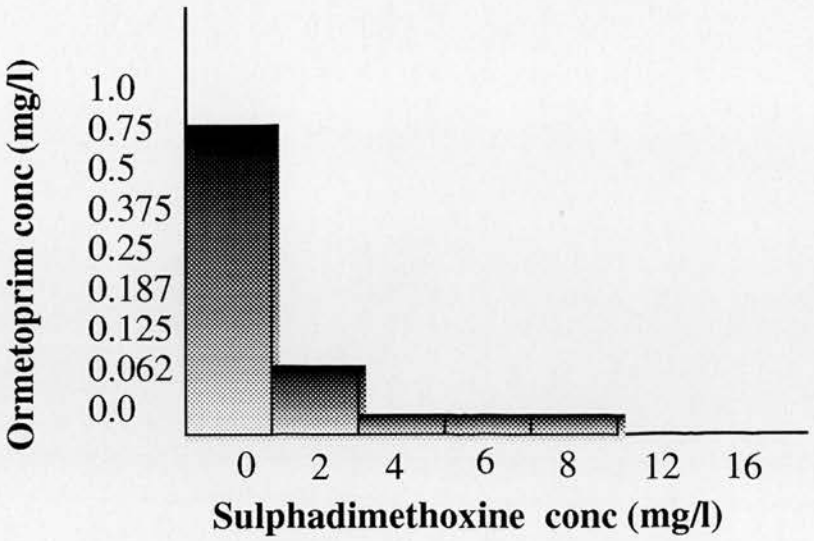


Figure 33. Isobologram from which fractional inhibitory concentrations of ormetoprim and sulphadimethoxine were determined aginst *A.salmonicida* MT729 (Su-sensitive).

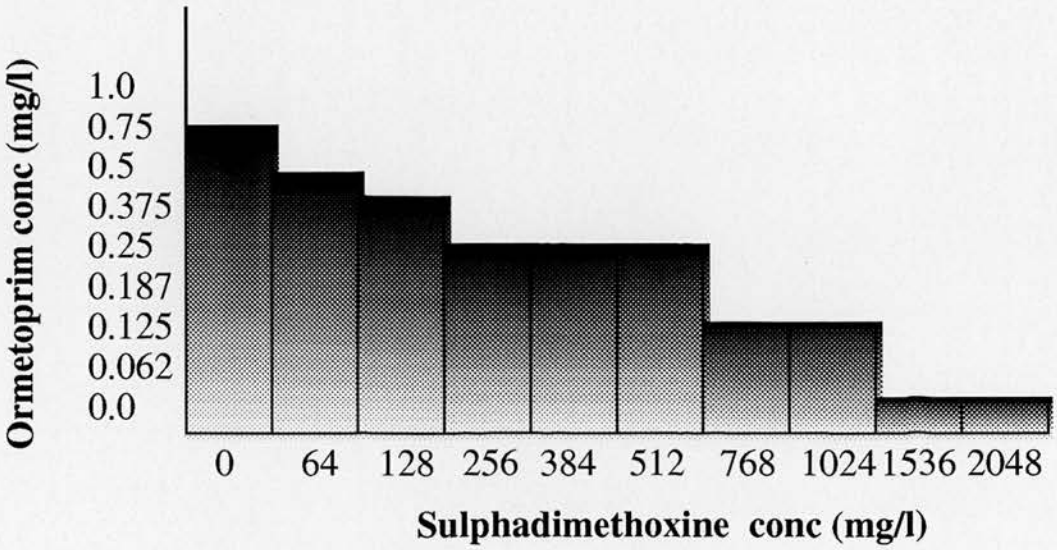


Figure 34. Isobologram from which FICs of ormetoprim and sulphadimethoxine were determined against *A.salmonicida* MT320 (Su-resistant).

3.5 RESISTANCE OF *AEROMONAS SALMONICIDA* TO AMOXYCILLIN.

Amoxycillin was licensed for use in Atlantic salmon in Scotland in September 1990. Potential resistance to this compound was therefore investigated.

3.5.1. *In Vitro* Activity of Amoxycillin Against Scottish Isolates of *A.salmonicida.* *In vitro* activity was assessed in terms of minimum inhibitory concentration against 80 isolates of *A.salmonicida.* The isolates could be divided into two phenotypes on the basis of their amoxycillin susceptibility; those which were amoxycillin susceptible and those which were amoxycillin resistant (table 28). MICs of amoxycillin ranged between 0.30mg/l and 1.50 mg/l against all of the *A.salmonicida* subsp. *salmonicida* tested. On the other hand, all of the *A.salmonicida.* subsp. *achromogenes* isolates tested were resistant to amoxycillin, having MICs in excess of 500mg/l.

A.salmonicida subspecies	No. of isolates	Range of MICs (mg/l)	MIC_{50} (mg/l)	MIC_{90} (mg/l)
salmonicida	71	0.30-1.50	0.30	0.50
achromogenes	9	>500.00	>500.00	>500.00

Table 27. Range of MICs of amoxycillin against *A.salmonicida* subspecies.

3.5.2. Mechanism of Amoxycillin Resistance. The nine resistant isolates were investigated further to characterize the nature of the observed resistance. Crude extracts prepared from amoxycillin resistant isolates were examined for β-lactamase activity by a nitrocefin spot test. All of the amoxycillin resistant isolates produced a β-lactamase enzyme (designated ASE-1). When focused according to its isoelectric point, the enzyme focused into multiple bands with the main band having a pI of approximately 8.0 (fig. 35).

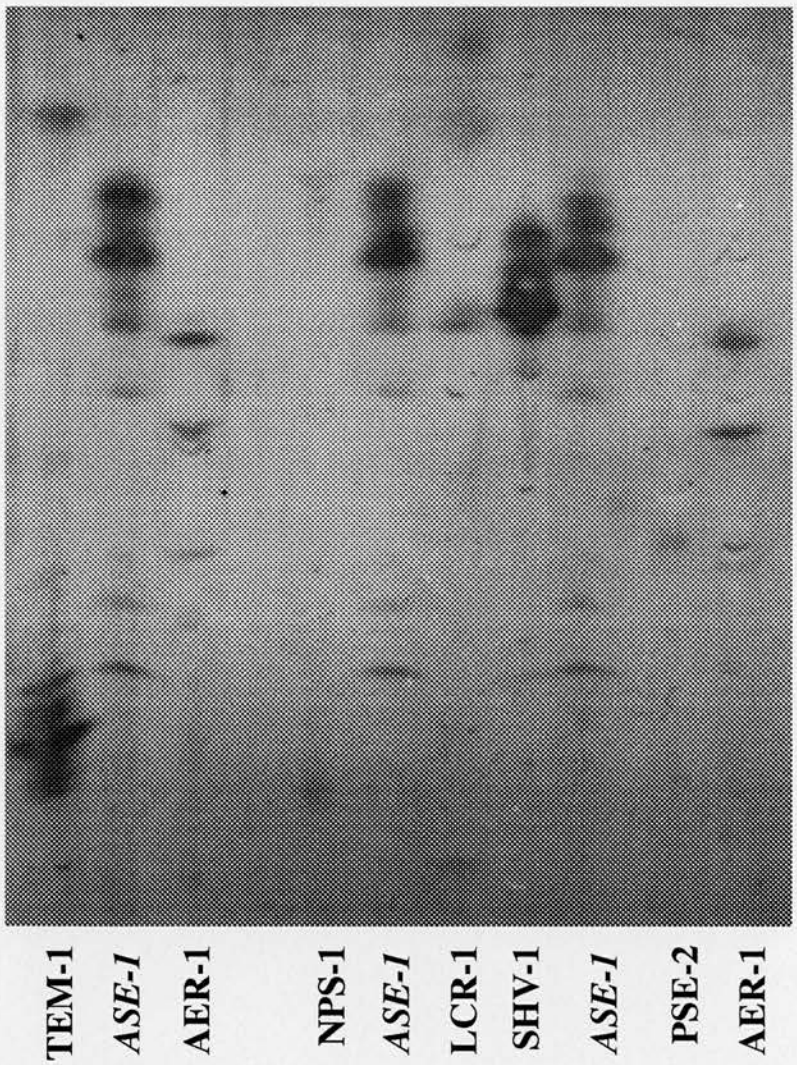


Figure 35. Polyacrylamide gel showing focused bands of β-lactamases, including ASE-1.

3.5.3. Spectrum of β-lactamase Activity. The rate of hydrolysis of a range of β-lactams was determined and compared to that of penicillin G. ASE-1 was found to hydrolyse ampicillin very rapidly, but had little activity against either carbenicillin or cefotaxime (table 29).

3.5.4. Susceptibility to Augmentin. Clavulanic acid is a β-lactamase inhibitor frequently administered with amoxycillin in human medicine. The combination (4:1 amoxycillin: clavulanate) is marketed as Augmentin. The susceptibility of the *achromogenes* isolates to Augmentin was therefore investigated. The MICs of the resistant isolates were significantly lower to Augmentin than to amoxycillin. All the isolates had MICs of amoxycillin in excess of 500mg/l, whereas MICs of Augmentin were 16.0 mg/l (table 30).

Enzyme	pI	Relative rate of hydrolysis*			Reference
		Amp	Carb	CTX	
TEM-1	5.4	106	10	<1	Hedges *et al.* (1974)
SHV-1	7.6	212	8	0	Petroceilou *et al.* (1974)
LCR-1	6.5	145	4		Simpson *et al.* (1983)
NPS-1	6.5	223	18	<1	Livermore & Jones (1986)
PSE-2	6.1	267	121	16	Matthew (1978)
AER-1	5.9	38	98	20	Hedges *et al.* (1985)
ASE-1	**8.0**	**182**	**3**	**0**	**This study**

Amp, ampicillin; Carb, carbenicillin; CTX, cefotaxime.
*The rate of hydrolysis for Penicillin G = 100.

Table 29. Relative initial rates of hydrolysis of β-lactamases.

3.5.5. Genetic Basis for Amoxycillin Resistance. During the plasmid curing experiments, no matter what concentration of ethidium bromide (EtBr) was used, the replica plates showed that there was never any loss of resistance to amoxycillin. This strongly suggests that the β-lactamase ASE-1 is encoded on the bacterial chromosome. ASE-1 was inducible with 10mg/l cefoxitin, the induced form hydrolysing nitrocefin at a rate of 0.227/μmole/min/g of protein compared to 0.0017/μmole/min/g protein for the uninduced form, a 163-fold increase in activity.

No. of isolates	Range of MICs (mg/l)	
	Amoxycillin	Augmentin
9	>500.0	16.0

Table 30. Range of MICs of amoxycillin and Augmentin against *A.salmonicida* subsp. *achromogenes*.

3.5.6. Conjugation with *E.coli* K12. Attempts to mobilise the gene(s) expressing β-lactamase ASE-1 from *A.salmonicida* were unsuccessful.

3.6. A SEAWATER INFECTION CHALLENGE MODEL.

Groups of 50 Atlantic salmon post smolts were infected with a virulent Scottish isolate of *A.salmonicida* (MT879). Inocula of 1 x 10^4, 1 x 10^5 and 1 x 10^6 cfu/ml were employed. Mortalities were removed daily and bacteriological samples taken to ascertain the cause of death.

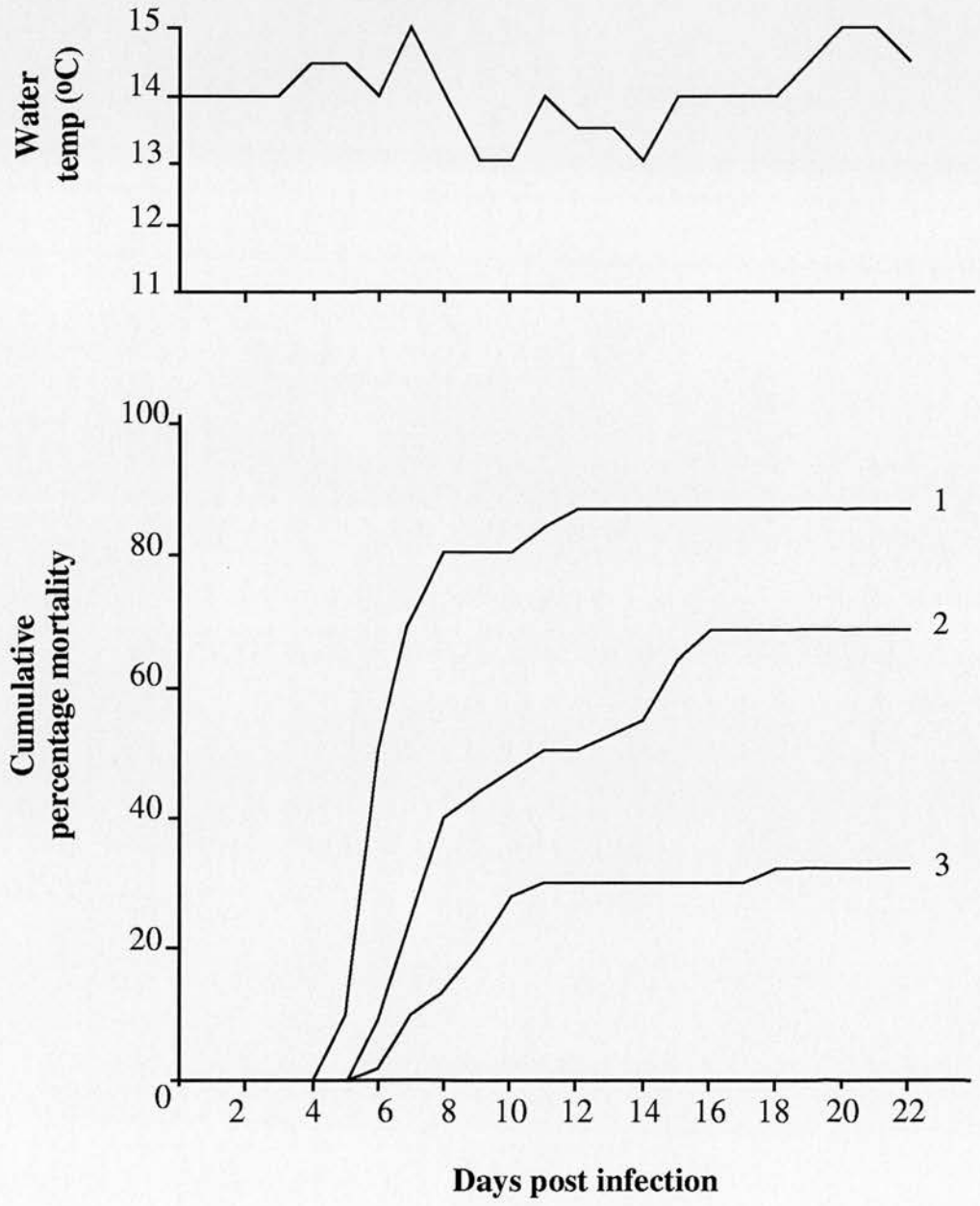


Figure 36. Cumulative mortalities resulting from furunculosis at three levels of infection challenge: Curve 1 shows challenge with 10^6 cfu/ml *A.salmonicida* MT879; curve 2 shows challenge with 10^5 cfu/ml; curve 3 shows challenge with 10^4 cfu/ml. Sea water temperatures over the challenge period are also shown.

At seawater temperatures of around 14°C (fig. 36), fish challenged with the highest concentration of *A.salmonicida* MT879 (10^6 cfu/ml) began dying 5 days post infection. Fish exposed to 10^5 or 10^4 cfu/ml began to die one day later. There was a close relationship between infection challenge dose and mortalities: mortality rates and total mortalities increased with exposure to increasing numbers of *A.salmonicida* (fig. 36). At the end of the challenge period (22 days), cumulative mortalities reached 31.9%, 66.7% and 87.9% in populations exposed to 1 x 10^4, 1 x 10^5 and 1 x 10^6 cfu/ml respectively. Pure cultures of *A.salmonicida* were reisolated from all fish which died, confirming all mortalities resulted from furunculosis.

3.7. A PILOT *IN VIVO* EFFICACY STUDY ON ROMET 30.

A study into the *in vitro* efficacy of the potentiated sulphonamide Romet 30 (refer to chapter 3.4), suggested that this compound was active against Scottish isolates of *A.salmonicida*. Previous work has demonstrated that Romet is effective in trout in freshwater (Maestrone, 1984), but, as yet, no data are available on the control of furunculosis in salmon with Romet.

These *in vivo* experiments were performed in a quarantined part of the SOAFD facility at Aultbea, Wester Ross, containing predominantly fresh water tanks but also some capable of being supplied with salt water. To achieve disinfection during live disease agent trials, iodine is added to the effluent water at a rate necessary to achieve a final concentration in the settlement sump of 5ppm. The residence time in the sump is flow dependent but not less than 30 minutes. Granular iodine is contained in 15cm diameter, 1 metre long perspex columns connected to a freshwater supply. Iodine concentration in the effluent of the columns has, at 100-150ppm, been measured to be relatively independent of flow rate through the column in the range 1 - 10 L/min (R. Johnstone, personal communication) as long as the height of the column is maintained by frequent addition of iodine. The output of the column is matched to the outflow of the tanks requiring disinfection in order to effect the required concentration. Previous experimentation by the SOAFD staff had verified the efficacy of these disinfection procedures under fresh water conditions only and under the maximum possible admixture of salt to fresh water conditions (ca 1:10).

3.7.1. Efficacy Study; Phase 1. Three replicate efficacy trials were performed.

Trial 1. Twenty-three days following exposure to *A.salmonicida* infection, cumulative mortalities in the control (untreated) population reached 29.4%. Bacteriological examination of dead fish indicated that 86.7% of the mortalities (ie. 25.5% of the total untreated population) resulted from furunculosis. During the same period, 2% of the fish in the population which had received a single five day oral treatment with Romet 30 died of furunculosis (figure 37). However, 22 fish in the Romet treated group died

from anoxia on day 18 when a sand-eel blocked the water supply inlet and cut off the water supply to the tank. *A.salmonicida* was not isolated from any of these fish, nor from one further fish found dead the next day.

Trial 2. Mortalities in the untreated population reached 32.6% after 23 days. In the population which had received a single five day oral treatment with Romet, 4% of the fish died during the same period (figure 38). Pure cultures of *A.salmonicida* were isolated from all fish which died.

Trial 3. A total of 39.6% of the control population died from furunculosis within the 23 day period following exposure to water-borne *A.salmonicida* infection. A further 3.9% mortality in this population was not attributable to *A.salmonicida* infection. A total of 3.9% of the fish which received a single treatment of Romet died within the same period (figure 39).

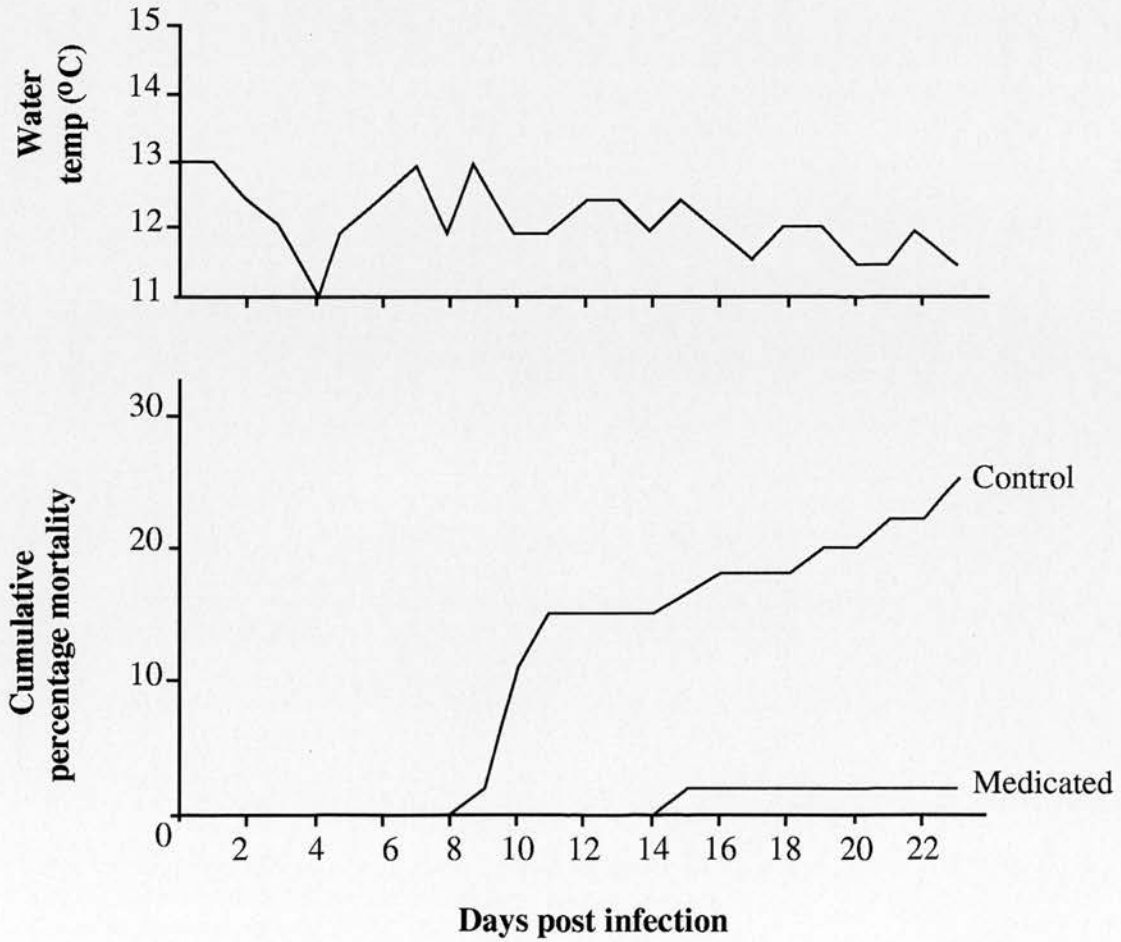


Figure 37. Efficacy of Romet in controlling furunculosis in Atlantic salmon held in sea water. Trial 1.

Taking trials 1,2 and 3 together, 32.7% of fish in the untreated populations died from furunculosis infection within the 23 day trial period. Cumulative furunculosis mortalities in the populations which had been administered a single five day oral treatment with Romet were 3.3% during the same period. The relative percentage survival of Romet treated fish compared with control (untreated) fish was 89.9%.

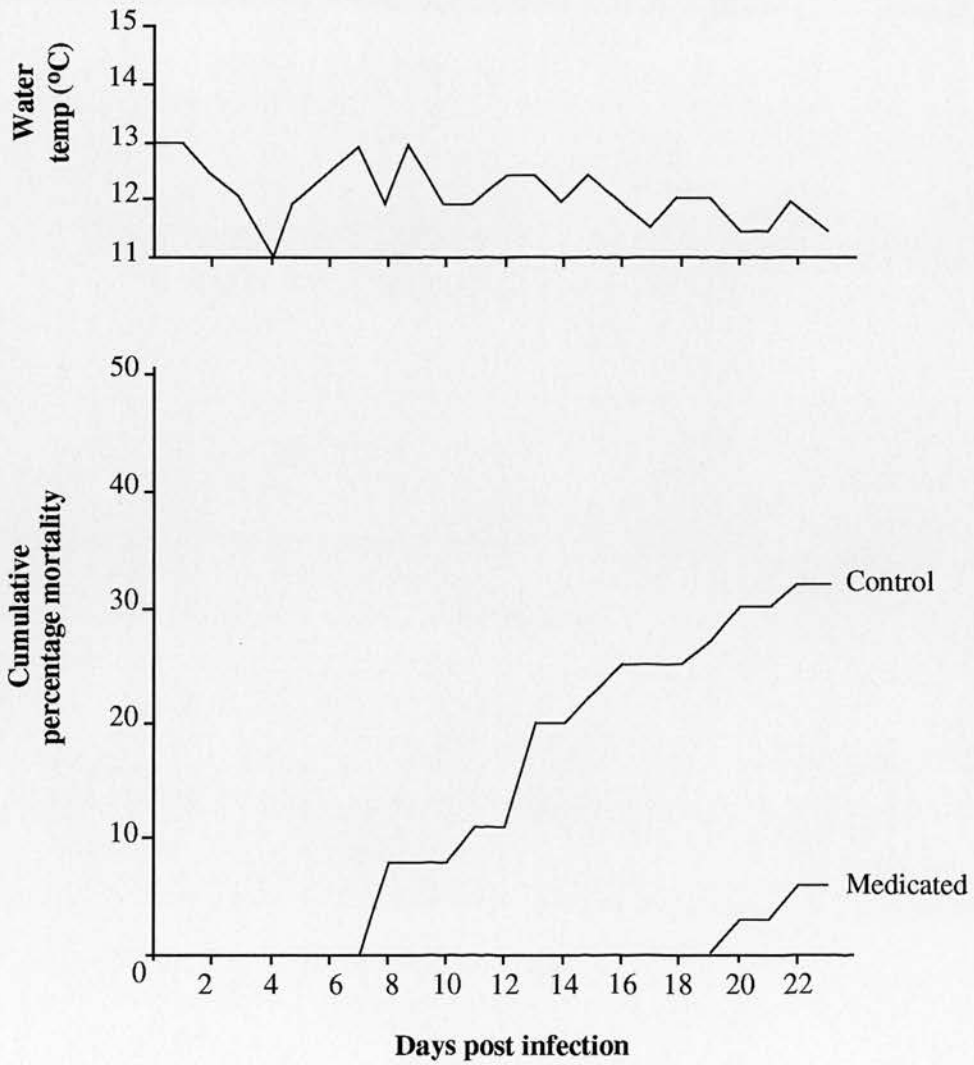


Figure 38. Efficacy of Romet in controlling furunculosis in Atlantic salmon held in sea water. Trial 2.

3.7.2. Efficacy Study; Phase 2. During the following period in which trials 1,2 and 3 received a second five day oral treatment with Romet, the treated populations suffered a total of 3.9% mortalities from furunculosis (an additional 0.6% was not attributable to furunculosis). During the same period, the corresponding control (untreated) populations suffered 9.3% mortalities caused by furunculosis. The results of trials 1,2 and 3 are summarised in figure 40.

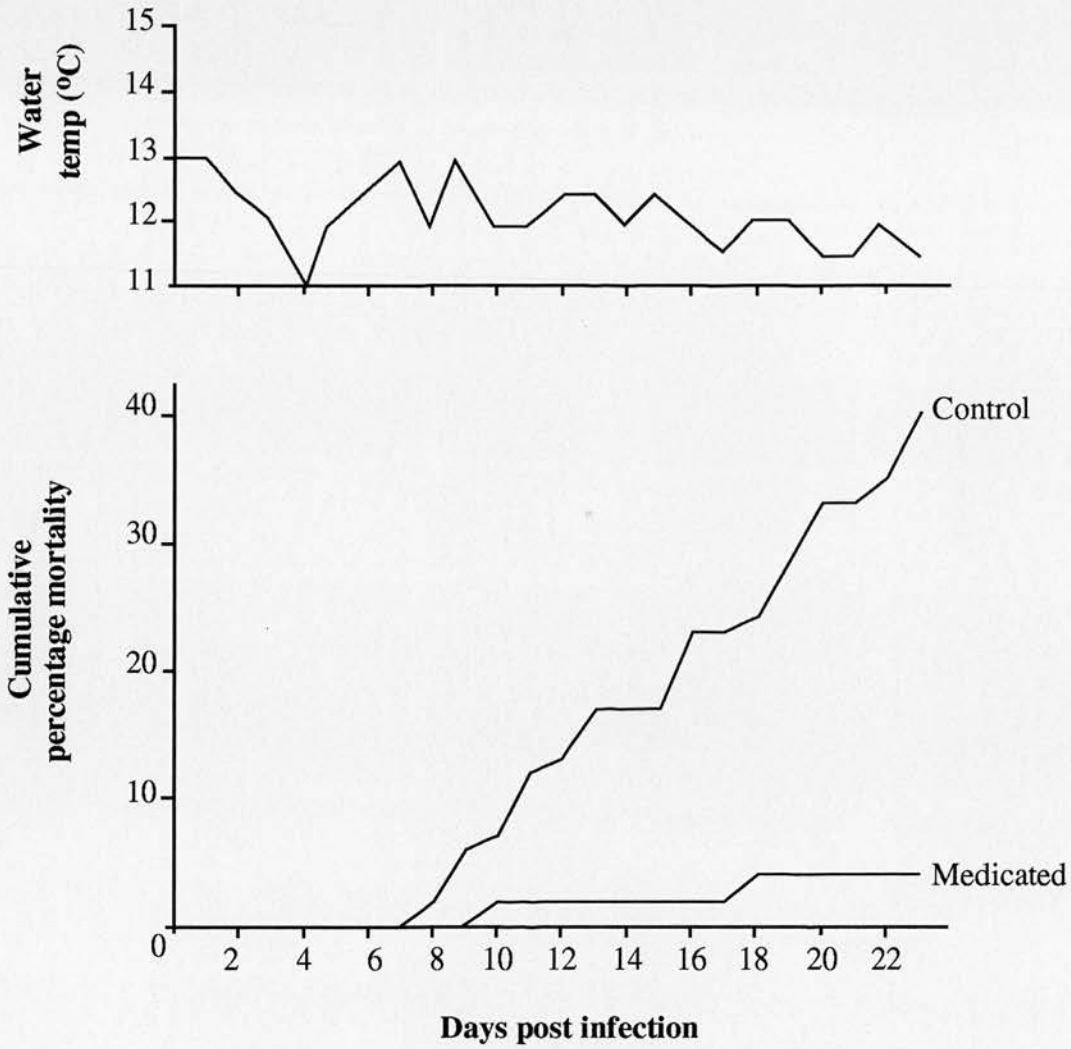


Figure 39. Efficacy of Romet in controlling furunculosis in Atlantic salmon held in sea water. Trial 3.

When surviving fish were sacrificed 30 days post initial infection, *A.salmonicida* was isolated from 7.7% of the Romet treated fish as compared with 6.0% of the control fish (table 31). Thus the prevalence of detected asymptomatic carriage of *A.salmonicida* amongst the surviving fish was similar in treated and untreated fish.

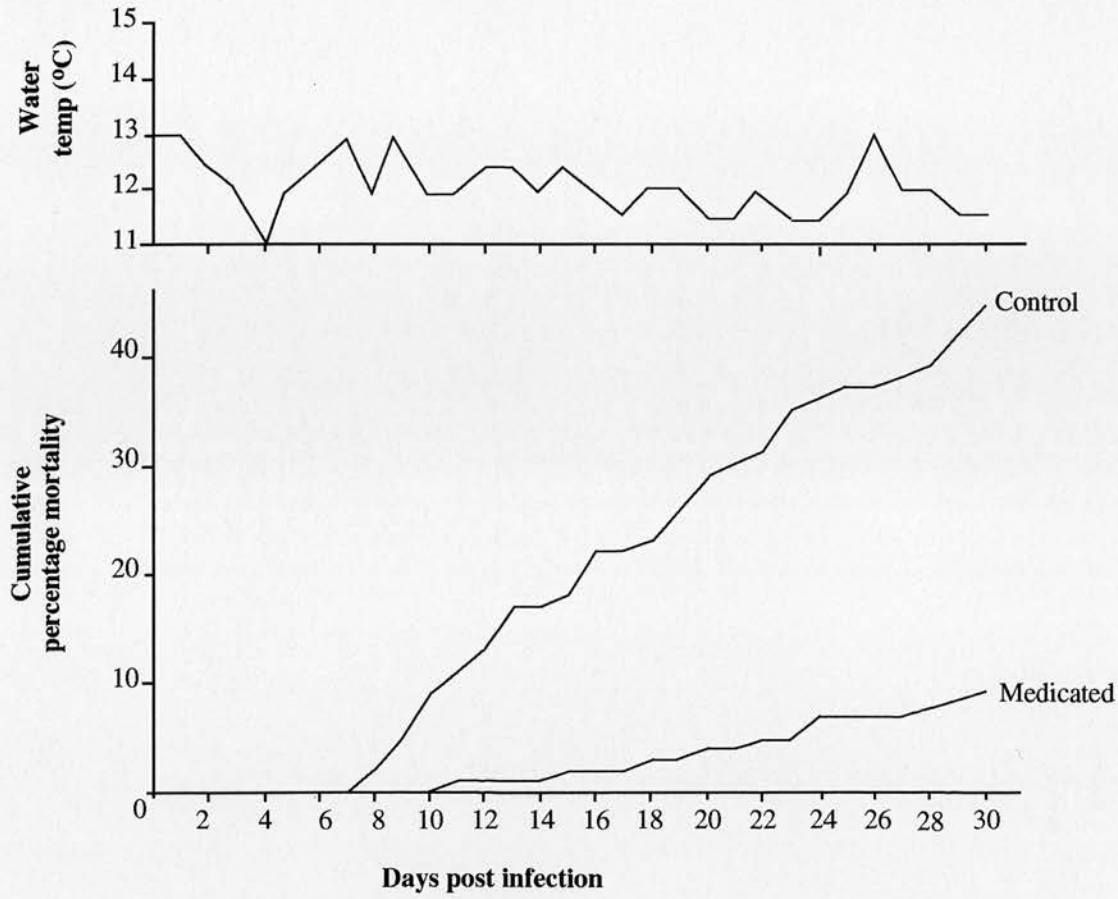


Figure 40. Cumulative mortalities due to furunculosis in Atlantic salmon held in sea water following two 5-day oral treatments with Romet. Combined results of trials 1, 2 and 3.

	Trial 1		Trial 2		Trial 3		Total	
	Romet	Control	Romet	Control	Romet	Control	Romet	Control
No. of survivors	27	33	45	28	45	22	117	83
No. of survivors in which *A.salmonicida* detected	0	0	7	2	2	3	9	5
Percentage of survivors in which *A.salmonicida* detected	0	0	15.5	7.1	4.4	13.6	7.7	6.0

Table 31. Detection of asymptomatic *A.salmonicida* infection in surviving fish.

3.7.3. Observations on the Palatability of Romet. The feeding response of the fish was closely monitored during oral administration of Romet medicated feed. Most, if not all, of the fish appeared to participate in feeding activity. There was no evidence of spitting out or regurgitation of feed pellets, and all of the medicated feed appeared to be taken each day.

3.8. EFFICACY OF IODINE AS A SEAWATER DISINFECTANT.

The previously reported *in vivo* experiments were performed in a quarantined part of the SOAFD experimental facility at Aultbea that contained fresh and salt water tanks. Iodine (final sump concentration 5ppm) was added to the effluent during the experiment to effect disinfection and the efficacy of this procedure has been verified by experiment. Site modifications and improvements dictated that an additional and extended series of seawater *in vivo* trials planned to be carried out towards the end of this project be conducted in a new sea water only quarantine facility. During its commissioning, however, the efficacy of iodine at the concentrations previously used in fresh water, as a disinfectant in undiluted sea water, or sea water/fresh water mixtures of around 1:10, was called into question. As a result, the planned *in vivo* studies were not performed. Instead, some preliminary investigations into the efficacy of iodine under sea water conditions were undertaken.

3.8.1. Bactericidal Activity of Iodine in Sump Seawater. Iodine was bactericidal in sump water, killing 99% of the bacteria after 20mins exposure at a concentration of 8ppm (fig. 41). However at 4ppm, close to the iodine concentration which may be achieved using a single column at Aultbea, 10% of the bacteria survived after 20 min (fig. 41).

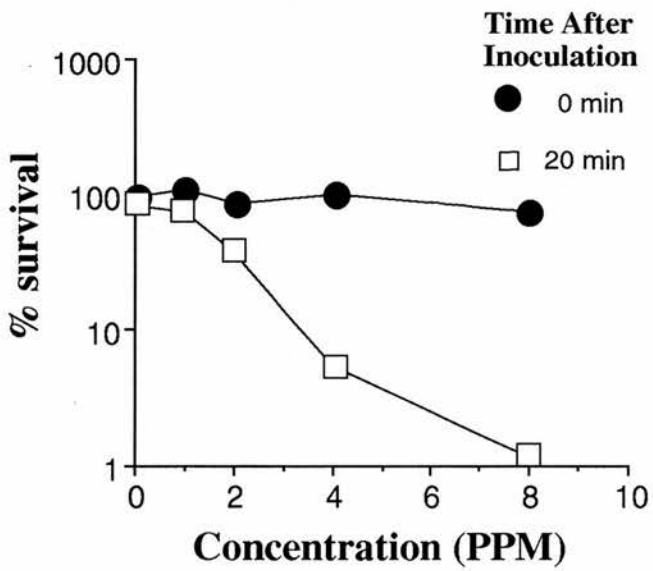


Figure 41. Bactericidal activity of iodine in sea water from the effluent sump at Aultbea.

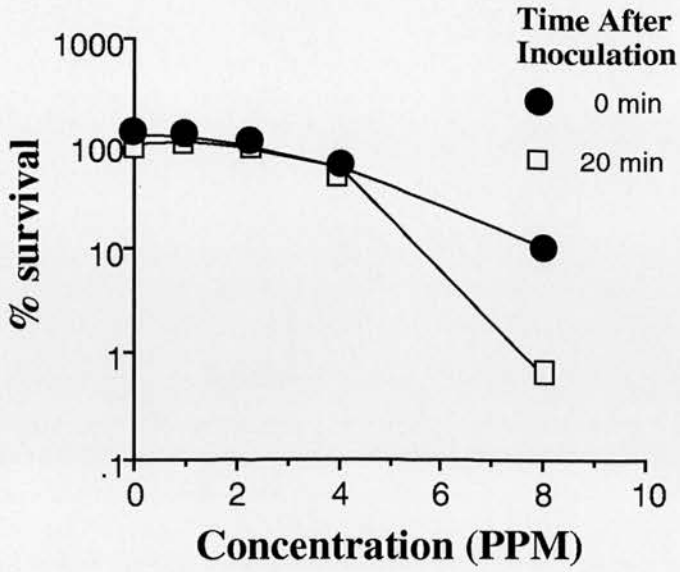


Figure 42. Bactericidal activity of iodine in artificial sea water.

3.8.2. Effect of Organic Load on Bactericidal Activity of Iodine. To determine whether the bacterial survival was due to organic loading or presence of free ions, the bactericidal acivity of iodine was investigated in artificial seawater (i.e. same ion balance as seawater, but no organic material). Iodine was no more bactericidal in artificial seawater than in

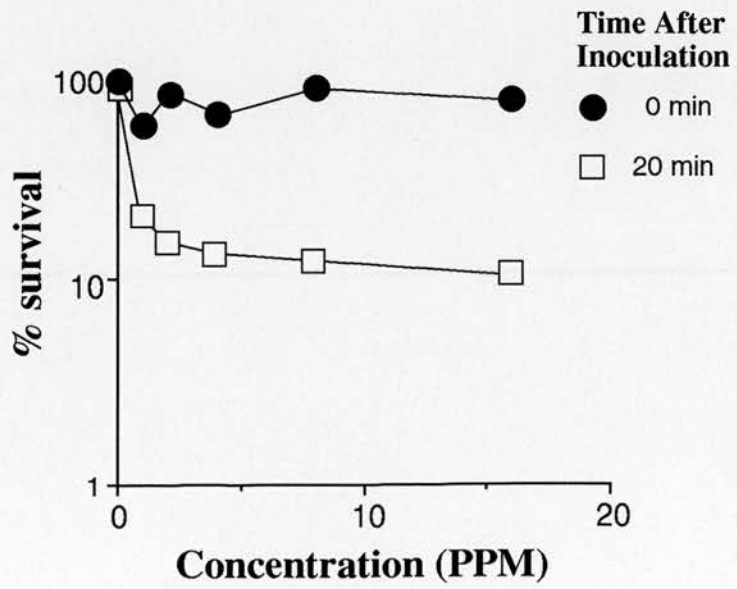


Figure 43. Bactericidal activity of iodine in effluent sea water with reduced organic load.

effluent seawater from Aultbea, with approximately 1% of the bacteria surviving 20 mins exposure to iodine at 8ppm (fig. 42). This suggests that any organic material in the seawater samples played little part in inhibiting the efficacy of iodine. To confirm this, the antibacterial activity of iodine was investigated in sump water with half the number of fish at half the normal feed rate to decrease the organic loading. This seemed to reduce further the bactericidal activity of iodine, with 10% of the organisms surviving even after 20mins at 16ppm (fig. 43).

3.8.3. Effect of pH on Killing Efficacy of Iodine. It has been reported that reduced pH can increase the disinfectant qualities of iodine. Thus the bactericidal activity of iodine in seawater at normal pH (which was found to be 7.20 on that day) was compared to activity in seawater with the pH corrected to 4.00 with HCl. At pH 7.20, Iodine was able to kill 99% of the initial inoculum (10^5 cfu/ml) after 20 mins exposure at 8ppm (fig. 44). At pH4.00, less than 0.01% of the bacteria survived after 20 mins at 8ppm (fig 44).

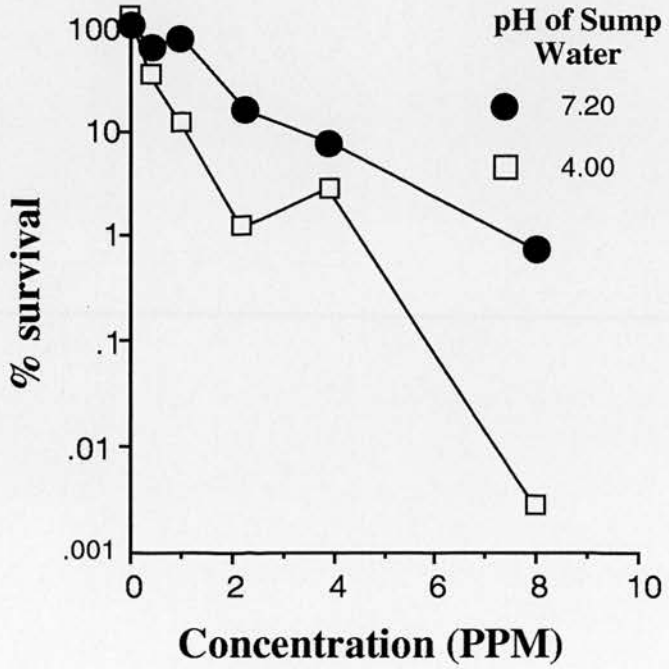


Figure 44. Effect of reduced pH on the bactericidal activity of iodine.

3.9. A POTENTIAL ALTERNATIVE TO INFECTION CHALLENGES.

In light of the results of the study into the bactericidal efficacy of iodine in undiluted seawater (section 3.8), *in vivo* efficacy studies involving infection could no longer be undertaken safely at Aultbea. Thus an alternative means of evaluating the quinolones in Atlantic salmon was developed. Fish were medicated and serum samples taken to determine the level of antibiotic in the fish serum. These serum samples were then used in a bactericidal assay against sensitive and resistant isolates of *A.salmonicida* to determine whether the achieved antibacterial levels were sufficient to inhibit or kill bacteria in the serum.

3.9.1. Serum Levels of Oxolinic Acid and Fluoroquinolones in Atlantic Salmon. The concentration of antibiotic required to inhibit the multiplication of, or to kill, bacteria *in vitro* is a useful means of comparing antibiotics in the laboratory. However, these results have little real meaning unless these levels can be achieved in fish during therapy. The attainable serum levels of oxolinic acid and four fluoroquinolones were therefore investigated in Atlantic salmon smolts.

Serum samples, taken from medicated fish, were analysed by bioassay with seven standards for each three samples, in triplicate. Inhibition zone sizes for samples and standards were read after 24 hours incubation at 22°C, and the logarithm of the standard sample concentration was plotted against zone diameter to give standard curves for each equation. Best fit polynomials (logarithmic) were calculated for each plate using Cricket Graph ™ v1.3 (Cricket Software Inc., USA), and the concentrations of antibiotic in the unknown serum samples were determined from these expressions (figs. 45-49, tables 32 & 33).

Serum levels of oxolinic acid ranged between 0.47 and 1.04 mg/l. There did not appear to be any particular pattern of serum level over the sampling period (ie. 25 hours). Similar serum levels were achieved with flumequine, and again, these were reasonably consistent over the sampling period. The highest serum levels were attained with enrofloxacin, with a maximum level

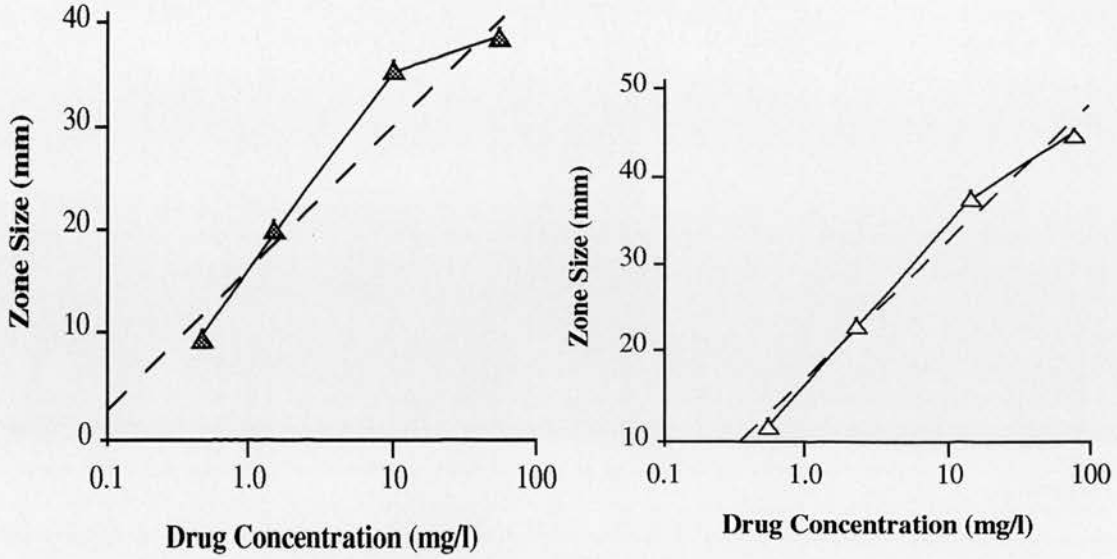


Figure 45. Graphs (a) and (b). Oxolinic acid standards from which equations (a) and (b) were derived.

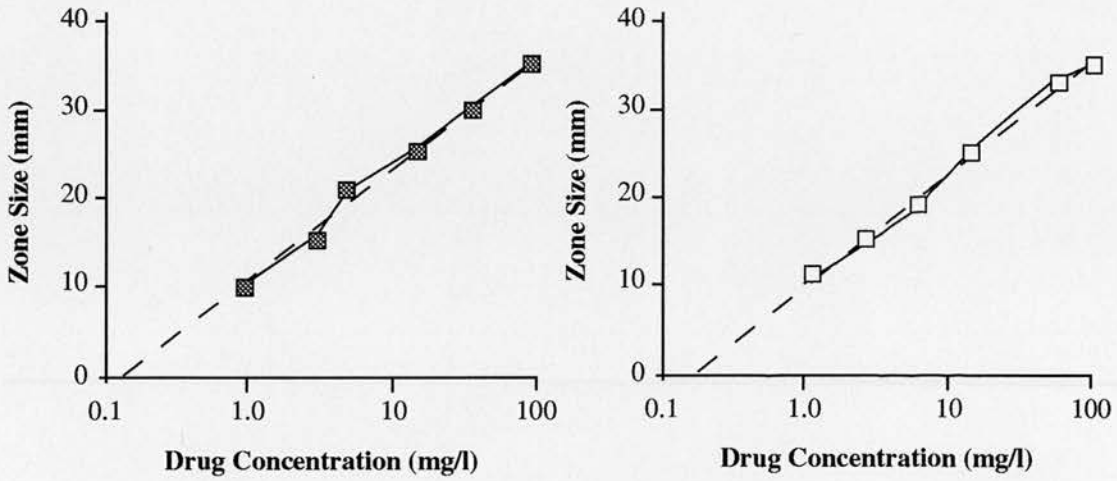


Figure 46. Graphs (c) and (d). Flumequine standards from which equations (c) and (d) were determined.

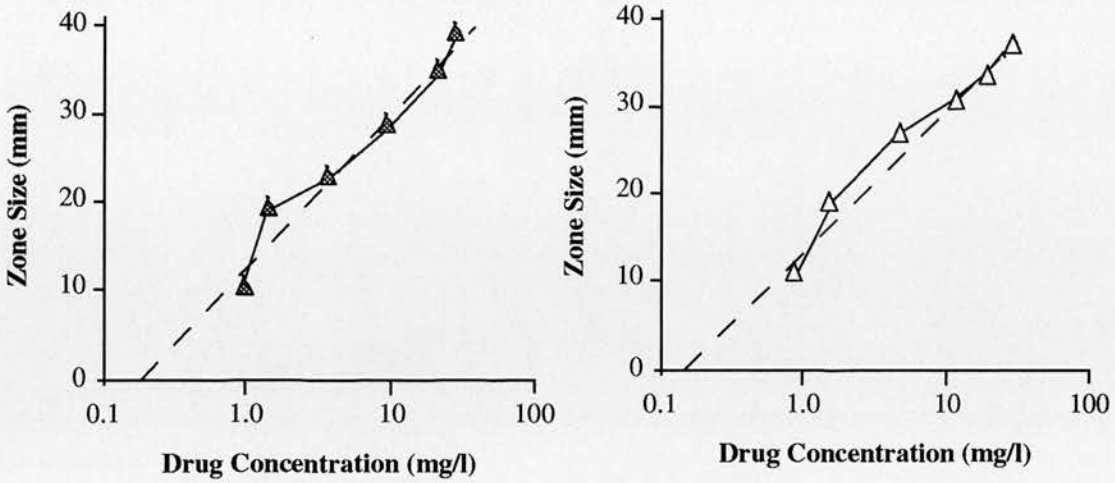


Figure 47. Graphs (e) and (f). Enrofloxacin standards from which equations (e) and (f) were determined.

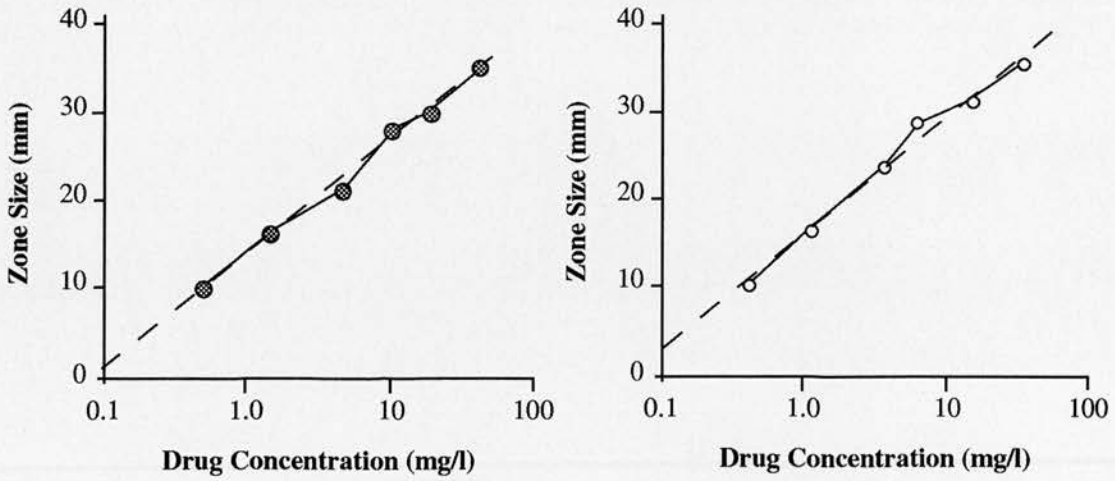


Figure 48. Graphs (g) and (h). Sarafloxacin standards from which equations (g) and (h) were determined.

of 2.22 mg/l observed 4 hours after the final feed. The level of enrofloxacin appeared to fall over the 24 hour sampling period reaching approximately 0.5 mg/l after 24h 45 min. Sarafloxacin was only detected in one of the pooled serum samples. The serum levels of Ro 09-1168 were low, with a

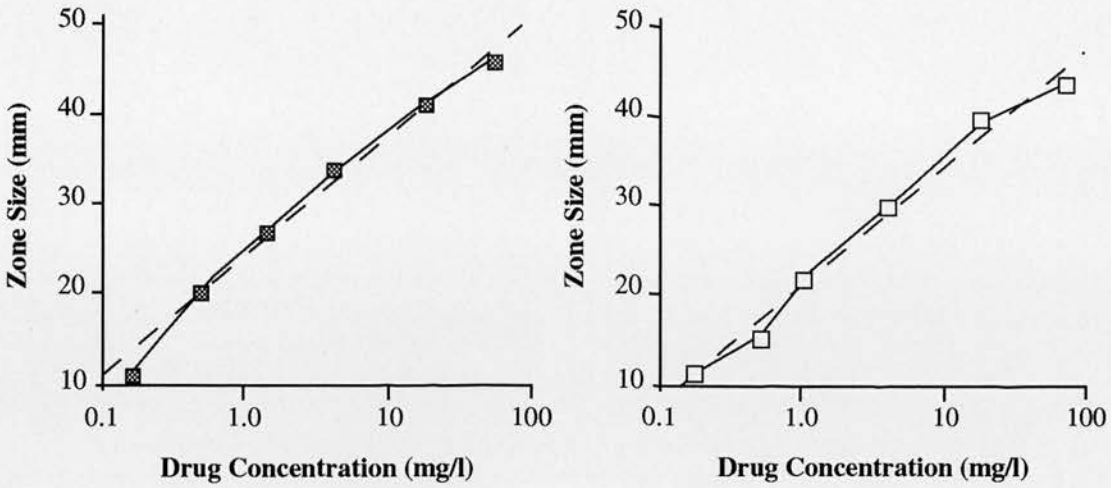


Figure 49. Graphs (j) and (k). Ro 09-1168 standards from which equations (j) and (k) were determined.

maximum level of 0.242 mg/l recorded 24h 15min after the final feed. This level, however, is in excess of the MICs recorded for all but the most resistant isolates of *A.salmonicida* for this drug (Appendix B).

Antibiotic	Tank	Equation	Polynomial (Best fit, logarithmic)
Oxolinic acid	97	(a)	y=16.263 + 13.856Log(x)
	98	(b)	y=16.708 + 15.676Log(x)
Flumequine	93	(c)	y=10.867 + 12.495Log(x)
		(d)	y=9.1694 + 12.973Log(x)
Enrofloxacin	94	(e)	y=12.901 + 12.756Log(x)
		(f)	y=15.073 + 12.653Log(x)
Sarafloxacin	95	(g)	y=13.955 + 12.173Log(x)
		(h)	y=14.186 + 12.314Log(x)
Ro 09-1168	96	(j)	y=23.490 + 12.464Log(x)
		(k)	y=21.626 + 12.911Log(x)

Table 32. Equations calculated from best fit lines of graphs (a) to (k).

Antibiotic	Tank	Sample Time (h.min, post last feed)	Mean Zone Size (mm)		Serum Level (mg/l) (calculated from equation)	
			Pool 1	Pool 2	Pool 1	Pool 2
Oxolinic acid	97	4.40	12.33	15.00	0.520 (a)	0.811 (a)
		8.05	14.00	11.67	0.687 (a)	0.466 (a)
		25.10	11.33	NT	0.466 (a)	NT
	98	3.05	NT	17.00	NT	1.044 (b)
		7.50	13.33	15.66	0.609 (b)	0.857 (b)
		24.05	12.33	14.00	0.526 (b)	0.672 (b)
Flumequine	93	4.25	10.67	10.00	0.964 (c)	0.852 (c)
		8.55	10.00	10.33	0.852 (c)	1.229 (d)
		25.00	10.00	11.33	1.467 (d)	1.159 (d)
Enrofloxacin	94	4.05	17.33	12.33	2.224 (e)	0.902 (e)
		8.40	15.00	12.33	1.469 (e)	0.607 (f)
		24.45	10.33	11.67	0.422 (f)	0.538 (f)
Sarafloxacin	95	3.45	9.00	10.67	ND (g)	0.537 (g)
		8.30	9.00	9.00	ND (g)	ND (h)
		24.35	9.00	9.00	ND (h)	ND (h)
Ro 09-1168	96	3.30	12.00	11.67	0.120 (j)	0.113 (j)
		8.20	11.00	12.67	0.100 (j)	0.202 (k)
		24.15	12.67	13.67	0.202 (k)	0.242 (k)

Table 33. Serum levels of antibiotics from pooled serum samples. Also shown are sampling times and code letters for equations from which levels were derived.

The pooled serum samples taken from fish medicated with Ro 09-1168 were sent to Dr Theodore Graser of F Hoffman la-Roche, Basle, Switzerland for analysis by High Pressure Liquid Chromatography (HPLC). This assay was performed with 0.4ml sample aliquots. The limit of quantification was 0.005

Sample Identification			Serum Conc. (mg/l) HPLC	Bioassay
Tank 96 (Ro 09-1168)	3h30min	Pool 1	0.105	0.120
		Pool 2	0.072	0.113
	8h20min	Pool 1	0.091	0.100
		Pool 2	0.215	0.202
	24h15min	Pool 1	0.123	0.202
		Pool 2	0.115	0.242

Table 34. HPLC results determined for quinolone Ro 09-1168.

mg/l. The results for serum levels of this drug were, in general, close to those recorded by our bioassay technique (table 34). The exceptions were the samples taken after 24.25h, for which the levels determined by HPLC were approximately 50 % of the values determined by bioassay. The relatively close correlation between the levels recorded by HPLC and by bioassay of the other two groups of samples, however, suggests that these results are acceptable.

	Oxolinic acid	Sarafloxacin	Enrofloxacin	Flumequine	Ro 09-1168
MT363 MIC (mg/l)	0.03	0.05	0.02	0.10	0.01
MT477 MIC (mg/l)	1.50	0.75	0.20	1.50	0.10
Concentration in serum determined by bioassay (mg/l)	1.04	0.54	2.22	1.47	0.24

Table 35. Concentration of quinolones in serum, and their respective MICs against two isolates of *A.salmonicida*.

3.9.2. Bactericidal Activity of Quinolones in Fish Serum. The bactericidal activity of the quinolones in the fish serum obtained from the pharmacokinetic studies was investigated against *A.salmonicida* MT363 (oxolinic acid-susceptible) and MT477 (oxolinic acid-resistant).

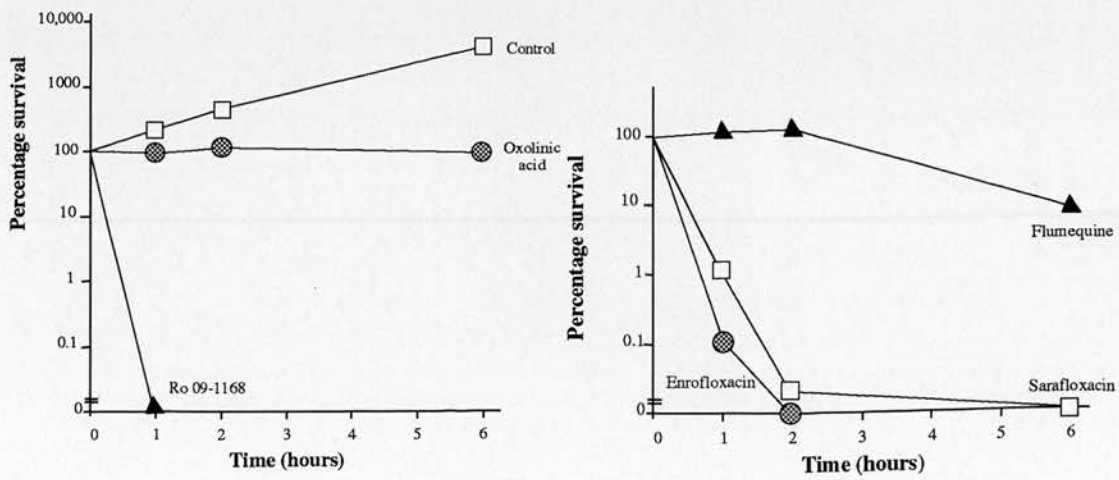


Figure 50. Bactericidal activity of quinolones in fish serum against *A.salmonicida* MT363 (oxolinic acid-susceptible).

When the antibacterial activity of the quinolones was assayed against MT363 (oxolinic acid-susceptible), the newer fluoroquinolones were rapidly bactericidal (fig. 50). Ro 09-1168, enrofloxacin and sarafloxacin all reduced the viable count to the limit of detection for this experiment (<10 cfu/ml for sarafloxacin and enrofloxacin; <100 cfu/ml for Ro 09-1168) within the 6 hour period of the experiment at the concentrations attained *in vivo* in the serum. Flumequine was less bactericidal, killing 90% of the bacteria after 6h exposure, whereas oxolinic acid was merely bacteriostatic (fig. 50).

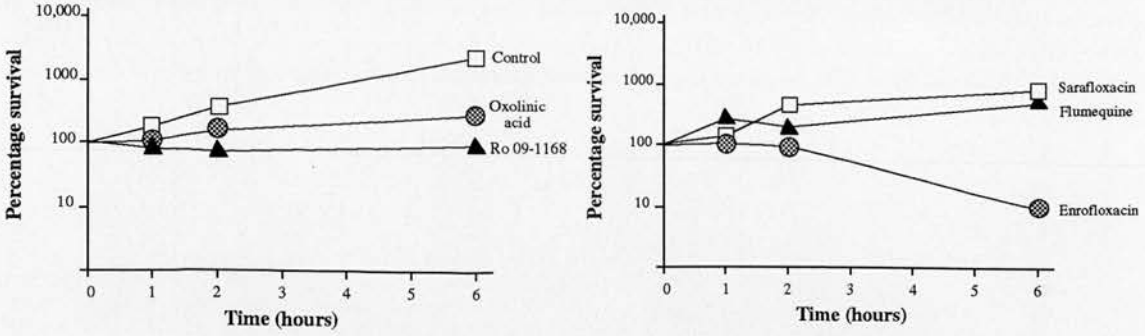


Figure 51. Bactericidal activity of quinolones in fish serum against *A.salmonicida* MT477 (oxolinic acid-resistant).

All the quinolones inhibited the growth of MT477 in serum (fig. 51). Sarafloxacin appeared to inhibit the growth of the bacteria in spite of the fact that the level of the drug in the serum was lower than the MIC against MT477 (table 35). Similarly the level of oxolinic acid in serum was lower than the MIC against MT477 yet the antibiotic still inhibited growth to a degree. (table 35, fig.51). The level of flumequine in serum was similar to the MIC against this isolate and the drug was able to inhibit growth.

In contrast, the MIC of enrofloxacin was ten times the MIC against MT477, yet the killing activity of the drug was very slight, even after 6 hours exposure (table 35, fig.51). In a similar manner, the concentration of Ro 09-1168 in serum was 20 times the MIC, yet the bactericidal activity was insignificant after 6 hours.

DISCUSSION

4.1. *IN VITRO* ACTIVITIES OF THE QUINOLONES.

Several quinolones have been evaluated as potential antibacterials for the control of furunculosis in terms of minimum inhibitory concentration (MIC), bactericidal activity and optimum bactericidal concentration (OBC).

4.1.1. Minimum Inhibitory Concentrations. The MICs of oxolinic acid ranged between 0.01 and 15.0 mg/l. This range differed somewhat from that reported by Tsoumas *et al.* (1989). In their study, oxolinic acid susceptibilities ranged between 0.00012mg/l and 4.0 mg/l. Tsoumas *et al.*, however, determined their MICs by a broth technique in microtitre wells. This technique can lead to erroneous results as the quinolones cause filamentation of bacteria. The filamentation results in increased light scattering by the cultures, normally associated with bacterial growth; however, the viability of the culture, when determined on solid media, decreases markedly (Smith, 1984a). This phenomenon tends to lead to over-estimated MICs rather than the low values determined by Tsoumas *et al.* (1989).

In a study in 1989, Tsoumas *et al.* divided their isolates into theree categories, based on their oxolinic acid MICs. Those with an oxolinic acid MIC $\geq$ 1.0mg/l were deemed oxolinic acid resistant; those with an MIC between 0.125 and 0.5 were deemed moderately susceptible; whilst those with MICs $\leq$ 0.0625 were deemed susceptible to oxolinic acid.

The isolates investigated in this study could be divided into two phenotypes based on their oxolinic acid MICs: according to a simplified adaptation of the criteria proposed by Tsoumas *et al.*, (1989), those isolates with an MIC less than 1.0 mg/l were deemed oxolinic acid susceptible, those isolates with MICs greater than or equal to 1.0 mg/l were deemed oxolinic acid-resistant. In our survey, 38 of the 83 isolates tested were resistant to oxolinic acid, that is over 45% of these randomly selected isolates recovered predominantly in 1989. This is a higher percentage than that reported by Inglis *et al.* (1991), who reported that 36.5% of their survey strains were oxolinic acid resistant. However, their isolates were obtained from a limited number of sites with

a narrow geographical distribution in Scotland. Furthermore, their survey relied upon disc susceptibility testing as opposed to determination of MICs, and little indication of the criteria they used to define resistance to oxolinic acid is given in their paper. This is particularly important as the relationship between zone size and MIC is not well defined. In a study in 1987, Hastings & McKay related zones of inhibition to MICs of oxolinic acid resistant isolates of *A.salmonicida.* They demonstrated that isolates with MICs ranging between 1.25 and 5.0mg/l each gave a zone of inhibition of 15mm around a 2μg oxolinic acid disk. Furthermore, 3 distinct isolates, each with an MIC of 1.25mg/l gave inhibition zone diameters of 15, 20 and 22mm. Thus it can be seen that disc susceptibility testing results do not necessarily correlate precisely with MIC. In a study in 1987, O'Grady *et al.* found MICs of oxolinic acid ranging between 0.012mg/l and 2.0mg/l in Irish isolates of *A.salmonicida.* In contrast to the findings of Hastings and McKay (1987), O'Grady *et al.* (1987) found reasonable correlation between MIC and Disc susceptibility results when the semi-quantitative Kirby-Bauer disc technique was employed.

In this study, the MICs of sarafloxacin related closely to those reported by Stamm (1989). Sarafloxacin was slightly more active than oxolinic acid in terms of MIC against both oxolinic acid susceptible and resistant isolates of *A.salmonicida.*

Enrofloxacin was also more active than oxolinic acid against susceptible isolates, and was very much more active than oxolinic acid against oxolinic acid resistant isolates. Bowser & House (1990) reported an MIC for enrofloxacin of 100mg/l against a strain of *A.salmonicida.* However the present study failed to find such high levels of enrofloxacin resistance in Scottish isolates. This difference may reflect the different techniques used to determine MICs (agar- as opposed to broth-dilution). Alternatively, their strain may have been atypical of quinolone resistant *A.salmonicida* as previous studies which determined MICs by broth dilution did not report such elevated levels of quinolone resistance (Bragg & Todd, 1988; Tsoumas *et al.,* 1989).

Three of the fluoroquinolones tested were considerably more active than oxolinic acid against both resistant and susceptible isolates. Ro 09-1168, PD127,391 & PD117,596 were at least 10-15 times more active than oxolinic acid against resistant and susceptible isolates. In contrast, CI934 was less active than oxolinic acid against oxolinic acid-susceptible isolates of *A.salmonicida,* and not significantly more active than oxolinic acid, in terms of MIC, against resistant isolates. Flumequine, was more active than oxolinic acid against resistant isolates.

With the exception of sarafloxacin, the quinolones were less active in terms of MIC at 10°C than at 22°C. This is in agreement with recent work by Martinsen *et al.* (1992), who reported that the MICs of quinolones at 4°C were 1.3-1.5 times higher than those recorded at 15°C. As some of the quinolones evaluated in the present study were bactericidal against non-dividing cells, and against cells in which protein and RNA synthesis was inhibited, a possible explanation of the increased MICs at lower temperatures is reduced accumulation of the drug within the bacterial cells. These observations may be significant, as Bowser & Babish (1991) reported that the bioavailability of enrofloxacin was only 23% at 10°C compared to 43% at 15°C. These two workers state that a drug must have a bioavailability of >30%, within the temperature range in which it will be used, before it will be useful in aquaculture. Unfortunately, they give no indication of how they calculate this figure. In spite of this, Bowser *et al.* (1990) reported successful field trials with enrofloxacin in water temperatures ranging between 7°C and 10°C.

A method of increasing the bioavailability of quinolones has been reported in cultured yellowtail and sea bream (Endo *et al.* 1987a, 1987b). Ultra fine size reduction of oxolinic acid resulted in uptake of the drug, administered by admixture with diet, increasing by 1.3 to 1.7 fold.

4.1.2. Effect of Seawater Ions on Antibacterials. It has been reported that the quinolones are susceptible to the effects of divalent cations (Smith & Lewin, 1988). Indeed, in human medicine, the quinolone ciprofloxacin is prescribed with strict instructions that milk and antacid compounds should be avoided during therapy. Similarly, uptake of oxytetracyline has also been reported to be affected by multivalent ions (Jun & Lee, 1980; Lunestad & Goksøyr, 1990)

The sea has a complex and varied ion make-up. However, magnesium ions may be present in concentrations in excess of 50mM in sea water of 35‰ salinity (Potts & Parry, 1964). The effect of this concentration of ions on aquaculture antibacterials was therefore investigated. Not surprisingly the MICs of the quinolones and oxytetracycline were increased by 20 to 30 fold on Bacto marine agar, which contains 50mM magnesium chloride. This effect appeared to result almost entirely from the presence of Mg^{2+}, as addition of 50mM $MgCl_2$ to TSA had a similar effect, whereas addition of 340mM NaCl (concentration in Bacto marine agar) to TSA had no effect on the MICs of the quinolones or oxytetracycline. Amoxycillin, and the components of the potentiated sulphonamide Romet, ormetoprim and sulphadimethoxine, were not affected by these concentrations of Mg^{2+} or Na^{+}.

These observations may be clinically relevant. When sea water fish are medicated with oxytetracycline or a quinolone, contact with sea water cations is unavoidable. This occurs when the surface coated pellet is in the water or in the gastrointestinal tract, as marine teleost fish are hypo-osmotic and must continually drink sea water to compensate for water loss. For example, Atlantic salmon maintained in sea water have been reported to drink 129ml/kg/day sea water, while those kept in fresh water drink none at all (Usher *et al.,* 1988). Thus the inside of the gut of marine fish can be envisaged as modified sea water.

In light of these results, susceptibility testing of marine fish pathogens may be more accurately undertaken on Bacto marine agar, or medium prepared with natural or synthetic seawater. Indeed, Lunestad and Goksøyr (1990) recommended that media be prepared with 70% sea water, as considerable underestimation of the levels of quinolone and oxytetracycline resistance may occur when media are prepared without using sea water. Use of sea water in media for susceptibility testing amoxycillin or potentiated sulphonamides, however, would appear, from these results, to be less important.

The problem of drug interaction with cations in sea water prior to entry into the fish may partially be avoided by encapsulation of the antibiotic in feed pellets such as aqualets® (Apothokernes Laboratorium AS, Oslo, Norway).

However, for the drug to be uptaken from the fishes' gut, it must be in the liquid phase, thus interaction with sea water ions at this stage is unavoidable.

4.1.3. Bactericidal Activity. In human medicine, it is often necessary merely to inhibit the multiplication of a pathogen to enable the immune system to deal with the infection. Current treatment regimens for furunculosis with the bacteriostatic drug oxolinic acid may only save those fish which are in early stages of *A.salmonicida* infection. However, as a result of resistance to both normal and immune serum (Munn & Tust, 1984), coupled with cytotoxicity of *A.salmonicida* to macrophages (Olivier *et al.,* 1992), the immune system of the fish may be rapidly compromised rendering it unable to control the diease. Thus a bactericidal antimicrobial may be of benefit under these circumstances.

The quinolones are rapidly bactericidal against *E.coli* (Smith, 1984a). However, it has been demonstrated that oxolinic acid is not bactericidal against *A.salmonicida* (Lewin & Hastings, 1990). On the other hand, two new fluoroquinolones, norfloxacin and ciprofloxacin were shown to be rapidly bactericidal against *A.salmonicida* (Lewin & Hastings, 1990). In the present study, it was confirmed that oxolinic acid is merely bacteriostatic against *A.salmonicida* after 6h exposure. However, the normal therapeutic period for treatment with oxolinic acid is 10 days, during which the level of oxolinic acid in the tissues should remain in excess of the MIC for sensitive isolates, provided the treatment regimen is adhered to. It would be interesting, therefore, to study the bactericidal activity of oxolinic acid over a longer period of time.

Flumequine, a fluoroquinolone which was initially developed over 20 years ago, was able to kill *A.salmonicida in vitro* after 3hours exposure.With the exception of CI934, the remaining fluoroquinolones tested, sarafloxacin, enrofloxacin, PD127,391, PD117,596 and Ro 09-1168, were rapidly bactericidal against both oxolinic acid-susceptible and resistant isolates of *A.salmonicida,* the activity being detected at concentrations just above the MIC after 1 hour exposure. This bactericidal activity may be of use in eliminating the asyptomatic carrier status of *A.salmonicida.* In this context, it is interesting to note that Scallan & Smith (1985) obtained promising

results with flumequine in reducing carriers in salmon and trout, and Markwardt & Klontz (1989b) had similar encouraging results with sarafloxacin. It should be noted, however, that in the study of Markwardt & Klontz (1989b), an artificial carrier status was induced by gastric intubation (Markwardt & Klontz, 1989a). It is questionable how well this reflects the carrier state in the natural situation.

Antibacterial	MIC (mg/l) (a)	OBC (mg/l) (b)	Ratio (b/a)
CI934	0.40	1.50	3.75
PD127,391	0.0075	0.03	4.00
PD117,596	0.015	0.05	3.33
Enrofloxacin	0.02	0.90	45.00
Sarafloxacin	0.05	1.50	30.00
Ro 09-1168	0.01	0.30	30.00

Table 36. Ratio of MICs and OBCs for fluoroquinolones against *A.salmonicida* MT363 (oxolinic acid-susceptible).

4.1.4. Optimum Bactericidal Concentrations (OBCs). Optimum bactericidal concentrations ranged from approximately three times the MIC to as much as 45 times the MIC (table 36). OBCs have not been determined against *A.salmonicida* previously, thus few comparisons can be drawn. However, the ratio of OBC/MIC for the three Parke-Davis compounds, PD127,391, PD117,596 and CI934, was very low compared to the other fluoroquinolones tested here, and low compared with 4-quinolones tested against other bacterial species (Smith, 1984a).

Optimum bactericidal concentrations could not be determined in every case because of the nature of the technique used to assess bactericidal activity. With extremely active compounds, even after dilution of the sample aliquots, a sufficient concentration of antibiotic is carried onto the viable count plates such as to continue killing the bacteria. Thus the curves can only be drawn to a certain concentration above the MIC and the biphasic pattern is not established.

4.1.5. Mechanism of Action. The three fluoroquinolones investigated, sarafloxacin, enrofloxacin and Ro 09-1168, were all active against a) non-dividing bacteria, b) bacteria in which protein synthesis was inhibited, and c) bacteria in which RNA synthesis was inhibited. This suggests that these three antibiotics act through mechanism B in addition to mechanism A, the latter being common to all quinolones. Mechanisms A and B appear to be very closely related. Mechanism A possibly represents reversible binding of the drug to the DNA-DNA gyrase complex, while mechanism B may represent irreversible binding of the drug to this complex (Lewin *et al.*, 1991).

4.2. RESISTANCE TO THE QUINOLONES.

Almost half the isolates tested were resistant to oxolinic acid according to the criteria used. Resistance to the quinolones was stable, as repeated passage on drug-free TSA had little effect on the MICs, any reduction being less than that required to render the organism susceptible. This is in agreement with the findings of Tsoumas *et al.* (1989), who reported that MICs could be reduced by up to 2.5 fold by serial passages on drug free medium. However, Tsoumas *et al.* (1989) concluded from their observations that quinolone resistance was unstable, in spite of the fact that the decrease in MIC did not render the isolates susceptible according their own criteria.

Resistance to one quinolone tends to lead to a biochemical resistance, ie elevated MIC, to other quinolones as they all have a similar mode of action. In this study, *A.salmonicida* isolates with elevated MICs of oxolinic acid tended to have elevated MICs to the other fluoroquinolones. In practical terms the significance of this phenomenon may be limited, as a slightly elevated MIC of a highly active quinolone may be insufficient to compromise its therapeutic efficacy.

4.2.1. Frequency of Chromosomal Mutation to Resistance. *A.salmonicida* exposed to the fluoroquinolones tested at 5 times their respective MICs all developed resistance at lower frequencies than to oxolinic acid. A previous study by Stamm (1989) did not find this difference in mutation frequency

between sarafloxacin and oxolinic acid. This discrepancy in results may be explained by the 7-day incubation period employed in the present study compared with only 48 hours incubation in the study by Stamm (1989), as mutants resistant to oxolinic acid were rarely seen after 48 hours incubation. The lower mutation frequencies of the fluoroquinolones combined with their increased activity against *A.salmonicida* suggests that resistance to these drugs may be less likely to developduring clinical use than to oxolinic acid.

4.2.2. Resistance Resulting from Alterations in Outer Membrane Proteins. Although there was some apparent variation in the relative concentrations of minor OMPs as revealed by SDS-PAGE, both quinolone-sensitive and highly quinolone-resistant isolates of *A.salmonicida* appeared to have very similar major OMP profiles. However, the OMP profiles of mutant strains exhibiting low-level resistance to quinolones, coupled with cross resistance to oxytetracycline, had markedly different major OMPs. The two cross resistant mutants isolated in this study had differing MIC profiles: the oxolinic acid MIC of AB174 was increased 30-fold compared to its parent strain (MT363), while the oxolinic acid MIC of AB176 was only increased 3-fold compared to its parent (MT744). Both of these mutants were found to express an additional protein, molecular mass 37 kDa, which was not present in the parent strains. However, AB174 did not express a 43 kDa protein which was expressed in its parent (MT363), in AB176, and in all the highly resistant or susceptible isolates examined. The complete absence of this protein in AB174 may explain why resistance to oxolinic acid and oxytetracycline in this strain was higher than in mutant AB176 (See below).

Both the 37 kDa and the 43kDa proteins were non-covalently associated with peptidoglycan suggesting that they may function as porins (Darveau *et al.* 1983). Indeed, Darveau *et al.* (1983) reported that a peptidoglycan associated protein of approximately 42kDa functions as a porin in *A.salmonicida*. The porin model is supported by the the observation that hydrophilic antibiotics such as oxolinic acid and nalidixic acid, which rely almost entirely on porins for their uptake, were affected to a greater extent by the OMP changes than the more hydrophobic antibiotics sarafloxacin,

enrofloxacin, amoxycillin and oxytetracycline, which may be taken across the membrane barrier by other routes. It would therefore appear that these two outer membrane proteins possibly play a role in antibiotic uptake in this species, as this and other studies (Wood *et al.,* 1986; Griffiths & Lynch, 1989) have found that alterations in these two proteins are associated with changes in susceptibility to a wide range of drugs. These two porins may operate in a similar manner to OmpF and OmpC mutants of *E.coli* K12. Mutants lacking the OmpF porin (which possesses slightly larger pores), retain the OmpC with its narrower channel, thereby decreasing the outer membrane permeability (Nikaido, 1989). It is also intriguing to speculate that, as these changes are pleiotropic, a mutation in a regulatory gene similar to *marA* in *E.coli* (George & Levy, 1983; Cohen *et al.,* 1988; Nikaido, 1989) may be involved, rather than a gene encoding a specific porin.

This phenomenon of OMP associated cross-resistance is not solely a laboratory phenomenon. A wild type isolate of *A.salmonicida,* MT464, also exhibited increased expression of the 37kDa protein. However, this is probably not the sole cause of oxytetracycline or quinolone resistance in this isolate. The oxytetracycline MIC of 40mg/l is 40 fold higher than the laboratory mutant (AB176) carrying the same mutation. Such high levels of oxytetracycline resistance are likely to be plasmid mediated (Aoki, 1988; Hedges *et al.,* 1985b). Furthermore, the MIC of oxolinic acid for MT464 (1.50mg/l), although similar to that of AB174, is 15 times greater than that of AB176, the mutant carrying the same OMP alteration. Therefore, MT464 may also contain an alteration in DNA gyrase. This is supported by the observation that, in other Gram-negative bacteria, resistance to the 4-quinolones is often associated with alterations in both OMPs and DNA gyrase (Aoyama *et al.,* 1987; Lewin, Allen & Amyes, 1990). Furthermore, it has been shown in *E.coli* that *mar* mutants exhibiting reduced drug accumulation can then mutate relatively easily to higher, clinically relevant levels of quinolone resistance (Cohen *et al.,* 1989).

Griffiths & Lynch (1989) reported that these outer membrane alterations are often associated with a loss of protease activity. One may speculate, therefore, that such isolates may be avirulent, as loss of virulence has been

associated with protease deficiency (Sakai, 1985). However, the isolation of a wild type *A.salmonicida* exhibiting such OMP changes from a clinical outbreak of furunculosis in Scotland suggests that some of these isolates may retain, or regain, their virulence.

The significance of this cross resistance phenomenon in the aquaculture industry is considerable. In an industry where relatively few antibiotics are licensed, a single mutation giving rise to resistance to all of them could have serious implications.

4.2.3. Probing for Alterations in the Gyrase A Subunit. Attempts to probe for mutations in DNA gyrase in *A.salmonicida* were unsuccessful. It must be noted however, that purification of plasmid DNA from *A.salmonicida* appears to have been unsuccessful in several instances, thus we cannot tell from figure 32 whether the plasmid pBP513 is present or not. It is impossible to tell from the data generated in this study whether the problem with the two *gyrA* probes was because the plasmids were not introduced into the cell, or whether the plasmids were introduced but not expressed. Further work is required to determine which of these two possibilities is the case. Either the plasmids could be introduced by electroporation, which would determine whether they were expressed, or, alternatively, the wild type *E.coli gyrA* which is incorporated into both of the probes used could be cloned into an *A.salmonicida* plasmid and introduced by transformation. Hopefully this would reveal whether the transformation techniques employed in this study were successful or not.

4.3. *IN VITRO* EFFICACY OF ORMETOPRIM AND SULPHADIMETHOXINE.

4.3.1. MICs of the Two Components Determined Separately. MICs of ormetoprim against *A.salmonicida* ranged between 0.5 and 1.0 mg/l. This differed from results determined against Japanese isolates, where Aoki *et al.* (1983) reported MICs ranging between 0.1 and 400mg/l in culture pond isolates, and 1.6-12.5 mg/l in river isolates. In the present study, concentrations of trimethoprim required to inhibit *A.salmonicida* were approximately half the ormetoprim MICs. MICs of sulphadimethoxine 6.0mg/l and >2048mg/

l. These values seem remarkably high when compared to the MICs of 7 other sulphonamides determined by McCarthy *et al.* (1974a). This may, of course, reflect the regular use of potentiated sulphonamides in Scottish aquaculture since tribrissen was licensed in 1987.

4.3.2. Ormetoprim and sulphadimethoxine in combination. At the points of maximum interaction of the two antibacterials, the FIC indices were lower than 0.7, indicating potentiation of the two antibiotics. However, in the cases of several of the sulphadimethoxine resistant isolates, the FIC indices were very close to 0.7 at the point of maximum interaction. In the case of MT320, for example, the FIC index was greater than 0.7 at either side of the point of maximum interaction. This stresses the importance of achieving the correct ratio of the drugs in *vivo* if potentiation of the drugs is to occur where it is required. An important addition to this series of experiments, therefore, would be to determine the levels of the two drugs achieved *in vivo* when administered at the recommended dose.

4.4. *IN VIVO* STUDIES

4.4.1. A Laboratory Sea Water Infection Challenge Model. A laboratory infection challenge model for furunculosis in Atlantic salmon was developed in this study. The model, based on a water borne exposure to *A.salmonicida* infection, was capable of inducing significant mortalities due to furunculosis in control fish. Exposure of fish to *A.salmonicida* at 1×10^4, 1×10^5, 1×10^6 cfu/ml resulted in 30%, 65% and 85% mortalities respectively. Our challenge did not appear to be particularly reproducible, however, as a similar challenge conducted during the investigation into the clinical efficacy of Romet 30 did not result in such high mortalities. However, this is likely to result from lower water temperatures and the use of larger fish in the second study. A search of the literature has not revealed any other attempts at furunculosis challenges in Atlantic salmon in seawater. There are, however, numerous reports of bath challenges in Atlantic salmon, and other species, in freshwater (Adams *et al.*, 1987; McCarthy, 1983; Michel, 1980).

4.4.2. *In Vivo* Efficacy of the Potentiated Sulphonamide Romet. In laboratory trials, a 5-day oral treatment with Romet 30 (50mg/kg/day i.e 15mg active drug/kg day) was found to be highly effective in controlling

furunculosis in Atlantic salmon held in sea water. This dose rate was lower than that employed for trout in the USA (50mg active agent/kg/day)(Maestrone, 1984). There have been anecdotal reports of palatability problems associated with Romet 30 in trout (D.Love, Pers.Comm). No palability problems or adverse reactions to Romet 30 medicated feed using the lower dose rate were observed during the trials in Atlantic salmon. At the dose rate used, Romet 30 did not appear to be effective at reducing carrier populations, as the prevalence of asymptomatic *A.salmonicida* infection detected by bacteriological sampling among surviving fish was similar in Romet treated and control (untreated) populations. A possible improvement to this experiment would be to test the survivors according to the method of McCarthy (1977). This technique involves intramuscular injection of prednisolone acetate followed by elevation of the water temperature to 18°C, and has been reported to be the most effective means of identifying carrier fish (McCarthy, 1977).

4.4.3. Efficacy of Iodine as a Sea Water Disinfectant. The *in vivo* challenge model, and the clinical efficacy study into Romet 30, were performed in the summer of 1990 and disinfection was effected by iodination of effluent sea water mixed with fresh water at a ratio of approximately 1:10. However, site modifications dictated that subsequent infection work be carried out in a new sea water only quarantine facility. As the effectiveness of iodination as a method for disinfection of undiluted effluent sea water is not known, its antibacterial activity in the sea water at Loch Ewe under these conditions was investigated.

Iodine was bactericidal in sea water, killing >90% of the bacteria at 4ppm within 20min. However, in infection challenges, cell concentrations of 10^5 or 10^6 cfu/ml were used. If ten percent of these survive, then 10^4 or 10^5 cfu/ml of a virulent fish pathogen would be discharged into the marine environment. Thus a means of improving the disinfection needed to be found before any further infection work could be undertaken.

Increasing the iodine concentration to 8ppm decreased the number of survivors to 1%. However, in a challenge experiment, this would still mean that 10^3 or 10^4 cfu/ml would remain viable. The problem did not appear to result from the organic loading of the sea water as similar results were obtained in artificial sea water, and effluent sea water with reduced fish

numbers and lower feed rate. The iodine was therefore likely to be binding to dissociated ions in the sea water, reducing its bactericidal efficacy. Reduction of the pH of the sea water is a reasonably inexpensive process, even on a large scale, by addition of hydrochloric acid. Although this greatly increased the bactericidal activity of iodine, 0.003% of the challenge still remained viable. In the 10^5cfu/ml sea water challenge model, this would mean that there was still a possibility of 30 organisms per ml remaining viable. This may seem insignificant, however, lower numbers of a virulent isolate of *A.salmonicida* can cause outbreaks of furunculosis (Dr D I McIntosh, personal communication). As a result of these findings, no further sea water infection work was undertaken.

These experiments may not, however, fully reflect the situation in a challenge in seawater tanks. Due to the hydrophobic nature of *A.salmonicida* (Munn & Trust, 1984) it is more likely to be associated with micro algae, dirt on tank or sump walls, in sediments, and in the lipid rich neuston at the surface of the water. A more thorough approach, therefore, may involve pumping effluent water from the disinfectant sump through tanks containing healthy fish during a challenge. Mortalities due to furunculosis would indicate incomplete disinfection. Furthermore, surviving fish could be stressed by the method of McCarthy (1977) to determine the percentage of carriers. Negative results from such an experiment, coupled with the data presented in the current study may provide further evidence on the safety of infection experiments. Future seawater challenges could involve a similar set up as a control.

Possible alternatives to iodine include chlorine, which is cheaper but there are handling difficulties associated with its use. Small infection facilities occasionally employ ozone for disinfection purposes. This method, however, is expensive and may not be suited to high rate flow-through conditions. Bullock and Stuckey (1977) reported that doses of UV light were capable of killing 99.99% of bacteria including *A.salmonicida* in filtered freshwater, and preventing the transmission of infection to Atlantic salmon. Their system was capable of handling 56.8 litres/min of effluent water, a sufficiently large capacity to conduct a moderately scaled infection study. This technique is worth further investigation.

4.4.4. An *in vivo* Efficacy Study Not Requiring an Infection Model. A potential alternative to infection models has been investigated in this study. A series of pharmacokinetic studies followed by a study into the ability of the drugs to kill the target pathogen in serum may give an indication of a drugs potential efficacy *in vivo* without the necessity of an infection challenge. Should infection models be required to back up these results, they may be performed provided adequate dilution of sea water with fresh water is effected prior to iodination.

The pharmacokinetic study was performed on a small scale, with limited numbers of fish and only three time periods were sampled. This precluded the calculation of an absorption rate constant. However, as a pilot study, some information on the levels of quinolones attained in fish serum following oral administration of the drugs at certain dose rates was determined. Results were determined from serum pooled from several fish, and therefore do not reflect variations between individual fish. Pooled samples were employed to provide sufficient volumes for the bactericidal assays.

The highest level of oxolinic acid reached was between 0.8 and 1.0 mg/l between 3 and5 hours after the final administration of feed. This differs from previous results (Hustvedt *et al.*, 1991) which reported peak levels of 1-3mg/l occuring 9-20 hours after administration. However, since samples were not taken between 9 and 20 hours post administration in the present study, a peak may have been missed at this time.

The highest serum concentrations of flumequine in this study ranged between 1.16 and 1.47 mg/l, attained 25 hours after the final oral dose.

Sarafloxacin was only detectable in one of the serum samples, with a level of 0.54 mg/l. This level corresponds with those determined by Abbott Laboratories (L. Brown, Pers.Comm.) who reported levels between 0.3 and 0.5 mg/l in serum.

The highest serum levels recorded for the other fluoroquinolones were varied. One of the pooled samples of serum from the fish treated with enrofloxacin had 2.2 mg/l of the drug present, however the level recorded in the other pool sampled at the same time was less than half this amount. In contrast, the serum concentration attained with Ro 09-1168 was very much lower, peaking at 0.24 mg/l after 24h 15min. The serum samples obtained from the Ro 09-1168 medicated fish were sent to F. Hoffmann La-Roche in Basle, Switzerland for validation by HPLC. The levels recorded by HPLC were close to the results determined by bioassay in the present study, supporting the validity of these results.

In this study, a level of enrofloxacin in excess of 2mg/l was detected in serum. In a study performed in rainbow trout fingerling (Bowser *et al.* 1992), a peak serum level of 0.35 mg/l was attained at the same dose rate (5mg/Kg/day). This may reflect poorer uptake of the drug in rainbow trout. Alternatively, as fewer fish were employed in this study, the results may not reflect the general situation in a large population of fish. The serum concentrations attained with enrofloxacin and Ro 09-1168 were well in excess of the MICs of most of the isolates examined during this study. This is encouraging as, at least in humans, quinolones tend to accumulate in the kidneys, urinary tract and other tissues, rather than the serum (Bergan, 1988). Thus if the levels in the serum are sufficient to inhibit *A.salmonicida,* the drug should be effective in therapy.

As a study into the efficacy of the quinolones *in vivo* was not possible at this stage of the study, the antibacterial activity of the serum samples obtained from the pharmacokinetic study was investigated. The quinolones merely inhibited the oxolinic acid resistant isolate, MT477. However, with the exception of oxolinic acid, they were able to reduce significantly viable numbers of a sensitive isolate of *A.salmonicida,* MT363. Enrofloxacin, Ro 09-1168 and sarafloxacin were able to kill >99% of the bacteria within the 6 hour duration of the experiment. This is particularly significant if they are to be used in the elimination of the asymptomatic carrier state of furunculosis.

Based on a recent report by Cipriano (1992) which states that the pathogen is more frequently isolated from the external mucus of Atlantic salmon than from the kidney or other internal organs of carrier fish, treatments based on bathing may be more successful than oral therapy. In this context, O'Grady *et al.* (1988) demonstrated that bath administration of flumequine to brown trout and Atlantic salmon was effective both therapeutically and prophylactically. This may not be so effective in seawater in light of the antagonism of fluoroquinolones reported in the present study.

Further work on this technique needs to be undertaken to correlate the results obtained here with true infection models, in order to verify how these serum bactericidal assays relate to clinical efficacy. This type of model may have future potential to reduce the need for infectious disease experiments for humanitarian reasons, or where lack of suitable containment facilities precludes the use of infection models.

4.5. AMOXYCILLIN RESISTANCE IN *A.SALMONICIDA*.

The MICs of amoxycillin against all the *A.salmonicida* subsp. *salmonicida* isolates tested were less than or equal to 1.5mg/l, whereas the *A.salmonicida* subsp. *achromogenes* isolates tested were resistant, having MICs in excess of 500mg/l.

Resistance to amoxycillin in the subsp. *achromogenes* appeared to result from the production of a β-lactamase enzyme with a major band of pI 8.0. This pI seems to be common amongst ß-lactamases of the *Aeromonas* species, as Bakken *et al.* (1988) reported three enzymes with this pI isolated from *A.sobria*, three isolated from *A.hydrophila,* and one from *A.veronii.*

In common with the enzymes isolated in the study Bakken *et al.* (1988), the β-lactamase in the present study was highly inducible, hydrolysing nitrocefin 163 times faster when induced with cefoxitin, suggesting the enzyme is chromosomally mediated. In contrast, however, the β-lactamase activity isolated in the present study was susceptible to clavulanic acid, as MICs of amoxycillin were greatly reduced in the presence of this compound. Although this is characteristic of enzymes of plasmid origin, ASE-1 is likely to be

chromosomally mediated as production of the enzyme was not affected by ethidium bromide, a plasmid curing agent (Bouanchaud *et al.,* 1969). Chromosomal location is supported by the inducibility, which is characteristic of chromosomally mediated ß-lactamase enzymes in Gram-negative bacteria.

The substrate profile of ASE-1 was similar, within the limits of experimental error, to SHV-1. A number of SHV enzymes have been identified, but all of them have extended-spectrum activity against cefotaxime and ceftazidime (Payne & Amyes, 1991). None of them match the pI or substrate profile of ASE-1, thus this enzme is believed to be novel.

The existence of amoxycillin resistant strains of *A.salmonicida* is cause for concern in view of the recent introduction of this antibiotic for control of furunculosis. However, resistance was only detected in the *achromogenes* subspecies which has seldom, if ever, been associated with disease in farmed salmonids in Scotland; nevertheless, this subspecies has caused outbreaks of furunculosis at Canadian (Harmon *et al.,* 1991) and Finnish salmon farm sites (Rintamäki & Valtonen, 1991). Furthermore, the β-lactamase enzyme responsible for resistance appears to be chromosomally mediated and is thus unlikely to be transferred to the more prevalent *salmonicida* subsp., unless it is borne on a transposon. This is unlikely as ASE-1 could not be mobilised with the *IncP* plasmid R702. It must be stressd that further experimentation involving alterations in the transfer protocol is required, as this route of transfer is not completely ruled out in the limited scope of this experiment. However, the use of amoxycillin may promote the selection of an increased number of outbreaks of disease resulting from infection by the *achromogenes* subsp. In addition, bearing in mind the plethora of transferable β-lactamase genes in environmental bacteria, caution should be excercised when using amoxycillin in furunculosis therapy as the potential for selecting β-lactamase producing strains is still present.

4.6 CONCLUDING OBSERVATIONS.

Aquaculture is an essential industry in Scotland, both as a source of employment in rural areas, and as a valuable source of revenue. Furthermore, the demand for high quality fish meat cannot continue to be fulfilled from wild fish stocks. Indeed, severe measures are being imposed on most EEC fisheries to limit the over-fishing of coastal and deep-sea fish stocks. Thus the demand for fish must be supplied, at least in part, by farming.

The rapid growth of the farming industry has brought with it the problems of disease. In Scottish salmon farming, a major problem is the bacterial disease furunculosis. Effective commercial furunculosis vaccines are not yet available thus the control of this disease is still largely dependent upon the use of antibacterial compounds. Currently, Scottish salmon farmers are faced with falling profits, increased disease problems, and an extremely limited arsenal of chemicals with which to combat furunculosis.

This study has investigated the potential of a range of new drugs which are being considered, or have recently been introduced for aquaculture use. *In vitro* the quinolones are very active in terms of MIC and bactericidal activity against the causative agent of furunculosis, *A.salmonicida* . However, they are adversely affected by the cations found in sea water, and to a lesser degree by the low temperatures associated with Scottish sea lochs. Nevertheless, reasonable levels can be attained in the serum, and these levels were found to be bactericidal against susceptible isolates of *A.salmonicida* and inhibitory against a resistant isolate.

Resistance to the quinolones may develop by spontaneous chromosomal mutation, which leads to either high level (possibly *gyrA* mediated) resistance, or lower level multiple antibiotic resistance mediated by alterations in outer membrane proteins. The frequency of spontaneous mutation to resistance to the fluoroquinolones, however, is much lower than that for oxolinic acid.

Of the quinolones tested in this study, PD117,596, PD127,391 and CI934 have been withdrawn for potential human use. Enrofloxacin has been

withdrawn by Bayer as the major metabolite of the compound is ciprofloxacin (Vancutsem *et al.,* 1990), currently the largest selling quinolone in human medicine. It is possible that the manufacturers are concerned about the spread of resistance to ciprofloxacin into human pathogens, as there has been a report of ciprofloxacin resistant *Campylobacter jejeuni* infections in humans resulting from chickens treated with enrofloxacin in the Netherlands (Endtz *et al.,* 1991).

The Roche quinolone, Ro 09-1168, is currently in the preliminary stages of investigation and is being investigated for veterinary (this study) or human (Shimma *et al.* 1991) use for the time being. Sarafloxacin is at an advanced stage of the licensing process, and may gain full licensing for use in aquaculture sometime this year (L. Brown, Pers. Comm.). Sarafloxacin is potentially a better drug than oxolinic acid, as it is more active in terms of MIC and bactericidal activity, and retains its activity in the absence of protein and RNA synthesis, and against non-dividing cells. Furthermore, the frequency of spontaneous chromosomal mutation to resistance to this compound is significantly lower than for oxolinic acid. These benefits put sarafloxacin in astrong position for aquaculture use. A disadvantage, however, is presented by the recent withdrawal of the Abbott fluoroquinolone temafloxacin (Teflox) from clinical use in humans due to severe adverse reactions (*Pharmaceutical Journal* **248**: 768). Side effects included hypoglycaemia, renal dysfunction, anaphylaxis and death. Although quinolones have been widely used in human medicine for 25 years with no severe adverse effects, the withdrawal of Teflox may create added difficulties in the future licensing of fluoroquinolones.

The potentiated sulphonamide, Romet 30, has been used since 1984 in the USA for the treatment of bacterial infections in salmonids (Meyer & Schnick, 1989). A study in our laboratory demonstrated that the components of this compound acted synergistically and were able to inhibit Scottish isolates of *A.salmonicida in vitro.* An *in vivo* trial conducted in this study demonstrated that low dose treatment with Romet 30 was effective in controlling a laboratory induced outbreak of furunculosis in Atlantic salmon in seawater, though it had little effect on asymptomatic *A.salmonicida* infection. It remains to be seen, however, whether this drug will provide an improved alternative to Tribrissen.

Amoxycillin was licensed for use in Scottish aquaculture in September 1990. In this study it has been shown that, as yet, high level β-lactamase mediated resistance to this compound only occurs in the atypical *A.salmonicida* subsp. *achromogenes.* Although this subspecies causes destructive fish kills in wild populations, it is rarely reported to cause outbreaks of furunculosis in farmed fish in Scotland.

This study has evaluated a number of potential chemotherapeutic agents for the control of furunculosis. Further work is required, not merely in the search for new compounds, but also into the environmental impacts associated with chemotherapy. Furthermore, to encourage the pharmaceutical companies to develop their compounds for aquaculture use, agreement needs to be sought among licensing authorities of fish farming nations to enable companies to recoup the costs of obtaining product licences. Finally, good husbandry and management strategies which reduce the prevalence of disease, and thus minimise the requirement for antibiotics, will become increasingly necessary if there is to be a future for Scottish aquaculture.

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APPENDIX

Appendix A. Culture collection: *Aeromonas salmonicida* isolates used in this study. Appendix lists subspecies, host, year of isolation, and country of origin. The source of isolates was the SOAFD Marine Laboratory, unless otherwise stated.

Appendix B. Minimum inhibitory concentrations (MICs)(mg/l) of antibiotics determined in this study against isolates of *A.salmonicida.*

Strain	Species	Subspecies	Year isolated	Host	Country of Origin
AB173	*Aeromonas salmonicida*	*salmonicida*	1990	Lab mutant (parent	
AB174	*Aeromonas salmonicida*	*salmonicida*	1990	Lab mutant (parent	
AB175	*Aeromonas salmonicida*	*salmonicida*	1990	Lab mutant (parent	
AB176	*Aeromonas salmonicida*	*salmonicida*	1990	Lab mutant (parent	
AB177	*Aeromonas salmonicida*	*salmonicida*	1990	Lab mutant (Parent	
AB178	*Aeromonas salmonicida*	*salmonicida*	1990	Lab mutant (parent	
AB179	*Aeromonas salmonicida*	*salmonicida*	1990	Lab mutant (parent	
AB180	*Aeromonas salmonicida*	*salmonicida*	1990	Lab mutant (parent	
AB181	*Aeromonas salmonicida*	*salmonicida*	1990	Lab mutant (parent	
AB182	*Aeromonas salmonicida*	*salmonicida*	1990	lab mutant (parent MT490)	
AB183	*Aeromonas salmonicida*	*salmonicida*	1990	Lab mutant (parent	
AB184	*Aeromonas salmonicida*	*salmonicida*	1990	Lab mutant (parent	
AB185	*Aeromonas salmonicida*	*salmonicida*	1990	Lab mutant (parent	
AB202	*Aeromonas salmonicida*	*salmonicida*	1990	Lab mutant (parent AB176)	
AB222	*Aeromonas salmonicida*	*salmonicida*	1990	Lab mutant (parent	SW Flumequine
AB223	*Aeromonas salmonicida*	*salmonicida*	1990	Lab mutant (parent 490)	SW Flumequine
AB224	*Aeromonas salmonicida*	*salmonicida*	1990	Lab mutant (parent 740)	sw flumequine
AB225	*Aeromonas salmonicida*	*salmonicida*	1990	Lab mutant (parent 744)	sw flumequine
Isolate C	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon	Abbott (UK)
Isolate C	*Aeromonas salmonicida*	*salmonicida*	1992	Passaged thro' Salmon	Abbott (UK)
MT004	*Aeromonas salmonicida*	*salmonicida*	1978	Atlantic salmon, sea water	Scotland (Unilever
MT1010	*Aeromonas salmonicida*	*salmonicida*	1990	Atlantic salmon	Norway
MT1011	*Aeromonas salmonicida*	*salmonicida*	1990	Atlantic salmon	Norway
MT1012	*Aeromonas salmonicida*	*salmonicida*	1990	Atlantic salmon	Norway
MT1013	*Aeromonas salmonicida*	*salmonicida*	1990	Atlantic salmon	Norway
MT1014	*Aeromonas salmonicida*	*salmonicida*	1990	Atlantic salmon	Norway
MT1015	*Aeromonas salmonicida*	*salmonicida*	1990	Atlantic salmon	Norway
MT1016	*Aeromonas salmonicida*	*salmonicida*	1990	Atlantic salmon	Norway
MT1017	*Aeromonas salmonicida*	*salmonicida*	1990	Atlantic salmon	Norway
MT1018	*Aeromonas salmonicida*	*salmonicida*	1990	Atlantic salmon	Norway
MT1019	*Aeromonas salmonicida*	*salmonicida*	1990	Atlantic salmon	Norway
MT194	*Aeromonas salmonicida*	*achromogenes*	1985	Atlantic salmon, seawater	Scotland
MT199	*Aeromonas salmonicida*	*salmonicida*	1985	Rainbow trout, freshwater	Scotland
MT273	*Aeromonas salmonicida*	*achromogenes*	1989	unknown	Scotland
MT320	*Aeromonas salmonicida*	*salmonicida*	1986	Atlantic salmon, seawater	Scotland
MT321	*Aeromonas salmonicida*	*salmonicida*	1986	Atlantic salmon, seawater	Scotland
MT324	*Aeromonas salmonicida*	*salmonicida*	1986	Atlantic salmon, seawater	Scotland
MT326	*Aeromonas salmonicida*	*salmonicida*	1986	Atlantic salmon	Scotland

Strain	Species	Subspecies	Year isolated	Host	Country of Origin
MT328	*Aeromonas salmonicida*	*salmonicida*	1986	Atlantic salmon	Scotland
MT329	*Aeromonas salmonicida*	*salmonicida*	1986	Atlantic salmon, Seawater	Scotland
MT335	*Aeromonas salmonicida*	*salmonicida*	1986	Atlantic salmon, seawater	Scotland
MT336	*Aeromonas salmonicida*	*salmonicida*	1986	unknown	unknown
MT337	*Aeromonas salmonicida*	*salmonicida*	1986	Unknown	Scotland
MT340	*Aeromonas salmonicida*	*salmonicida*	1987	Atlantic salmon	Scotland
MT350	*Aeromonas salmonicida*	*salmonicada*	1987	Atlantic salmon	Scotland
MT361	*Aeromonas salmonicida*	*salmonicida*	1987	Atlantic salmon	Scotland
MT362	*Aeromonas salmonicida*	*Salmonicida*	1987	Atlantic salmon, seawater	Scotland
MT363	*Aeromonas salmonicida*	*salmonicida*	1987	Atlantic salmon	Scotland
MT364	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon, sea water	Scotland
MT366	*Aeromonas salmonicida*	*salmonicida*	1986	Atlantic salmon, Seawater	Scotland
MT367	*Aeromonas salmonicida*	*salmonicida*	1987	Atlantic salmon, seawater	Scotland
MT368	*Aeromonas salmonicida*	*salmonicida*	1987	Atlantic salmon, seawater	Scotland
MT404	*Aeromonas salmonicida*	*salmonicida*	1987	Atlantic salmon	Scotland
MT427	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, seawater	Scotland
MT429	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, seawater	Scotland
MT431	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, seawater	Scotland
MT438	*Aeromonas salmonicida*	*salmonicida*	1988	salmon parr	Scotland
MT446	*Aeromonas salmonicida*	*salmonicida*	1988	Salmon parr, freshwater	Scotland
MT448	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, seawater	Scotland
MT455	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, seawater	Scotland
MT456	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon	Scotland
MT458	*Aeromonas salmonicida*	*salmonicida*	1988	Salmon parr	Scotland
MT459	*Aeromonas salmonicida*	*salmonicida*	1988	Salmon parr	Scotland
MT460	*Aeromonas salmonicida*	*achromogenes*	1988	Sea trout	Scotland
MT461	*Aeromonas salmonicida*	*salmonicida*	1988	salmon smolt	Scotland
MT462	*Aeromonas salmonicida*	*salmonicida*	1988	Salmon parr	Scotland
MT463	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, seawater	Scotland
MT464	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, seawater	Scotland
MT466	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, seawater	Scotland
MT469	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, seawater	Scotland
MT471	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, seawater	Scotland
MT472	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, seawater	Scotland
MT473	*Aeromonas salmonicida*	*achromogenes*	1988	Atlantic salmon, seawater	Scotland
MT474	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, seawater	Scotland
MT475	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, seawater	Scotland
MT476	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, seawater	Scotland

Strain	Species	Subspecies	Year isolated	Host	Country of Origin
MT477	*Aeromonas salmonicida*	*salmonicida*	1987	Atlantic salmon, seawater	Scotland
MT478	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, seawater	Scotland
MT479	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon, seawater	Scotland
MT479	*Aeromonas salmonicida*	*achromogenes*	1988	mature salmon, freshwater	Scotland
MT481	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon	Scotland
MT482	*Aeromonas salmonicida*	*achromogenes*	1988	Mature salmon, freshwater	Scotland
MT487	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, seawater	Scotland
MT488	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, seawater	Scotland
MT489	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, seawater	Scotland
MT490	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, seawater	Scotland
MT491	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, Sea	Scotland
MT492	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, seawater	Scotland
MT493	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon,seawater	Scotland
MT494	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, seawater	Scotland
MT495	*Aeromonas salmonicida*	*salmonicida*	1988	Atlantic salmon, seawater	Scotland
MT529	*Aeromonas salmonicida*	*achromogenes*	1989	Atlantic salmon, sea water	Scotland
MT533	*Aeromonas salmonicida*	*achromogenes(EL*	1980	Atlantic salmon, seawater	Iceland (B.
MT596	*Aeromonas salmonicida*	*unknown*	1989	Mature salmon, freshwater	Scotland
MT600	*Aeromonas salmonicida*	*salmonicida*	1989	salmon parr	Scotland
MT616	*Aeromonas salmonicida*	*salmonicida 152 6G*	1986	unknown:A -, no high Mw	(D. Evenberg)
MT674	*Aeromonas salmonicida*	*salmonicida*	1989	Salmon fry	Scotland
MT675	*Aeromonas salmonicida*	*achromogenes*	1989	Sea Trout, freshwater	Scotland
MT728	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon, seawater	Scotland
MT729	*Aeromonas salmonicida*	*salmonicida*	1989	Isolated from an unusual	
MT730	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon, seawater	Scotland
MT731	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon, seawater	Scotland
MT732	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon, seawater	Scotland
MT733	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon, seawater	Scotland
MT736	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon,seawater	Scotland
MT737	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon, seawater	Scotland
MT738	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon, seawater	Scotland
MT739	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon, seawater	Scotland
MT740	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon, sea water	Scotland
MT743	*Aeromonas salmonicida*	*salmonicida*	1989	Salmon parr	Scotland
MT744	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon, seawater	Scotland
MT746	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon, Seawater	Scotland
MT747	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon, seawater	Scotland
MT748	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon, seawater	Scotland

Strain	Species	Subspecies	Year isolated	Host	Country of Origin
MT750	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon, seawater	Scotland
MT752	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon, seawater	Scotland
MT753	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon, seawater	Scotland
MT754	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon, seawater	Scotland
MT755	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon, seawater	Scotland
MT756	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon, seawater	Scotland
MT757	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon, seawater	Scotland
MT879	*Aeromonas salmonicida*	*salmonicida*	1989	Atlantic salmon	Scotland

Strain	Oxolinic acid	Flumequine	Saraflo xacin	Enrofl oxacin	Roche	PD127, 391	PD117, 596	CI934	Oxytetra cycline	Amoxy cillin
AB174	1.00	NT	0.40	0.15	NT	0.10	0.15	2.0	1.50	0.75
AB175	0.075	NT	0.20	0.075	NT	0.05	0.075	2.00	0.75	NT
AB176	0.10	NT	0.20	0.10	NT	0.05	0.15	2.00	1.00	1.00
AB177	1.00	NT	1.00	0.15	NT	0.05	0.15	2.00	0.50	NT
AB178	1.00	NT	1.00	0.15	NT	0.05	0.15	2.00	0.40	NT
AB179	0.04	NT	0.075	0.04	NT	0.075	0.075	1.00	1.50	NT
AB180	0.10	NT	0.30	0.15	NT	0.10	0.15	3.00	0.75	NT
AB181	0.10	NT	0.30	0.15	NT	0.10	0.15	2.00	0.75	NT
AB182	0.10	NT	0.30	0.15	NT	0.10	0.15	2.00	0.75	NT
AB183	1.00	NT	1.00	0.15	NT	0.05	0.15	3.00	0.40	NT
AB184	0.04	NT	0.075	0.04	NT	0.03	0.04	1.00	1.50	NT
AB185	0.10	NT	0.30	0.15	NT	0.10	0.15	3.00	1.00	NT
AB202	1.50	NT	0.30	0.10	NT	NT	NT	NT	1.00	0.75
AB222	NTNT	NT	NT	NT	NT	NT	NT	NT	NT	NT
AB223	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
AB224	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
AB225	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
MT004	0.075	0.075	0.02	0.02	0.01	0.005	0.015	0.075	0.30	0.50
MT1010	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
MT1011	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
MT1012	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
MT1013	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
MT1014	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
MT1015	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
MT1016	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
MT1017	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
MT1018	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
MT1019	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
MT194	0.03	NT	0.01	0.005	0.01	0.002	0.004	0.20	0.30	>500.00
MT199	0.20	0.075	0.15	0.15	0.01	0.03	0.075	1.00	0.30	0.50
MT273	NT	0.075	NT	NT	NT	NT	NT	NT	0.30	>500.00
MT320	7.50	0.75	1.50	0.40	NT	0.10	0.20	3.00	40.00	0.075
MT321	0.04	0.15	0.075	0.03	0.10	0.0075	0.015	0.50	40.00	0.50
MT324	0.03	NT	1.00	0.015	NT	0.0075	0.0075	0.30	NT	NT

Strain	Oxolinic acid	Flumequine	Saraflo xacin	Enrofl oxacin	Roche	PD127, 391	PD117, 596	CI934	Oxytetra cycline	Amoxy cillin
MT326	0.03	NT	0.075	0.02	NT	0.01	0.01	0.40	NT	NT
MT328	7.50	NT	4.00	1.00	0.20	0.15	0.40	7.50	0.30	0.40
MT329	0.03	0.075	0.05	0.03	0.01	0.0075	0.015	0.50	50.00	0.50
MT335	5.00	NT	4.00	1.50	0.10	0.75	0.75	3.00	50.00	0.75
MT336	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
MT337	NT	0.075	NT	NT	0.01	NT	NT	NT	0.30	0.50
MT340	0.75	NT	0.50	0.15	NT	0.04	0.075	1.50	NT	NT
MT350	0.04	0.075	0.05	0.02	0.01	0.0075	0.015	0.40	40.00	0.50
MT361	2.00	NT	0.75	0.20	0.10	0.04	0.075	4.00	150	0.50
MT362	5.00	NT	2.00	0.75	0.40	0.150	0.30	4.00	0.30	0.30
MT363	0.03	0.10	0.05	0.02	0.01	0.0075	0.015	0.40	0.20	0.30
MT364	7.50	0.75	3.00	1.00	0.01	0.10	0.30	3.00	0.30	0.50
MT366	0.03	NT	0.075	0.05	0.01	0.015	0.015	0.40	NT	0.30
MT367	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
MT368	5.00	NT	4.00	1.50	NT	0.30	0.50	10.00	NT	NT
MT404	NT	0.10	NT	NT	0.01	NT	NT	NT	40.00	0.50
MT427	NT	NT	NT	NT	0.075	NT	NT	NT	40.00	0.40
MT429	3.00	NT	2.00	0.50	0.01	0.10	0.20	7.50	30.00	0.40
MT431	5.00	2.00	2.00	1.00	0.20	0.15	0.30	3.00	0.30	0.40
MT438	1.50	NT	1.00	0.20	0.20	0.04	0.075	3.00	0.30	0.50
MT446	2.0	NT	0.20	0.05	NT	0.015	0.05	0.40	NT	NT
MT448	NT	0.075	NT	NT	NT	NT	NT	NT	40.00	0.50
MT455	0.03	0.10	0.075	0.02	0.01	0.01	0.01	0.40	30.00	0.75
MT456	0.03	0.10	0.075	0.03	0.01	0.01	0.015	0.50	40.0	0.50
MT458	1.00	NT	0.75	0.30	0.075	0.075	0.015	1.50	50.00	0.50
MT459	2.00	NT	1.50	0.30	0.10	0.075	0.10	2.00	0.30	0.50
MT460	0.40	0.075	0.30	0.04	0.01	0.015	0.05	1.50	0.30	>500.00
MT461	0.03	0.075	0.075	0.03	0.01	0.01	0.015	0.50	0.30	0.30
MT462	2.0	NT	1.00	0.20	0.075	0.04	0.075	3.00	100.00	0.30
MT463	10.00	NT	4.00	1.50	0.20	0.20	0.75	10.00	0.30	0.75
MT464	1.50	1.00	1.00	0.20	0.10	0.05	0.10	3.00	40.00	0.30
MT466	2.00	NT	0.75	0.20	0.075	0.04	0.10	3.00	75.0	0.50
MT469	NT	0.10	NT	NT	0.01	NT	NT	NT	0.30	0.50
MT471	3.00	NT	1.50	0.50	0.075	0.04	0.10	3.00	0.30	0.40

Strain	Oxolinic acid	Flumequine	Saraflo xacin	Enrofl oxacin	Roche	PD127, 391	PD117, 596	CI934	Oxytetra cycline	Amoxy cillin
MT472	3.00	NT	2.00	1.00	0.20	0.10	0.20	2.00	0.30	0.30
MT473	0.40	NT	0.20	0.15	0.01	0.075	0.05	0.75	0.30	>500.00
MT474	NT	0.075	NT	NT	NT	NT	NT	NT	0.30	1.50
MT475	3.00	NT	2.00	0.75	NT	0.10	0.15	3.00	NT	NT
MT476	2.00	NT	0.75	0.20	0.075	0.04	0.10	3.00	0.30	0.50
MT477	1.50	0.50	0.75	0.20	0.10	0.04	0.075	3.00	0.30	0.50
MT478	NT	NT	NT	NT	0.01	NT	NT	NT	NT	NT
MT479	10.00	NT	4.00	1.00	0.20	0.10	0.75	7.50	0.30	0.40
MT479	NT	0.075	NT	NT	NT	NT	NT	NT	0.30	>500.00
MT481	3.00	NT	2.00	0.75	NT	0.10	0.15	3.00	NT	NT
MT482	0.01	0.075	0.02	0.003	NT	0.0075	0.015	0.15	0.30	>500.00
MT487	5.00	NT	2.00	1.00	0.10	0.10	0.20	2.00	0.30	0.50
MT488	NT	0.50	NT	NT	0.075	NT	NT	NT	0.30	0.50
MT489	3.00	NT	1.50	0.30	0.20	0.10	0.15	3.00	0.30	0.50
MT490	0.03	0.075	0.04	0.02	0.01	0.0075	0.015	0.40	0.30	0.30
MT491	0.04	0.075	0.075	0.05	0.01	0.015	0.015	0.40	0.30	0.75
MT492	0.03	NT	0.075	0.02	NT	0.0075	0.0075	0.40	0.30	1.50
MT493	3.00	0.75	1.50	0.50	1.00	0.15	0.30	3.00	0.30	1.00
MT494	0.03	0.075	0.05	0.02	0.01	0.0075	0.015	0.40	30.00	0.30
MT495	0.03	0.075	0.04	0.02	0.01	0.0075	0.015	0.50	0.30	0.40
MT529	0.03	NT	0.015	0.0075	0.01	0.0075	0.003	0.15	NT	>500.00
MT533	NT	0.075	NT	NT	0.01	NT	NT	NT	0.30	>500.00
MT596	0.03	0.075	0.03	0.015	0.01	0.0075	0.015	0.40	0.30	0.40
MT600	0.03	NT	0.05	0.02	NT	0.01	0.01	0.40	NT	NT
MT616	0.015	0.075	0.0075	0.004	NT	0.003	0.004	0.04	0.30	1.00
MT674	7.50	5.00	3.00	1.00	0.10	0.10	0.20	10.00	50.00	0.40
MT675	0.015	0.10	0.01	0.005	NT	0.005	0.005	0.30	0.30	>500.00
MT728	0.03	0.10	0.05	0.02	0.01	0.0075	0.015	0.40	0.30	0.50
MT729	0.40	NT	0.20	0.075	0.01	0.015	0.04	1.50	NT	NT
MT730	0.04	0.075	0.05	0.02	0.01	0.0075	0.015	0.30	0.30	0.30
MT731	0.015	NT	0.03	0.03	0.01	0.0075	0.015	0.15	30.00	0.30
MT732	0.015	0.100	0.200	0.15	0.01	0.03	0.03	0.15	0.30	0.50
MT733	15.00	NT	1.00	0.50	0.50	0.15	0.20	3.00	40.00	0.30
MT736	0.015	0.500	0.04	0.04	0.01	0.01	0.015	0.30	100.00	0.75

Strain	Oxolinic acid	Flumequine	Saraflo xacin	Enrofl oxacin	Roche	PD127, 391	PD117, 596	CI934	Oxytetra cycline	Amoxy cillin
MT737	3.00	NT	2.00	0.75	NT	0.15	0.20	3.00	NT	NT
MT738	0.03	0.10	0.075	0.02	NT	0.01	0.01	0.40	0.30	0.30
MT739	3.00	NT	1.50	0.50	0.30	0.10	0.15	3.00	0.30	0.30
MT740	1.50	NT	1.00	0.20	0.10	0.03	0.075	3.00	NT	NT
MT743	0.03	0.075	0.075	0.02	0.01	0.01	0.01	0.40	75.0	0.50
MT744	0.03	0.075	0.05	0.015	0.01	0.0075	0.015	0.40	0.30	0.30
MT746	3.00	NT	2.00	0.75	NT	0.15	0.20	3.00	0.30	1.00
MT747	0.03	0.075	0.075	0.02	0.01	0.0075	0.015	0.40	0.30	0.50
MT748	0.03	0.10	0.03	0.02	0.01	0.0075	0.015	0.40	40.00	0.75
MT750	0.03	NT	0.05	0.02	0.01	0.0075	0.015	0.400	NT	0.40
MT752	NT	NT	NT	NT	NT	NT	NT	NT	0.30	1.50
MT753	0.03	NT	0.05	0.02	NT	0.0075	0.01	0.40	NT	NT
MT754	0.03	0.075	0.075	0.02	NT	0.01	0.01	0.40	40.00	0.30
MT755	4.00	NT	2.00	0.75	0.15	0.075	0.10	3.00	40.00	1.50
MT756	0.03	NT	0.04	0.02	0.01	0.0075	0.0075	0.30	NT	0.30
MT757	0.04	0.075	0.05	0.03	0.01	0.0075	0.015	0.50	30.00	0.40
MT879	7.50	5.00	5.00	0.50	0.10	NT	NT	NT	NT	NT