BACTERIAL ASSOCIATIONS WITH SALMONID EGGS

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A thesis submitted for the

degree of

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SUMMARY

Rainbow trout eggs *Salmo gairdneri* , Richardson, were incubated under a range of different environmental conditions. Recovery of bacteria from egg surfaces revealed that increased water temperature, slow water flow rates and high egg density all significantly increased egg surface bacterial populations. Live eggs were mainly colonized by *Cytophaga* sp., *Pseudomonas fluorescens* and *Aeromonas hydrophila*. In contrast, dead eggs supported considerable numbers of fluorescent *Pseudomonas* sp.

Analysis of potential nutrient sources for bacteria colonizing live egg surfaces revealed that small amounts of amino acids, phosphate and potassium may be lost by incubating eggs. Subsequently these nutrients were shown to be capable of supporting limited bacterial growth and reproduction. Dead eggs "leaked" increased amounts of the above nutrients which in turn supported higher bacterial numbers. In addition, biochemical analysis of eggs revealed amino acids and fatty acids that might be utilized by bacteria colonizing dead egg surfaces.

Assessment of adhesion properties of bacteria frequently recovered from egg surfaces revealed high cell surface hydrophobicity as an important factor in successful egg colonization.

Analysis of egg mortalities from groups of rainbow trout and brown trout (*S. trutta* L.) eggs maintained under two different incubation systems revealed that potentially a close correlation existed between egg surface bacterial numbers and mortalities in the eggs during incubation. Inoculation of newly-fertilized eggs with bacteria demonstrated that groups of eggs supporting high numbers of *P. fluorescens* suffered significantly higher mortalities during the early part of their incubation.

Exposure of incubating eggs to oxolinic acid, chlortetracycline and chloramphenicol demonstrated that numbers of bacteria on egg surfaces could be significantly reduced. However, as no corresponding increase in egg hatching success was revealed, the treatment of incubating eggs with antibiotics or antimicrobial compounds can not be recommended.

In commercial hatcheries bacteria are only likely to be responsible for egg deaths during incubation when environmental conditions are unfavourable. High water temperatures, slow water flow rates and high egg density all lead to increased bacterial numbers on egg surfaces, reduced water circulation and low levels of dissolved oxygen. Under such circumstances sufficient amounts of dissolved oxygen may not be available to support developing embryo's.

Key words :
Rainbow trout, Salmonid, Egg quality, Bacterial flora, Fish-farming.

To my mother and father for their encouragement

and support over so many years.....

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CHAPTER ONE

GENERAL INTRODUCTION

Since 1983 the United Kingdom has certified each year the importation of in excess of 50 million eyed rainbow trout eggs (*Salmo gairdneri*) Richardson, rising to approximately 61.5 million in 1987 (Bromage & Cumaranatunga 1988). Annual importation of potentially ever increasing numbers of eyed eggs into the U.K. is undesirable on grounds of cost, genetic strategy, and in addition leaves the industry vulnerable to the introduction of non-endemic diseases such as viral haemorrhagic septicaemia (V.H.S.) and infectious haemopoietic necrosis (I.H.N.), both of which are established as being transmitted in association with salmonid eggs (Vestergard-Jorgensen 1970 ; Wolf 1976). Precise reports of total egg numbers incubated in the U.K. per annum are currently not collated, however, a relatively accurate estimate may still be derived using material drawn from a number of sources. In the region of 18,000 tonnes of half pound fish were produced in 1988 (Gordon 1989) which equates with a current annual production of around 80 million individual fish. In addition to successfully reared adults many alevins, fry and eggs are lost during the rearing process. Bromage & Cumaranatunga (1988), using data from commercial trials, estimate that for a given batch of good quality eggs the following percentage survivals would be expected at each of five successive stages of development : fertilization 90%, eyeing 80%, hatching 75%, swim up 60% and after 4 months 35%.

In the south of the U.K farmed rainbow trout may reach half pound size in around 9 months and take just over a year in the north. Unfortunately no estimate exists in the scientific literature of mortalities expected between 4 months and one year. If, however, a relatively conservative estimate of 5% mortality is assumed then it is possible to further speculate that only 30% of eggs survive to adult half pound fish. This in turn suggests that to produce 80 million individual fish around 215 million eyed eggs were probably required in 1987 by the U.K. trout industry. However, of these eyed eggs 61.5 million were imported and thus the home industry probably produced approximately 153.5 million eyed eggs. Furthermore, again utilizing the percentage

survival figures outlined by Bromage & Cumaranatunga (1988) it can be calculated that the U.K. industry in total probably produced over 191.9 million green eggs. Thus, it is likely that total losses of the U.K.'s own eggs from stripping to hatching could amount to 48 million eggs (assuming 25% losses of an original 191.9 million stripped eggs). By following a similar exercise it can be calculated that about 3.1 million imported eyed eggs are also lost before hatching (assuming 5% mortalities from eyeing to hatching amongst 61.5 million imported eyed eggs). Therefore, overall in 1987 the U.K. industry might potentially have lost over 51 million eggs during incubation (48 million home produced eggs + 3.1 million imported eggs).

Even assuming some inaccuracy within these figures, a reasonable indication is outlined above of the considerable egg losses occurring within the U.K. fish farming industry. A reduction in such losses through a general improvement in fish husbandry techniques and, in particular, a decrease in egg mortality within U.K. hatcheries, would in turn reduce importation costs and lower the risk of introducing non-endemic diseases. Although, causes of egg death during incubation are not fully understood and are likely to be many and varied, two main areas can be identified as potentially important in influencing numbers of successfully hatched fry. One area of direct concern is the quality of husbandry practices observed in the hatchery and in particular the provision of optimum incubation conditions for developing embryos (to be described in full later). The other area encompasses other factors that might influence the initial quality of eggs produced by broodstock and in turn these can be further sub-divided into three main sections (to be described below) ;

1) *Broodstock age*. Individual fish generally produce eggs of uniform size (Bagenal 1969a ; Zonova 1973 ; Larsson & Pickova 1978), which will be determined in part, by the genetic make up of the parent. However, the age (and therefore size) of the fish are also important, for older and larger females tend to produce larger (but fewer) eggs when compared to younger, smaller females (Gall 1974 ; Bromage & Cumaranatunga 1988). Larger eggs contain more water than smaller eggs but, in

addition also contain a relatively larger yolk, which was once considered to provide fry with a nutritional advantage over those from smaller eggs. A higher percentage survival of fry from large eggs has been clearly demonstrated under conditions of deliberate fry starvation (Blaxter & Hempel 1963 ; Bagenal 1969b). Therefore, larger egg size would be expected to be an advantage in feral stocks if suitable nutrient sources were scarce. However, under commercial farming conditions scarcity of food is unlikely to occur and thus fry from smaller eggs are unlikely to suffer any disadvantage (Reagan & Conley 1977 ; Bromage & Cumaranatunga 1988).

2) *Broodstock diet and egg composition.* Studies on the biochemistry of the egg have shown great variation in chemical composition between both eggs of individual fish of the same strain and between eggs from fish of different strains. Chemical components of the egg have to support fry for the first 50 days of their lives (from fertilization to first feed at 10 °C). Therefore, it has been argued that by altering broodstock diets, to proportionally change the chemical components of the egg, egg quality, itself, would be altered. The largest component of salmonid eggs by dry weight is protein. Philips, Hammer Edmonds & Hosking (1964) studied protein levels in brown trout eggs and reported that "the best quality eggs" came from fish fed low protein diets. In contrast, Satia, Donaldson, Smith & Nightingale (1974) found that eggs containing a high percentage of protein were more likely to survive to the yolk sac absorption stage. Smith, Osbourne, Piper & Dwyer (1979) studied three types of protein diet, high, intermediate and low, over a three year period and found no significant difference in quality of eggs produced. However, Takeuchi, Watanabe, Ogino, Satio, Nishimura & Nose (1981) in agreement with Philips *et al* (1964), demonstrated that fish fed low protein but high energy diets did indeed produce better quality eggs. However, further work by Roley (1983) using four levels of protein diet ; 27%, 37%, 47%, and 56%, all with the same calorific value, again found no overall significant difference in egg quality between groups fed the above four diets. Thus, it would seem that a balanced feed containing adequate (but not excessive) levels of protein will promote egg and fry survival.

The second largest component of eggs by dry weight is fat. Eggs frequently vary in the proportion of fat they contain but a mean figure of 19% is not unusual, of which 70% - 80% will be used as an energy source during stages of egg development (Hayes 1949). Surprisingly, commercial diets generally contain a relatively low lipid level of between 6% to 14% by dry weight (Hilton & Slinger 1981). Most fat in eggs (and protein) can be found in the yolk, which until first feeding is the main nutrient supply for developing embryos. Fat and protein occur in the yolk as phosvitin and lipovitellin, both of which are components of vitellogenin. In turn, vitellogenin is manufactured in the liver and after secretion into the blood stream is taken up by maturing oocytes. Potentially, low levels of vitellogenin might be responsible for poor quality eggs, although as yet, no direct correlation has been demonstrated (Bromage & Cumaranatunga 1988).

Hirao, Yamada & Kikuchi (1954) and Takeuchi *et al* (1981) have clearly demonstrated the importance of trace elements in broodstock diet. Broodstock fed a diet without trace elements (not unexpectedly) produced eggs of an inferior quality (Hirao *et al* 1954 ; Takeuchi *et al* 1981). One group of trace elements once thought to be directly linked to egg quality is the carotenoids. They are responsible for giving a pink colouration to flesh and a deep orange colour to eggs (often regarded as an indication of good egg quality). Rainbow trout are unable to synthesize carotenoids and they are thus deliberately added to commercially produced feed. Developmentally the role of carotenoids is not fully understood but, they are a source of provitamin A. Originally it was considered that they may act as a chemotaxic attractant for rainbow trout sperm, in turn increasing levels of fertilization. However, Quantz (1980) increased dietary levels of carotenoids but, subsequently found no proportionally significant increase in sperm chemotaxis or in egg fertilization rate. Further work has since confirmed that increases in dietary carotenoids do not in turn lead to increased fertilization rates (Torrissen 1984 ; Tveranger 1986).

3) *Time of egg stripping*. Eggs of rainbow trout are ovulated into the coelomic cavity where they are held (under farmed conditions) until they are "manually" stripped.

Once in the cavity the eggs undergo a progressive reduction in viability, termed "overripening" (Sakai, Nomura, Takashima & Oto 1975 ; Hirose, Ishida & Sakai 1977 ; Lam, Nagahama, Chan & Hoar 1978 ; Craik & Harvey 1984 ; Springate, Bromage, Elliott, & Hudson 1984). Egg overripeness is a problem of great importance amongst farmed salmonid fish (Lam *et al* 1978) and is accompanied by physical changes to the egg, involving the aggregation and fusion of oil droplets and migration of the cortical alveoli to the animal pole (Nomura, Sakai & Takashima 1974). Nomura *et al* (1974) divided overripening into four separate stages ; stage 1 describes normal eggs up to 6 days after ovulation ; stage 2 begins at about 10 days after ovulation, when eggs begin to show early signs of overripeness, although up to 100% fertilization and a high percentage to eyeing is still possible ; stage 3 begins at about the 30th day after ovulation and eggs show all the classic signs of overripeness (as described) and stage 4, starting at the 35th day after ovulation includes eggs showing extreme signs of overripeness including an irregular shape and a reduction in material content. Both stage 3 and 4 eggs are of such poor quality that fertilized eggs will not reach the eyed stage. Egg overripening can be overcome by increased frequency of manual stripping and Sakai *et al* (1975) recommended that broodstock should be checked every 7 days when held at a temperature of 10 °C. Increased water temperatures greatly accelerate overripening and more frequent stripping is therefore essential (Escaffre & Billard 1979).

The second general area affecting numbers of successfully hatched fry is that of hatchery management, which is further sub-divided below into two main sections ; 1) fertilization and 2) hatchery environmental conditions. In addition, a third section is included outlining the potential role bacteria may play in causing "premature" egg mortality during incubation. This is the subject which is investigated in depth during this present study.

1) *Fertilization*. Successful egg fertilization is essential to maximize the number of fry produced from the total number of eggs incubated. Fertilization rates will of course be dependant on egg quality (as described) but, also on the quality of milt, a factor often given less emphasis than deserved. Milt quality is frequently related to sperm motility and is assessed microscopically under a magnification of about 50X. Good quality milt will contain spermatozoa that are vigorously active and that are swimming, more or less, in straight lines. Milt of lesser quality will contain spermatozoa which are swimming generally in tight circles (as opposed to straight lines) but, if used immediately,will still allow high fertilization rates to be achieved. In contrast, milt containing little spermatozoa movement will be of poor quality in turn leading to low fertilization rates and thus should be discarded. Obviously, assessment of sperm motility is subjective but, even so does seem to be indicative of, as yet, other unidentified properties of spermatozoa that are important in egg fertilization (Moccia & Munkittrick 1987). Careful fertilization of eggs with good quality milt can lead to fertilization rates as high as 90% under commercial conditions (Bromage & Cumaranatunga 1988). However, significantly lower fertilization rates will occur (even with good quality eggs and milt) if certain hatchery practises are not scrupulously enforced. For example, if water is allowed to come into contact with newly stripped eggs before fertilization it will pass through the egg micropyle into the perivitelline fluid in turn, causing the micropyle to move slowly towards one side and to decrease in size. Spermatozoa can only enter the egg through the micropyle and therefore, prospects of egg fertilization become progressively poorer as the micropyle opening gets smaller (Leitritz & Lewis 1976). Similarly, if water is allowed to come into contact with milt, spermatozoa motility will cease after only about one minute, ensuring subsequently poor fertilization rates.

Generally, salmonids produce a relatively high amount of milt, approximately 5×10^{12} spermatozoa / Kg body weight (Billard 1983a, b). Munkittrick & Moccia (1987) calculated that milt from one male could potentially fertilize the eggs from 3000 females and thus commercially there has been a tendency to limit the stockholding of

males. Consequently, if the males initially selected to fertilize eggs prove to have poor sperm quality, it is thus possible that there may only be a few remaining males that possess milt of sufficient quality. Subsequent fertilization rates may therefore be poor and in addition genetic diversity can be compromised. Thus, greater care must be taken to ensure that high quality milt is available from a reasonably wide range of males (ideally a ratio of milt from one male to three females) over the entire egg stripping period. At present, until cryopreservation techniques are more widely available, fertilization of "out of season" eggs may also necessitate photoperiodic manipulation of males in order to ensure availability of high quality milt.

2) *Hatchery environmental conditions*. Provision of optimum egg incubation conditions is of great importance in order to limit egg mortalities to the lowest possible level. The first basic essential for successful salmonid egg incubation is water flow rate. In addition, water quality, including pH, dissolved oxygen and suspended solids are all important factors to consider (Leitritz & Lewis 1976). Ideally, hatchery water should be neutral or slightly alkaline (pH 7-8) and, acid conditions (pH 6 or below) should be avoided (Stevenson 1980). Dissolved oxygen levels should be between 5-9 ppm (Leitritz & Lewis 1976). A fall in dissolved oxygen levels (for example, due to increased water temperature) to 3 ppm could potentially be insufficient to support large numbers of developing embryos. Salmonid embryos can withstand large fluctuations in amounts of suspended solids but, at high concentrations such solids may "coat" eggs in turn impairing sufficient availability of oxygen to embryos.

Two common methods of egg incubation are currently being used. The first, "vertical upwelling incubation" is less frequently used in the U.K., but has the advantages of taking up limited floor space and the potential for incubating vast numbers of eggs. However, a major disadvantage of this method includes limited access to eggs during "sensitive stages" of incubation and thus checks on fungal parasites and egg mortalities cannot be made. In addition, development of air pockets can lead to physical disturbances of eggs and also poor distribution of water through the eggs. The second method of egg incubation, "horizontal trays in hatchery troughs"

is, probably, the most frequently utilized method of egg incubation in U.K. hatcheries. Horizontal trays provide for access to eggs at all times and therefore allow regular monitoring of egg progress throughout incubation to be made (Springate & Bromage 1985).

After fertilization, water hardening and "laying down", eggs remain fairly resilient for a period of up to 48 hours at 10 °C but, after this time, are sensitive until eyeing. An extremely critical period of egg sensitivity is known to occur between the 7^{th} day and the 9^{th} day of incubation (at 10 °C), after which time the egg blastopore is closed (Leitritz & Lewis 1976). Physical disturbance of eggs before eyeing, for example, by flow rates that are too turbulent, air pockets (especially vertical incubators) or physical knocks to hatchery troughs can result in high numbers of egg mortalities. Eggs must also be protected from exposure to light, failure to do so can result in death in only a few minutes if direct exposure to sunlight is allowed to occur. Presence of dead eggs in trays frequently results in colonization by aquatic moulds such as those of the *Saprolegniaceae*, which can subsequently cause the death of nearby "healthy" living eggs (Smith, Armstrong, Springate & Barker 1985). To minimize the effects of such moulds, malachite green treatment should be administered daily (ideally at a concentration of 2 ppm for one hour) and pockets of dead eggs should be carefully removed (Stevenson 1980).

3) *Bacteria*. Whether bacteria can directly influence cultured fish egg losses is uncertain. However, historically the possibility that bacteria could be responsible for cultured egg losses began with a series of studies on marine fish. Dannevig (1919) demonstrated that during incubation, cod eggs (*Gadus callarias*) became overgrown with an organism resembling *Leptothrix*, and speculated that such an organism might in turn be responsible for lowering egg hatching rates. Spencer (1952) in a study of marine algae used a series of antibiotics to reduce contaminating populations of bacteria. His success in lowering bacterial numbers in samples of sea water led to a study by Oppenheimer (1955) relating bacteria in sea water to the hatching success of some marine fish eggs. Oppenheimer (1955) incubated batches of eggs from the Pacific

sardine (*Sardinops caerulea*), the Norwegian codfish (*G. callarias*) and a turbot (*sic, Pleuronichthys* sp. probably *ritteri)* in normal sea water and treated sea water containing various antibiotic solutions. By monitoring bacterial numbers in incubation waters and by recording egg hatching success, the author demonstrated a close relationship between bacterial numbers and egg death.

Studies on the effects of bacteria on stream incubated salmonid eggs began when Hunter (1959) reported that salmonid eggs could be recovered still undergoing decay up to two years after their original deposition into spawning beds. Similarly, McNeil, Wells & Brickell (1964) showed that pink salmon eggs (*Onc orynchus gorbuscha*) were still decaying 18 months after their original deposition in the spawning beds of a small southeastern Alaska stream. These authors discussed the possibility that decaying eggs (and adult carcasses) could in turn lead to a localized high biological oxygen demand in spawning beds and perhaps insufficient oxygen levels to support incubating eggs. Bell (1966) however, considered that in addition to lowering oxygen levels decaying eggs might also act as a source of microbial infection of healthy eggs. The above author therefore incubated newly fertilized pink salmon eggs in permeable plastic tubes placed into a salmon redd of the Big Qualicum River, Canada. Bacterial populations of egg surfaces were monitored throughout incubation and predominant bacteria were found to be *Pseudomonas* sp. and *Flavobacterium* sp. A more detailed study by Bell, Hoskins & Hodgkiss (1971) conducted at the same site showed that the state of salmonid eggs could markedly influence the composition of bacterial populations on the egg surfaces. *Cytophaga* sp. predominated in floras of living eggs, whilst fluorescent *Pseudomonas* sp. formed the bulk of populations associated with dead eggs. These same authors considered it unlikely that enzymes or toxins produced by bacteria on live egg surfaces could be the cause of the mortalities in the incubating eggs.

Trust (1972) investigated the bacterial populations at the egg surface of coho salmon eggs (*O. kisutch*), chum salmon eggs (*O. keta*) and rainbow trout eggs (*Salmo gairdneri*) placed in vertically stacked incubation trays of a commercial hatchery and

showed *Cytophaga* sp., *Pseudomonas* sp. and *Aeromonas hydrophila* were the predominant bacteria on the egg surfaces. Under conditions of poor water circulation Trust (1972) suggested that large bacterial populations on the egg surfaces might reduce available oxygen to a level insufficient for egg survival.

Investigations of bacteria located inside salmonid eggs have also been carried out. However, many of these studies have not been concerned with the deaths of incubating eggs but, with the transmission of specific bacterial pathogens within eggs. Parisot & Wood (1960) described the presence of an acid fast bacillus in the yolk of diseased chinook salmon (*O. tshawytscha*). Furthermore, the authors considered that the egg yolk might be the primary site of infection for the above bacterium, which might in turn be responsible for the disease "fish mycobacteriosis". Lund (1967) in a study of furunculosis caused by the bacterium *Aeromonas salmonicida*, described a positive isolation of the bacterium from the interior of fertilized eggs derived from naturally infected mature fish. In addition, the bacterium has also been isolated from both the ovary and testis of diseased mature fish suffering from furunculosis (Lund 1967 ; McDermot & Berst 1968). However, McCarthy (1980) did not recover *A. salmonicida* from the interior of experimentally infected salmonid eggs and thus considered that "vertical" transmission (transmission via eggs) of *A. salmonicida* "is not a significant route of transmission for furunculosis".

In contrast, bacterial kidney disease (B.K.D.) of salmonid fish caused by a small Gram +ve bacterium (*Renibacterium salmoninarum*) may be spread by vertical transmission. Bullock & Stuckey (1978) disinfected egg surfaces contaminated by *R. salmoninarum* with iodophor disinfectants. However, despite determined disinfection, some *R. salmoninarum* cells still appeared to remain active. Evelyn, Prosperi-Porta & Ketcheson (1981) examined eggs from a naturally B.K.D. infected salmon *(O. kisutch)* which had a high coelomic fluid count of *R. salmoninarum.* Eggs were removed, disinfected with iodophors and subsequently checked microbiologically for any active remaining cells. Eggs whose surfaces had been successfully sterilized were aseptically cut open and their contents removed for further bacteriological examination. 104 eggs

were surface sterilized and 6 eggs (5.8%) were subsequently found to contain viable cells of *R. salmoninarum*. A further study by Evelyn, Ketcheson & Prosperi-Porta (1984) estimated that the pathogen might exist in approximately 11.6% to 15.1% of the total eggs of each infected fish.

Other significant reports of bacteria inside salmonid eggs include a Gram +ve *Lactobacillus* sp. discovered by Cone (1982). Cone studied 14 diseased rainbow trout at a Newfoundland hatchery after post stripping mortalities were running unacceptably high (1-5 fish / day). Mixed infections of *A. hydrophila, P. fluorescens* and a *Lactobacillus* sp. were found in the diseased trout. Histological sections cut through 35 eggs from 5 of the above fish revealed contamination by *Lactobacillus* sp. in 33 eggs. As no bacteria were discovered in developing oocytes the authors concluded that passive contamination of eggs had probably occurred through the micropyle after eggs had been shed into the coelomic cavity. However, although contaminated eggs were of a normal size, these eggs had remained in the coelomic cavity since first stripping (a period of up to 5 months) and were thus extremely overripe. The nature of these eggs therefore compromises the more widespread value of this report.

Recently, a study by Sauter, Williams, Meyer, Celnik, Banks & Leith (1987) has revealed a wide range of bacteria inside eggs of healthy chinook salmon (*O. tshawytscha*). The authors also considered whether the presence of specific bacteria within eggs could subsequently influence "early life stage death" of incubating eggs and fry. However, although no definite conclusions could be drawn, the following bacterial genera were implicated ; *Vibrio, Listeria, Corynebacterium* and *Staphylococcus.*

All the aforementioned examples show that there is potentially a variety of associations between salmonid eggs and bacteria. The overall purpose of the present study is to examine a number of these associations under carefully controlled conditions. The following investigations therefore begin with a detailed characterization of the egg surface microflora of rainbow trout eggs incubated under conditions relevant to most U.K. hatchery facilities. During initial investigations, rainbow trout eggs were

examined microbiologically at stripping, and during incubation at two different densities under a horizontal flow of water. Eggs were also subjected to a wide range of varying environmental conditions including water temperature and water flow rate. Throughout incubation the effects of these parameters on the developing microflora of the eggs was regularly monitored, leading to the prospect of a general understanding of the "typical" bacterial numbers and genera that might be found colonizing incubating salmonid eggs within the U.K. Subsequently, an analysis of factors influencing the success of selected bacterial species on egg surfaces was undertaken, concentrating on identifying potential nutrient sources for bacteria colonizing egg surfaces. In addition, properties of bacterial adherence that might be important for egg colonization were also considered.

The possible relationships between "premature mortalities" of incubating eggs and their associated bacterial flora was also investigated. Rainbow trout egg mortalities were compared with those of brown trout eggs in conjunction with an assessment of bacteria both from inside eggs and located on egg surfaces. Consequently, bacterial genera implicated as potentially important in influencing egg mortalities were inoculated onto surfaces of newly-fertilized rainbow trout eggs and the subsequent effect on egg hatching success rate closely monitored. Finally, the "normal" bacterial flora of incubating eggs was reduced by prophylactic treatment to determine whether a corresponding increase in egg hatching success rates could be obtained.

CHAPTER TWO

THE SURFACE BACTERIAL FLORA OF INCUBATING RAINBOW TROUT EGGS UNDER VARYING ENVIRONMENTAL CONDITIONS

INTRODUCTION

Artificial rearing of rainbow trout has now been practised for at least half a century and a great deal of advice and information has been published on general aspects of fish farming leading to improvements in both efficiency and productivity. However, the area of hatchery management has often been overlooked and is poorly covered in the literature, with few notable exceptions (Leitritz & Lewis 1976 ; Springate & Bromage 1985). Frequently, fundamental decisions in hatchery management concerning, for example ; egg density and water flow rate, will be decided on a basis of "past experience" or "rule of thumb", leading to differences between farms in the environmental conditions provided for egg incubation. A poor environment could potentially be the cause of many "premature" egg losses and thus, proper "scientific" control of hatchery environmental conditions could prove to be an important factor in maximizing the production of healthy fry.

Bell (1966) completed the first detailed study of egg surface bacteria on stream incubated pink salmon *(Onc orynchus gorbuscha)* eggs and found that compared to glass beads, eggs supported a bacterial flora of different character comprising mainly of *Pseudomonas* sp. and *Flavobacterium* sp. A further study of greater depth (Bell, Hoskins & Hodgkiss 1971) conducted at the same site showed that the state of salmonid eggs could markedly influence composition of egg surface bacterial populations. *Cytophaga* sp. predominated in floras of living eggs while fluorescent *Pseudomonads* formed the bulk of populations associated with dead eggs.

This present study investigates the effects of a variety of carefully controlled environmental conditions on the egg surface microflora of rainbow trout eggs incubated under a horizontal flow of water as commonly occurs in most U.K. hatchery facilities. In the final choice of environmental parameters for investigation particular attention was focused on those environmental factors most likely to prevail in commercial enterprises

where pressures of numbers and time might not allow adequate monitoring and sufficient control of the hatchery environment.

Dissolved oxygen level is important in the successful incubation of salmonid eggs. In turn dissolved oxygen is influenced by a variety of factors which commercial fish farms often fail to control or monitor due to pressures of numbers or inadequate procedures and facilities. Although, certain enterprises expend considerable energy in their efforts to ensure that eggs are evenly distributed, it is not uncommon to observe accumulation of eggs often many layers deep. Close packing of eggs or reduced water flow rates, due to inadequate water supply, may reduce oxygen availability to a level that is insufficient to support egg maturation. Slow flow rates may also be insufficient for removal of toxic waste products associated with egg metabolism (ammonia and carbon dioxide) which if allowed to accumulate would impair egg development. In contrast fast water flow rates will allow greater volumes of water to pass over eggs increasing their oxygen availability and removing waste products. In addition, fast water flow rates are often accompanied by surface disturbance ("bubbling / frothing") which in turn, generates further aeration and subsequently increased levels of available oxygen for incubating eggs.

Another factor influencing availability of dissolved oxygen is water temperature - viewed simply, as water temperature increases levels of dissolved gasses (including oxygen) decrease. Most commercial hatcheries in the U.K. incubate eggs under water taken directly from a spring or borehole, usually at a constant temperature around 8 °C - 10 °C. However, other hatcheries rely on diverted rivers and streams where water temperature will be influenced by prevailing ambient temperatures. Under certain circumstances, such as the rearing of early, late or "out of season eggs" as produced by photoperiodic manipulation of broodstock (Bromage, Elliott, Springate & Whitehead 1984 ; Duston & Bromage 1987) water at temperatures above 10 °C may be common place with correspondingly reduced oxygen availability for developing embryos.

Although, flow rate, water temperature and egg density all have the potential to markedly affect oxygen tension and thereby egg maturation these same parameters may also influence bacterial populations. Fast water flow rates could potentially inhibit initial bacterial colonization, for (depending on the flow rate) water passing over eggs might prevent bacteria from remaining in contact with egg surfaces. In contrast, slow flow rates might permit bacteria to remain in contact with eggs thus facilitating colonization. High water temperature (15 °C) could potentially increase bacterial growth rate encouraging proliferation of numbers and further colonization of egg surfaces. Increased egg density might also encourage enhanced bacterial colonization of egg surfaces by allowing bacteria to move readily from one adjacent egg to another. In contrast, changes in levels of dissolved oxygen with flow rate, temperature and egg density may affect salmonid egg survival but, would be unlikely to greatly influence bacterial numbers. Although, egg surface bacteria such as *Cytophaga* sp. are facultatively anaerobic, *Pseudomonas* sp. are strictly aerobic but will withstand large fluctuations in oxygen availability (Palleroni 1984) and individual cells could certainly tolerate lower oxygen levels than developing salmonid embryos.

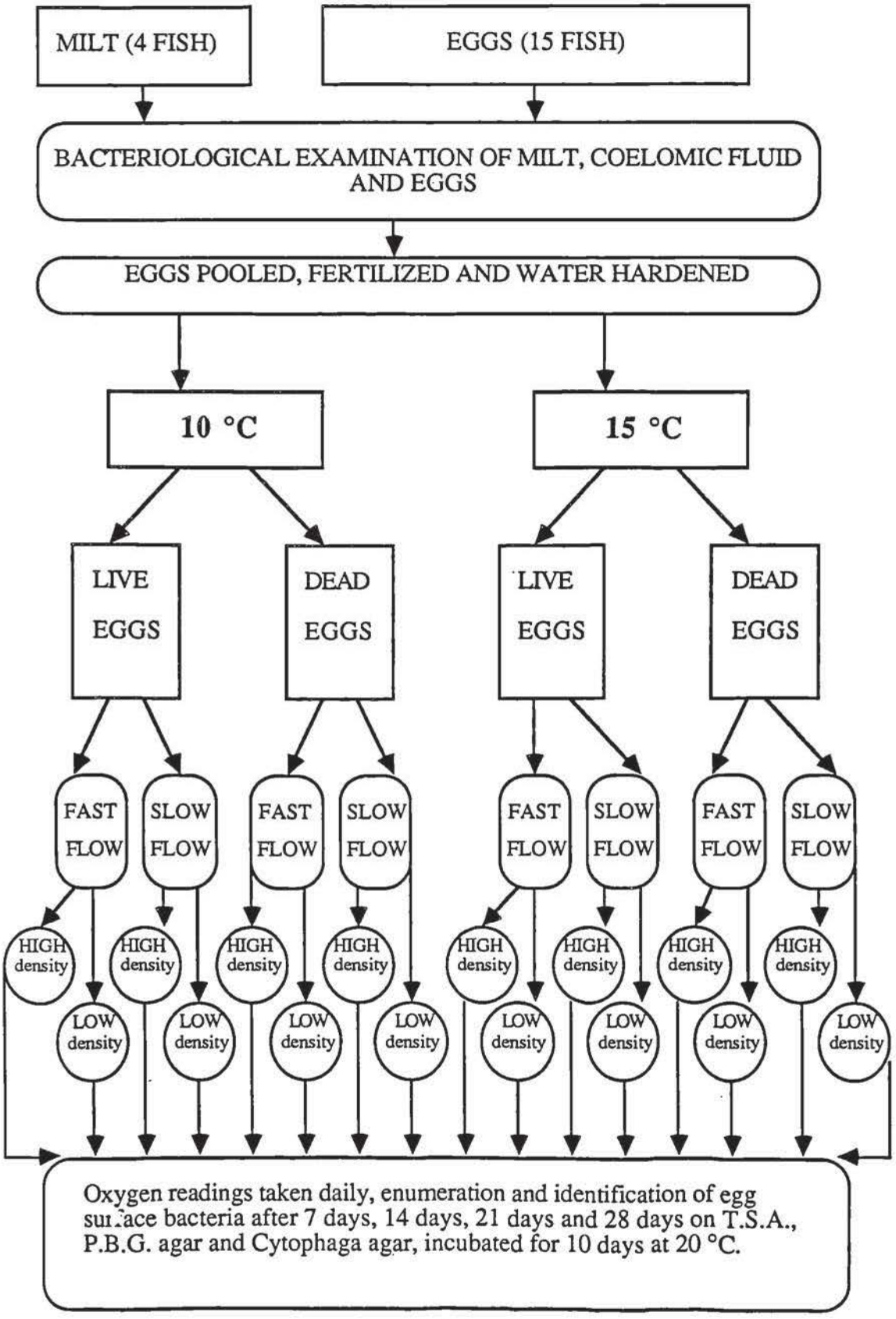
Thus, flow rate, water temperature and egg density, all with the potential to markedly affect levels of dissolved oxygen, were selected as parameters to be manipulated during the following study, and their subsequent effects on the developing egg surface bacterial flora of both live and dead eggs recorded. In addition, oxygen levels in incubation troughs were directly monitored throughout the incubation period.

Various standard techniques exist for the enumeration of bacterial cells and for the quantification of bacterial biomass. Generally, these techniques can be divided into three separate groups ; 1) direct - for example, counting stained bacterial cells (epifluorescence / immunofluorescence), 2) indirect - for example, plate counts, most probable number determinations and 3) chemical methods - for example, lipopolysaccharide (L.P.S.), muramic acid, and adenosine triphosphate (A.T.P.) measurements (Costerton & Geesey 1979 ; Fry 1988). All the above have well documented advantages and disadvantages (Buck 1979) and are intended to suit slightly

differing purposes. In this study a method was required to estimate total numbers of bacteria present on incubating egg surfaces and in addition to provide colonies for subsequent identification. Direct counting is, probably, the most accurate method of obtaining estimates of total bacterial numbers. Bacteria on some surfaces (for example, leaves of aquatic plants) can be counted directly by phase contrast or bright field microscopy. Unfortunately, salmonid eggs do not lend themselves well to this technique as both their round shape and large diameter hinders precise microscope focusing. Ultimately, it was decided that to obtain both an estimation of bacterial number, and colonies for subsequent identification, it would be necessary to use plate count techniques. Limitations and problems with this technique have been well discussed (Buck 1979), even so, it's usefulness in aquatic biology under certain circumstances is still acknowledged in many recent publications (Baxa, Kawai & Kusuda 1987 ; Sako 1988 ; Hirsch & Rades-Rohkohl 1988 ; Austin & Al-Zahrani 1988). However, in order to use plate count techniques effectively, bacteria first had to be dislodged from egg surfaces. A variety of methods were assayed to remove bacteria from egg surfaces. Initially, eggs were macerated and the ensuing homogenate, diluted and plated out. However, no differentiation could then be made between bacteria that were adhered to egg surfaces and any that might have been located inside the egg. Furthermore, homogenization also released large amounts of oils and other materials that subsequently interfered with plating techniques. Thus, it was eventually decided to remove bacteria from egg surfaces using a method adapted from Evelyn, Ketcheson & Prosperi-Porta (1984) (to be described later). Further analysis of this chosen technique suggested that over 80% of all bacteria could be removed from egg surfaces.

A schematic outline of the experimental protocol followed during the trial is summarized in Figure 2 : 1.

FIGURE 2:1 Flow diagram of experimental protocol.



MATERIALS AND METHODS

Experimental design

Incubation systems. Two identical egg incubation systems were constructed in parallel, maintained at 10 °C ± 1°C and 15 °C ± 1°C respectively. Each system comprised of a trough containing two aluminium trays (to support eggs) with a separate water inlet for each tray. One water inlet supplied 1500 l of water / day (slow flow rate) and the second supplied 5800 l / day (fast flow rate) (Figure 2 : 2). Water passed over eggs and was directed through a gravel filter bed (as a biological filter) before being pumped and recycled over eggs. A small volume of fresh "make up" water (approximately 5% per day) was added to allow for spillage and evaporation (Figure 2 : 3).

Collection of eggs and milt

Stripping and egg incubation. Eggs were obtained from 15 ripe rainbow trout from a single commercial fish farm. Eggs of each fish were stripped into individual sterile containers. The first eggs from each fish were discarded to avoid contamination from the surrounding water or ventrolateral surfaces of the female. Milt also collected in sterile containers was obtained from 4 sex-reversed females (i.e. fish with female genotype, masculinized by treatment with male steroids added to feed). Both milt and eggs were immediately put on ice and returned to the laboratory. Samples of eggs, coelomic fluid and milt from each container were removed for bacteriological examination. The remaining eggs were pooled and fertilized. After 5 minutes excess milt was washed off and eggs allowed to water harden for 45 minutes. Eggs were divided into two equal groups. One group of eggs was "shocked" by siphoning from a

FIGURE 2 : 2. Diagram of incubation systems

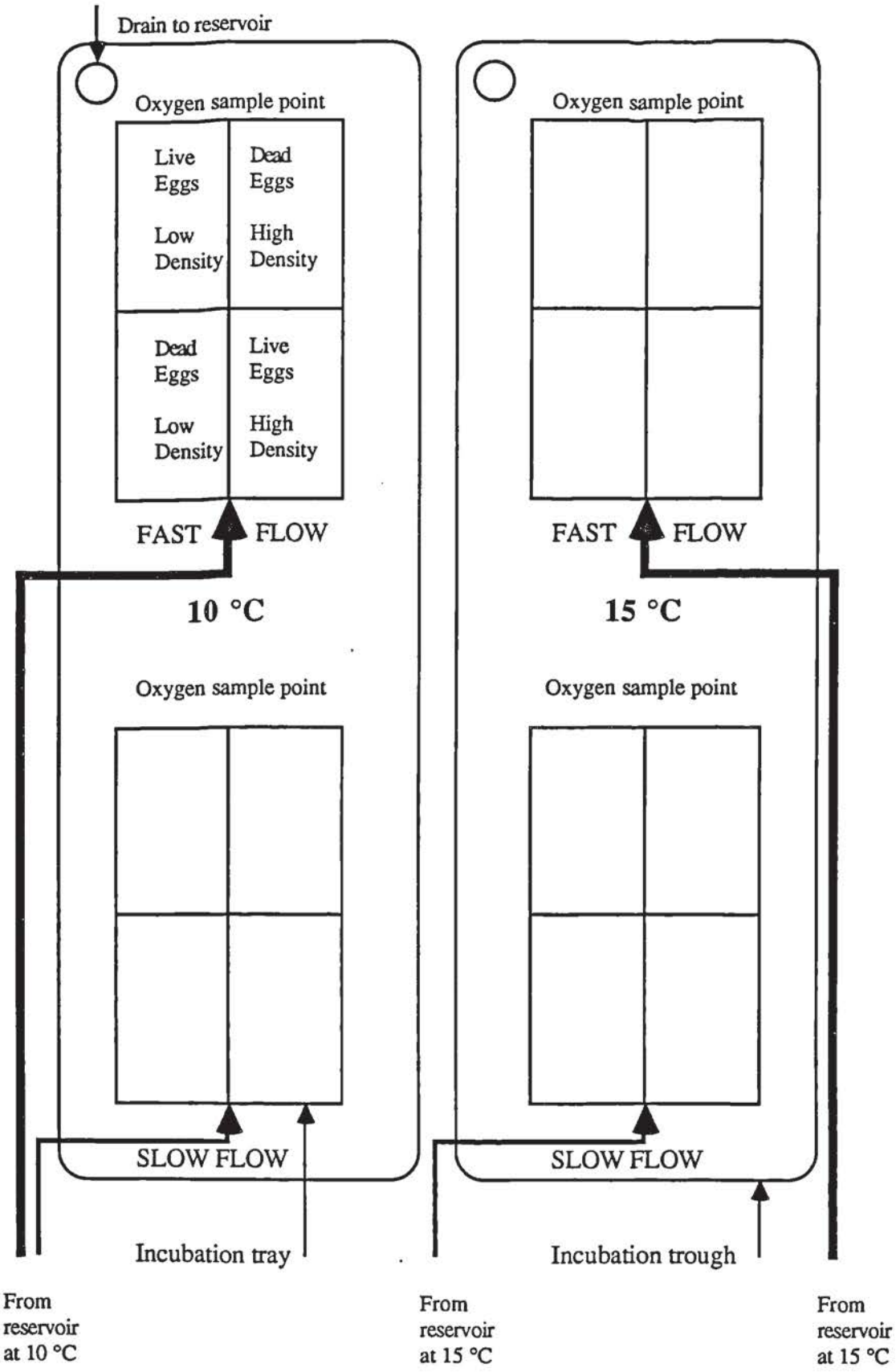
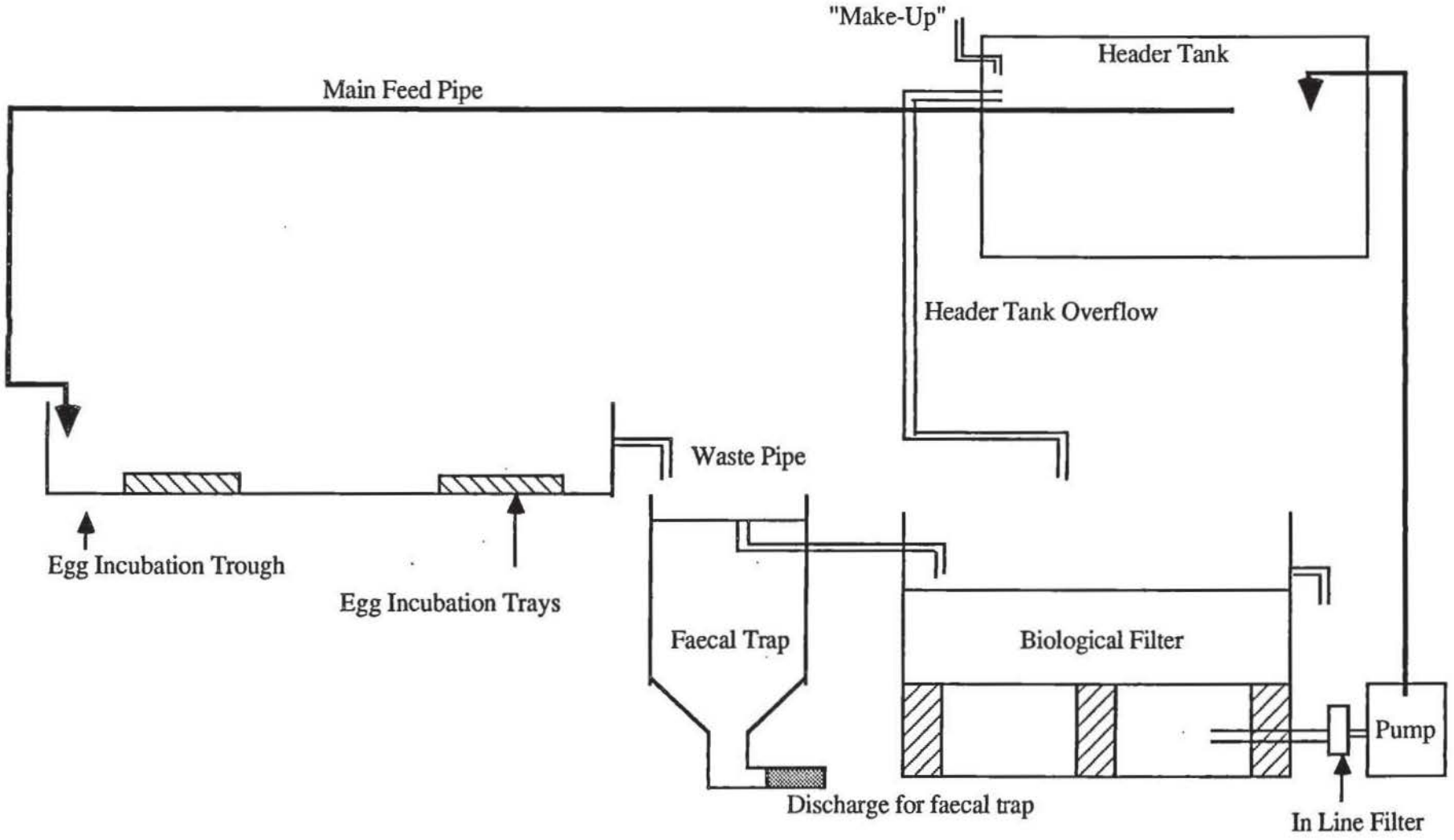


FIGURE 2:3 Pumped recirculatory incubation system



height of 2 m into a sterile beaker. Live eggs and shocked (dead) eggs were subsequently placed into individual aluminium trays at either high or low stocking densities. Low density eggs were defined as a single monolayer of non touching eggs, whereas high density eggs comprised of at least a bilayer of touching eggs.

Eggs were treated three times a day with malachite green oxalate (Difco, Surrey) at a working concentration of 2 ppm. Typically, eggs are only treated once a day with such a concentration of malachite green. However, as dead eggs were deliberately incubated (at high temperature and high density) a far stricter prophylactic regime was considered necessary to control levels of *Saprolegnia* sp.

Bacteriological examination

Coelomic fluid and milt. Aliquots of coelomic fluid and milt were serially diluted and inoculated in 0.02 ml amounts (drop inoculation method) onto : -

1) *Cytophaga* agar (Anacker & Ordal 1959) for enhanced recovery of *Cytophaga* sp.

2) Peptone beef extract glycogen agar (P.B.G. - McCoy & Pilcher 1974) for enhanced recovery of *Aeromonas* sp. and

3) Tryptone soya agar (T.S.A., Oxoid, Basingstoke) to recover as many bacteria as possible (Appendix 2 : 1).

Plates were incubated at 20 °C for up to 10 days and enumeration performed only from drops where total separation of colonies occurred.

Eggs. Eggs were sampled at the green stage (unfertilized) and during incubation after 7 days, 14 days, 21 days and 28 days after fertilization. It was not possible to sample live eggs incubated at 15 °C for 28 days as hatching had already occurred by this stage. For each sampling time / treatment three separate lots of 10 eggs were removed at random from appropriate sections. Samples were rinsed in 4 changes

of sterile water and added to 5 ml of sterile diluent, before shaking vigorously on a vortex stirrer for two minutes, as previously described. Three replicates, a total of 30 eggs were taken at each sampling. The number of viable cells transferred to the 3 different media was determined by plate count. Dividing by 10 gave the average count per egg and by taking into account the dilution factor, the volume of diluent and the egg surface area, the number of cfu's / mm^2 egg surface could be estimated.

Dissolved oxygen. Oxygen levels were measured using an oxygen / temperature meter (pHOX Systems Ltd Shefford, Bedfordshire). Measurements were taken daily at a standard point behind each egg incubation tray, ensuring a comparison between the two water temperatures and the two water flow rates (Figure 2 : 2). At each sampling time the oxygen probe was left to equilibrate for a period of 10 minutes before readings were taken.

Identification of bacteria. Bacteria were isolated by a variety of techniques, procedures and schemes outlined by Krieg and Holt (1984), Cowan (1974), the tables of Allen, Austin & Colwell (1983) and Stanier, Palleroni & Doudoroff (1966). Some Gram negative bacteria, especially *A. hydrophila*, were more easily identified using API 20E and 20NE identification strips (API laboratory products, Basingstoke). However, it was not possible to use the identification strips more frequently as many of the bacteria isolated would not tolerate the relatively high incubation temperatures (37 °C and 30 °C) recommended by the manufacturers of A.P.I. strips.

Statistical analysis. Numbers of colony forming units (cfu's) per mm^2 egg surface were compared at each sampling time (for live and dead eggs separately) using 3 way analysis of variance (Ridgman 1975; Snedecor & Cochran 1980). Differences between levels of dissolved oxygen were analysed using a "t" test.

RESULTS

Coelomic fluid, milt and eggs. Bacteria were isolated from the coelomic fluid of 7 fish during this trial. Genera recovered included ; *Pseudomonas, Aeromonas, Staphylococcus, Corynebacterium* and *Flavobacterium.* A similar range of bacterial genera were recovered from all milt samples but at higher concentrations. Removal of the testis and subsequent handling by farm staff (Woodroffe & Shaw 1974), a process necessary with sex-reversed fish, may have contributed to the greater bacterial loading of milt compared to coelomic fluid. Few bacteria were adhered to green (unfertilized) egg surfaces, although *Staphylococcus epidermis* was isolated in low numbers.

Incubating egg surfaces. Mean cfu's recovered from live egg surfaces are summarized by Figures 2 : 4 to 2 : 7. These figures show that although numbers of cfu's recovered vary markedly with incubation regimes, bacterial populations, especially those of an abundant nature, increase with time before undergoing a decline. Notable exceptions appear to be populations associated with slow flow / high density / 10 °C and slow flow / high density / 15 °C, which respectively either increase gradually or decline gradually with time.

Mean cfu's recovered from live egg surfaces after 7 days incubation are summarized in Figure 2 : 4, from which it can be seen that increased incubation temperature (15 °C compared to 10 °C) in turn gives rise to a very significant increase in numbers of egg surface bacteria ($P < 0.01$). Water flow rate and egg density are also revealed as significant factors in influencing egg surface bacterial numbers ($P < 0.01$) and ($P < 0.05$) respectively (Appendix 2 : 2). Within each specific incubation temperature (10 °C or 15 °C), fewest bacteria were recovered from eggs placed under a fast flow rate and at low density. In contrast, greatest numbers of bacteria were supported by egg surfaces incubated under slow flow rates and at high density. After 14 days incubation (Figure 2 : 5) flow rate is now seen as the most important factor affecting egg surface bacterial populations ($P < 0.05$). In contrast to the 7 day results

FIGURE 2:4 Mean colony forming units recovered from live egg surfaces after 7 days incubation

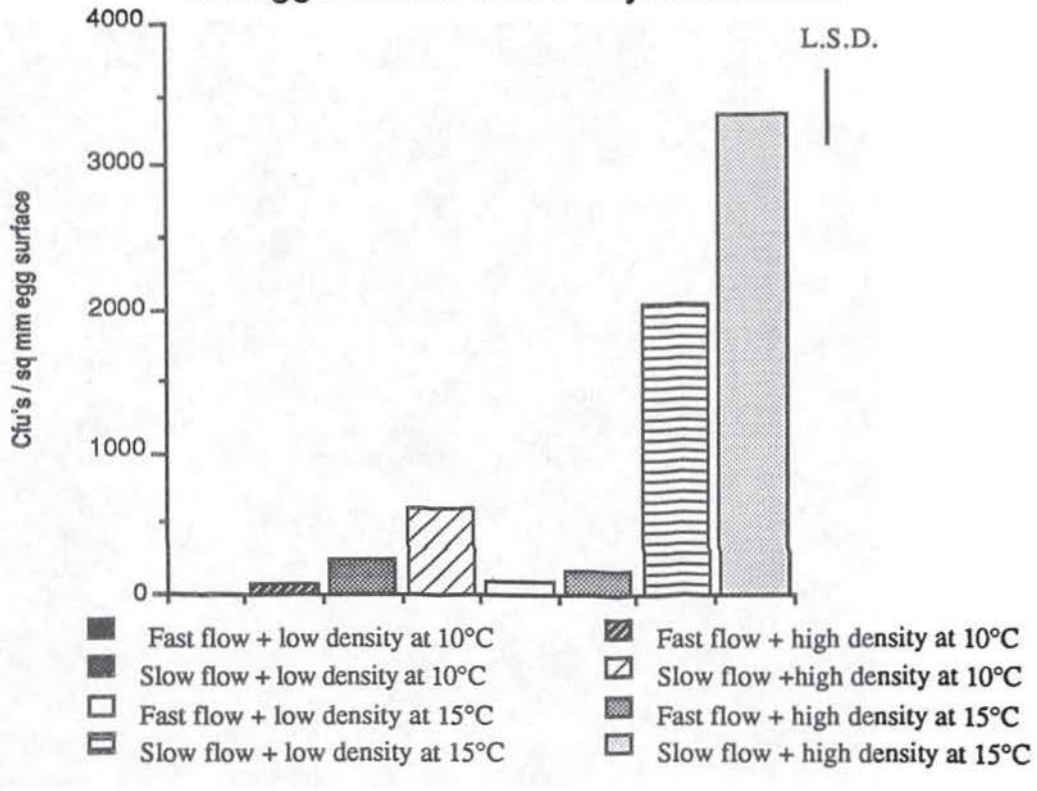


FIGURE 2:5 Mean colony forming units recovered from live egg surfaces after 14 days incubation

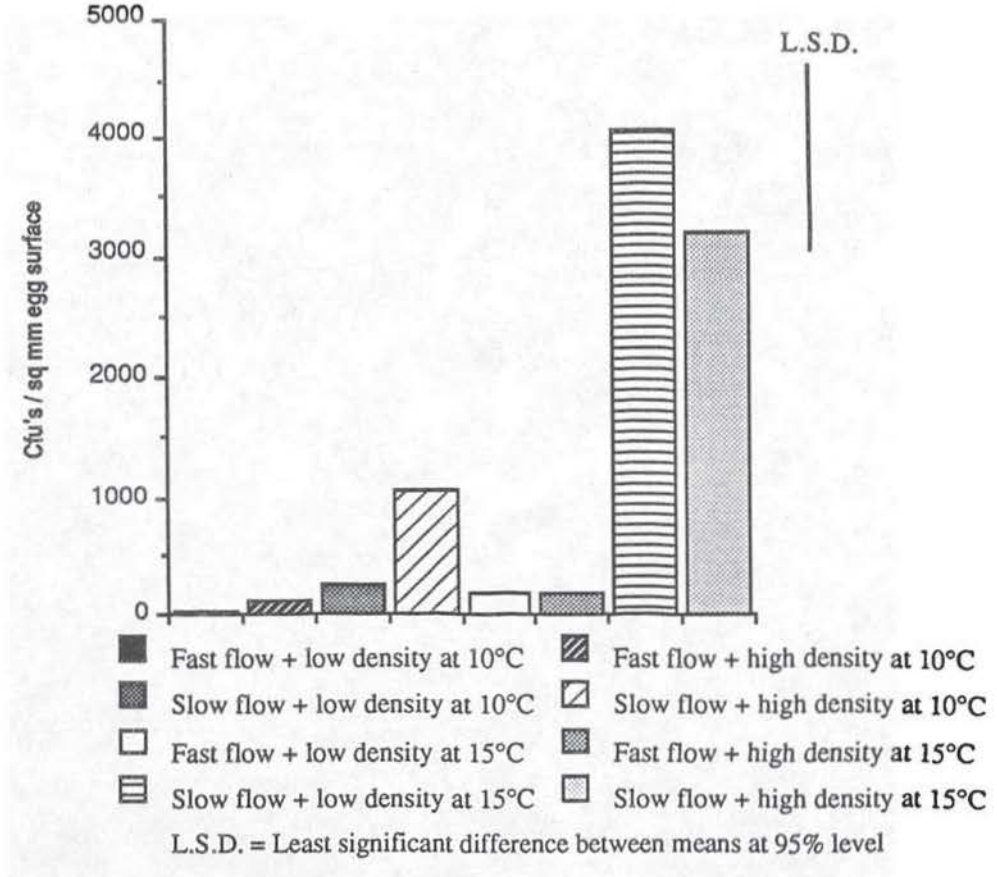


FIGURE 2:6 Mean colony forming units recovered from live egg surfaces after 21 days incubation

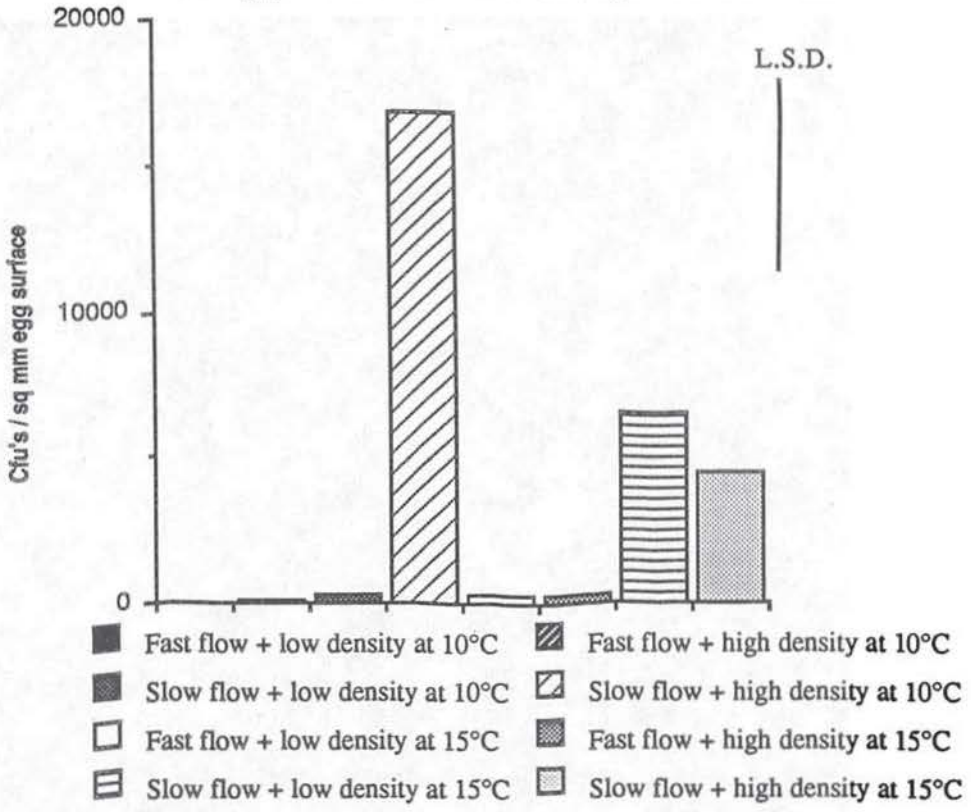
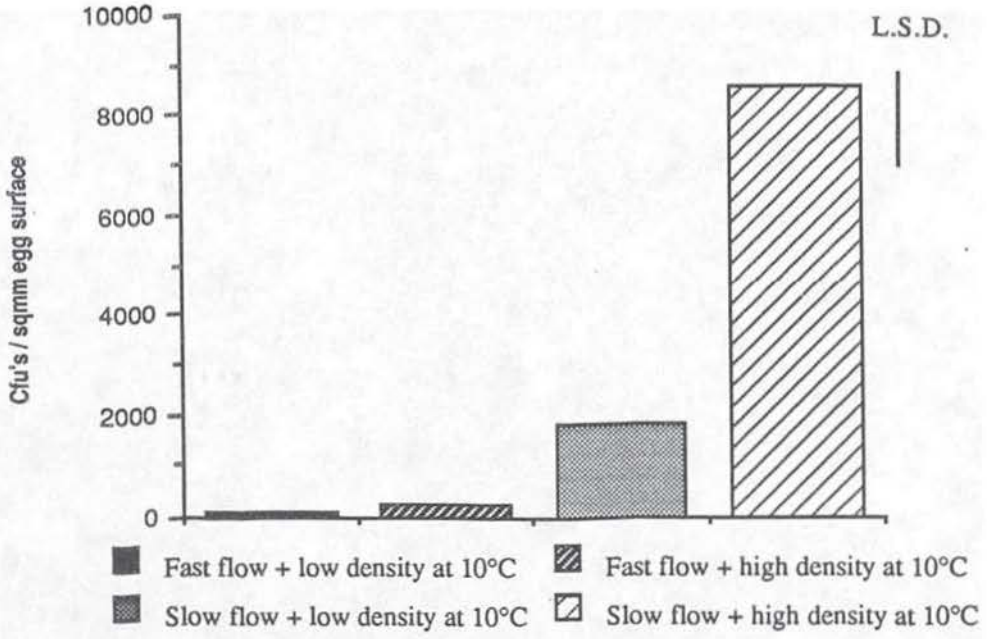


FIGURE 2:7 Mean colony forming units recovered from live egg surfaces after 28 days incubation



Live eggs incubated at 15°C had already hatched by 28 days

L.S.D. = Least significant difference between means at 95% level

FIGURE 2:8 Mean colony forming units recovered from dead egg surfaces after 7 days incubation

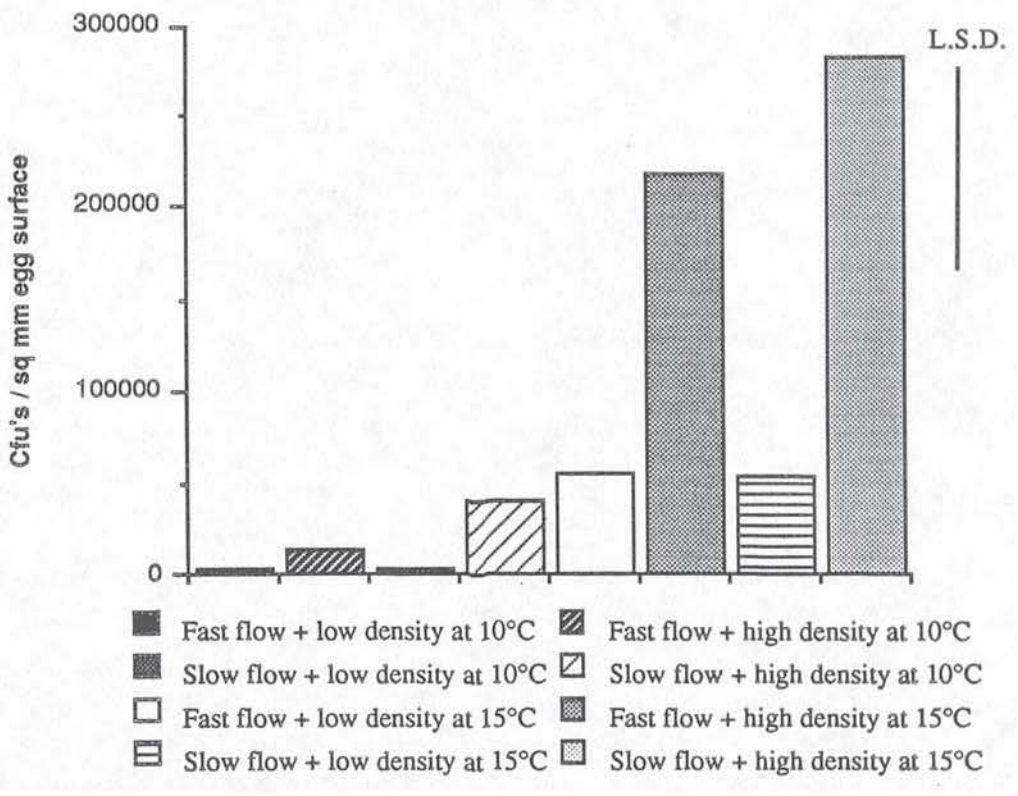
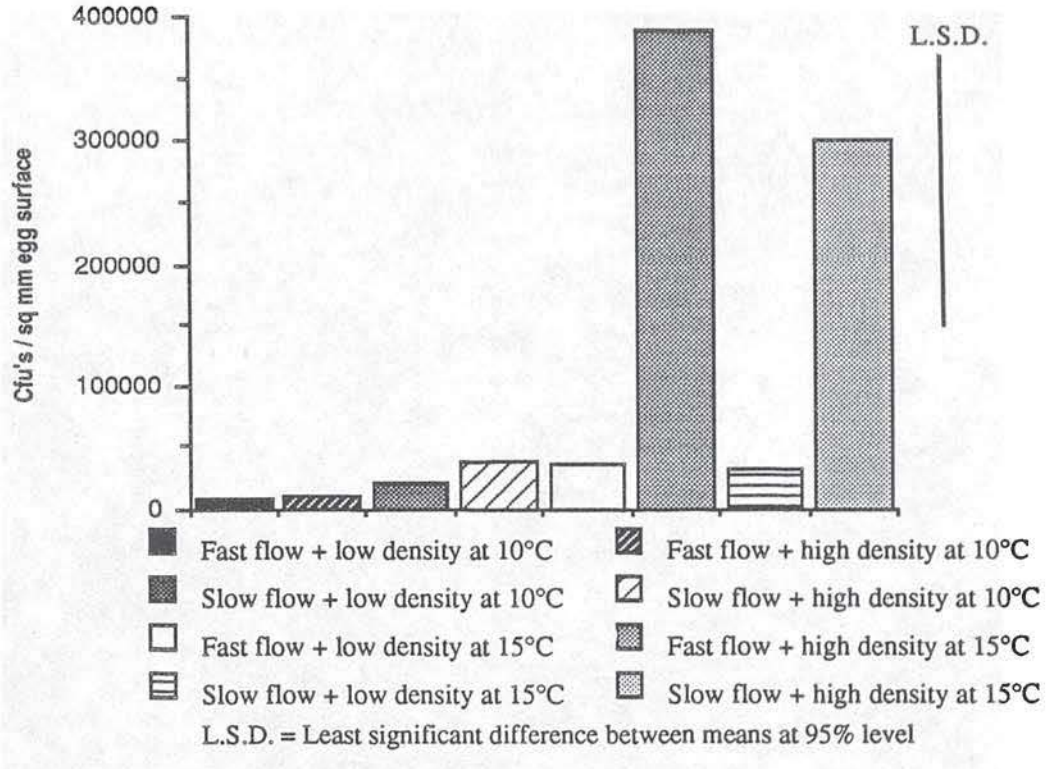


FIGURE 2:9 Mean colony forming units recovered from dead egg surfaces after 14 days incubation



L.S.D. = Least significant difference between means at 95% level

FIGURE 2:10 Mean colony forming units recovered from dead egg surfaces after 21 days incubation

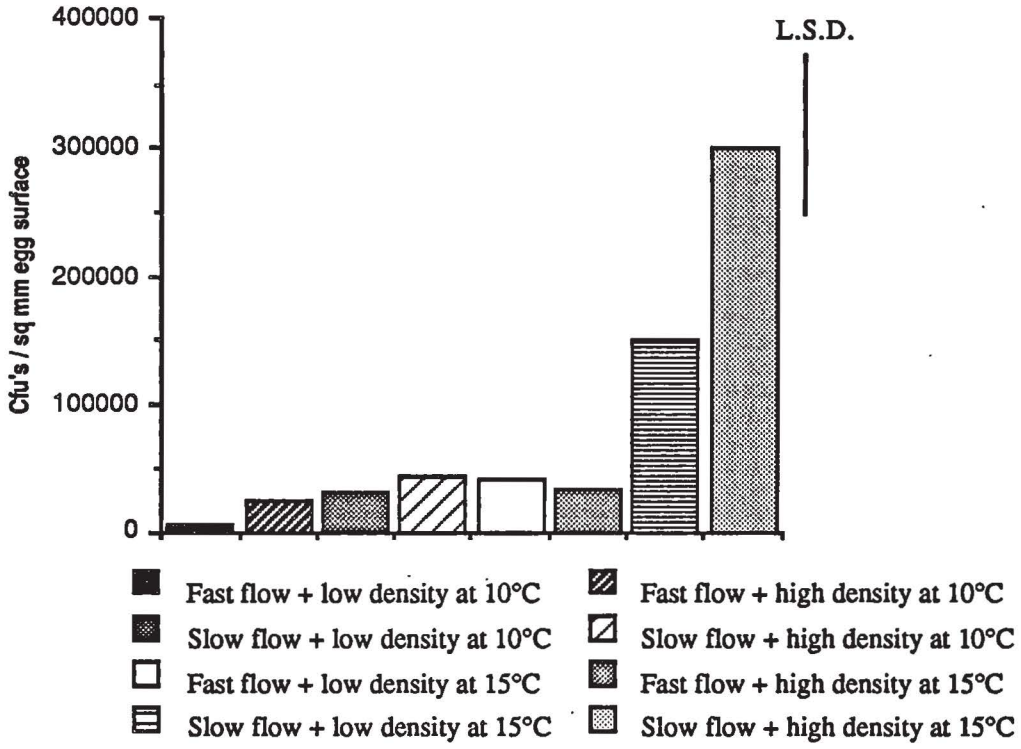
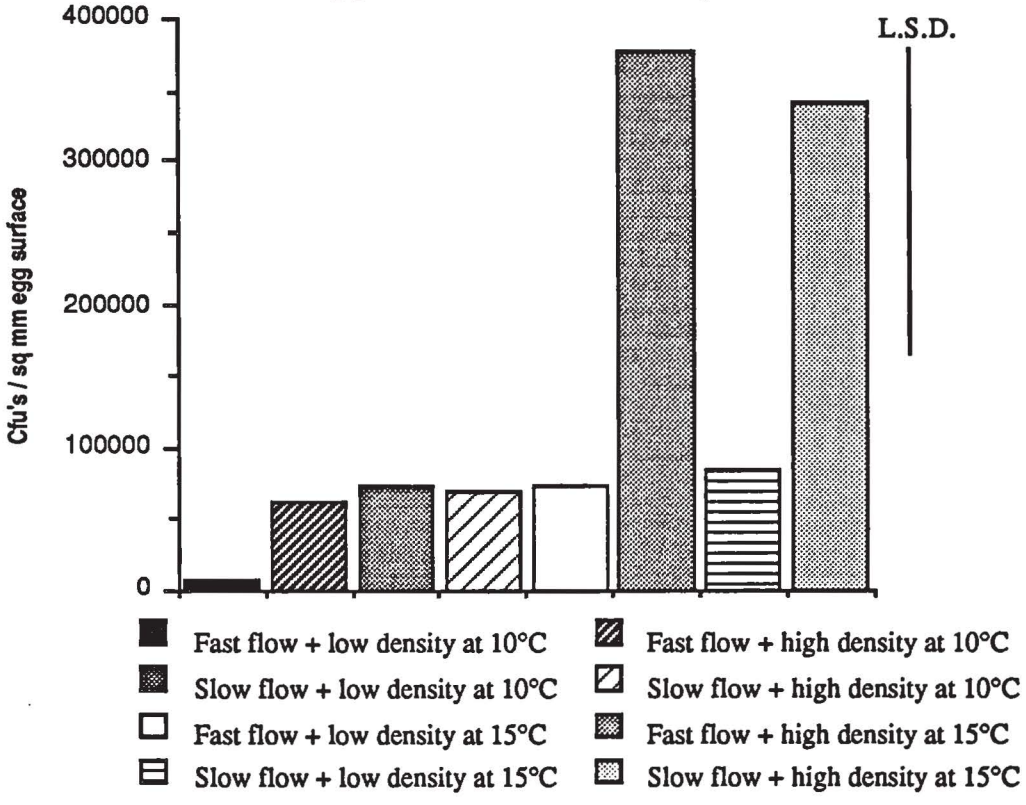


FIGURE 2:11 Mean colony forming units recovered from dead egg surfaces after 28 days incubation



L.S.D. = Least significant difference between means at 95% level

above, egg density and water temperature (alone) are no longer of significance ($P > 0.05$) (Appendix 2 : 3). After 21 days incubation (Figure 2 : 6) flow rate remains a significant factor ($P < 0.05$), but water temperature is not significant ($P > 0.05$), due mainly to the recovery of prodigious number of cfu's from eggs incubated under slow flow / high density / 10 °C. In addition, egg density was not a significant factor in influencing numbers of live egg surface bacteria after 21 days incubation (Appendix 2 : 4). After 28 days incubation it was no longer possible to sample live eggs incubated at 15 °C as hatching had already occurred. However, within the egg groups incubated at 10 °C (Figure 2 : 7), it can be seen that flow rate and egg density are very significant factors ($P < 0.01$) in influencing egg surface bacterial numbers (Appendix 2 : 5).

Mean cfu's recovered from dead egg surfaces are summarized in Figures 2 : 8 to 2 : 11. These figures show that dead eggs in comparison to live eggs supported far higher numbers of bacteria (one or two orders of magnitude higher). In contrast to live eggs, the numbers of bacteria on dead eggs tended to reach a "plateau" after 14 days incubation and did not greatly increase even after 28 days incubation. On live egg surfaces statistical analysis revealed that bacterial numbers were influenced by water flow rate. In contrast overall on dead egg surfaces water flow rate was of less significance in influencing bacterial numbers.

Mean cfu's recovered from dead egg surfaces after 7 days incubation are summarized in Figure 2 : 8, from which it can be seen that in common with live eggs water temperature was a significant factor ($P < 0.05$) in influencing numbers of egg surface bacterial populations. Similarly, egg density also proved to be a factor of significance ($P < 0.05$) but, in contrast to live eggs, flow rate was found to be unimportant ($P > 0.05$) (Appendix 2 : 6). Furthermore, at both temperatures (10 °C and 15 °C), eggs incubated under a fast flow and at high density supported greater numbers of bacteria than those under a slow flow rate and at low density. After 14 days incubation (Figure 2 : 9) no factors significantly influenced dead egg surface bacterial numbers (Appendix 2 : 7). After 21 days and 28 days overall "trends" of factors

FIGURE 2:12 Combined percentage species of bacteria present on live egg surfaces under fast + slow flow rate / 10 °C + 15 °C / high + low density

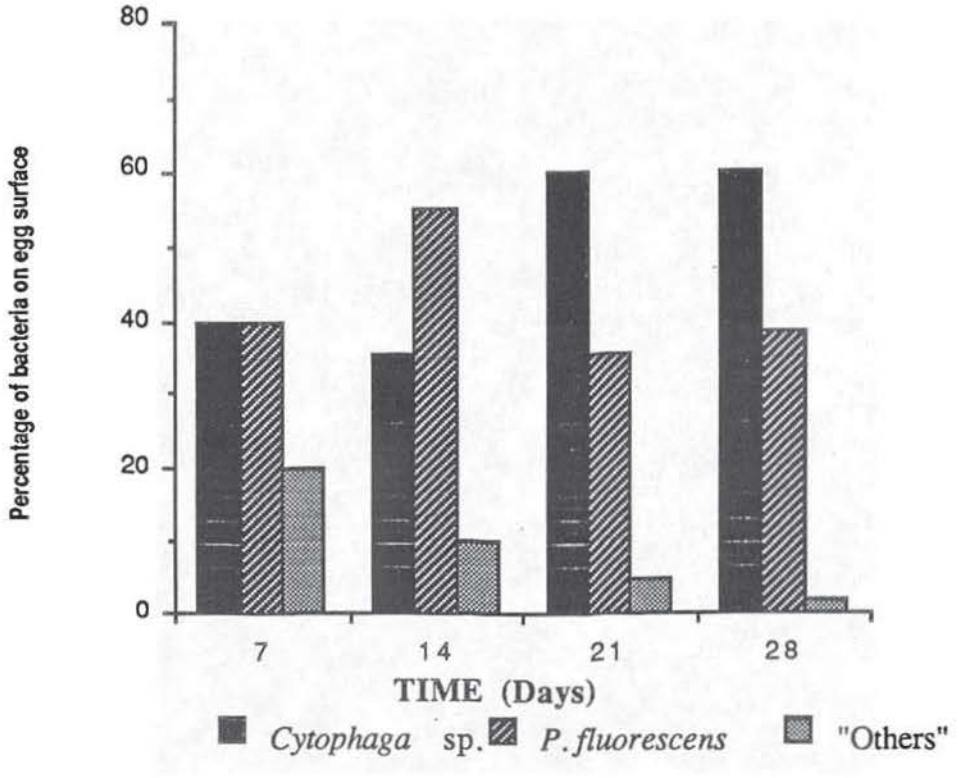


FIGURE 2:13 Combined percentage species of bacteria present on dead egg surfaces under fast + slow flow rate / 10 °C + 15 °C / high + low density

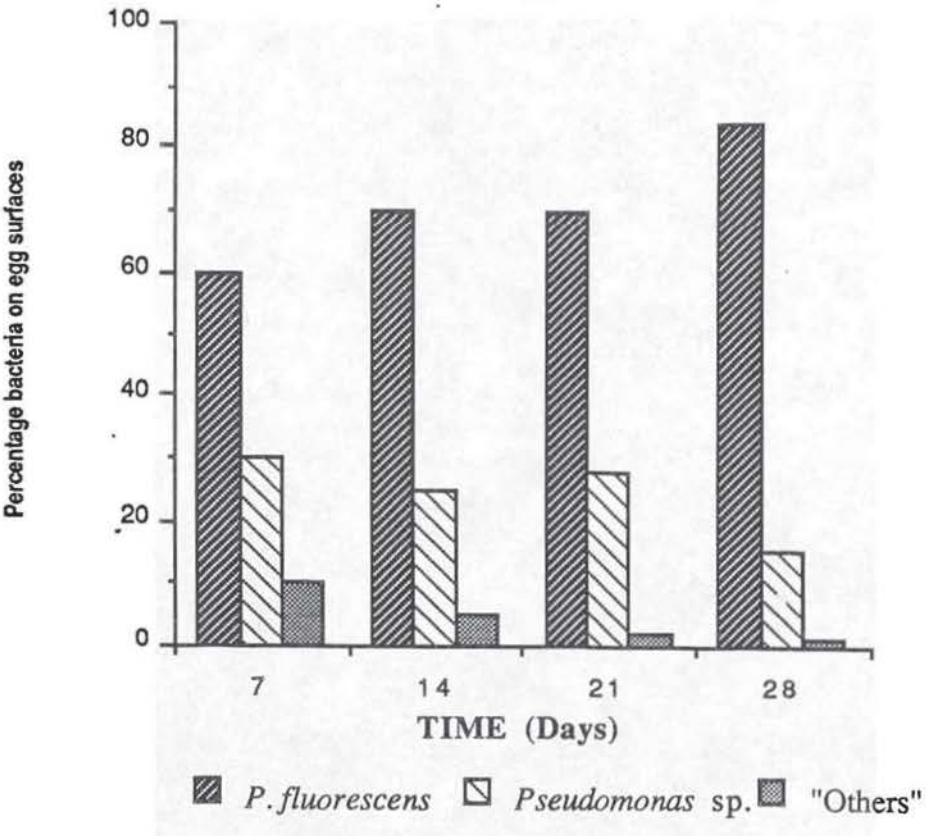


TABLE 2 : 1 Daily dissolved oxygen figures (parts per million)

	10 °C		15 °C	
Days	Fast Flow	Slow Flow	Fast Flow	Slow Flow
1	8.86	8.06	7.22	7.05
2	9.05	7.88	7.61	7.03
3	9.05	8.26	6.93	7.30
4	9.00	8.02	7.17	7.00
5	8.99	8.01	7.06	6.64
6	9.24	7.53	5.66	6.10
7	7.75	7.05	6.60	4.27
8	8.01	7.32	6.10	5.75
9	8.37	7.15	6.04	4.01
10	8.76	7.89	6.75	5.89
11	8.82	7.95	6.45	6.00
12	8.42	7.35	5.84	6.55
13	8.04	6.68	5.96	5.97
14	8.12	6.83	5.02	5.17
15	8.12	7.34	6.19	5.69
16	7.74	6.35	5.32	5.22
17	7.91	7.04	6.00	5.45
18	8.04	7.63	6.06	5.56
19	8.13	6.02	6.10	5.78
20	8.38	7.49	6.14	6.38
21	8.00	6.64	6.00	5.49
22	8.32	7.34	5.80	4.32
23	8.02	6.56	Commencement of hatching	
24	7.67	6.82		
25	8.45	7.58		
26	8.92	8.20		
27	8.56	8.14		
28	8.67	8.19		
29	Commencement of hatching			

influencing egg surface bacterial numbers are hard to differentiate. After 21 days incubation (Figure 2 : 10), flow rate remains of little significance, but in contrast, to results obtained after 14 days incubation (above), water temperature and egg density were found to be significant and ($P < 0.05$ and $P < 0.05$) respectively (Appendix 2 : 8). Finally, after 28 days incubation (Figure 2 : 11) water temperature, flow rate and egg density were found to be significant factors ($P < 0.05$) (Appendix 2 : 9).

An analysis of bacterial species dominating live and dead egg surfaces is summarized in Figures 2 : 12 and 2 : 13. from Figure 2 : 12 it can be seen that *Cytophaga* sp. (Appendix 2 : 10) tended to predominate on live egg surfaces throughout incubation. In addition, *Pseudomonas fluorescens* (Appendix 2 : 11) was also frequently isolated and on occasions exceeded numbers of *Cytophaga* sp. Other bacteria isolated included *Aeromonas hydrophila* (Appendix 2 : 12) and *Pseudomonas* sp. (Appendix 2 : 13). Environmental parameters, in particular egg density and water flow rate, did not seem to markedly affect species of bacteria isolated, although *Cytophaga* sp. may have occurred in slightly higher numbers (compared to *P. fluorescens*) on surfaces of eggs at high density. At higher water temperatures (15 °C) *P. fluorescens* was seen to frequently rival the numbers of *Cytophaga* sp. isolated from live egg surfaces. In comparison, dead eggs were almost exclusively dominated by *P. fluorescens* and other *Pseudomonas* sp. and environmental parameters (temperature, flow rate, egg density) were of little importance in influencing bacterial genera isolated from dead egg surfaces.

Dissolved oxygen. Oxygen levels recorded throughout the trial are summarized in Table 2 : 1, from which it can be seen that marked differences occurred in dissolved oxygen levels between the four sample positions. Higher levels of dissolved oxygen were recorded at water temperatures of 10 °C compared to 15 °C. In addition, fast water flow rates contained highly significantly greater levels of dissolved oxygen compared to water at slower flow rates at 10 °C ($P < 0.001$), and significantly greater levels at 15 °C ($P < 0.05$).

DISCUSSION

Coelomic fluid obtained with eggs from 7 out of 15 fish utilized during this trial was found to contain bacteria in the range of 10^1 to 10^2 cfu's / ml. Few other reports exist in the scientific literature concerning the bacterial content of coelomic fluid taken from healthy fish, although Sauter, Williams, Meyer, Celnik, Banks & Leith (1987) isolated *Aeromonas hydrophila* and *Corynebacterium* sp. from 5 out of 30 samples of coelomic fluid taken from healthy chinook salmon, *(Oncorhynchus tshawytscha)* Walbaum. In contrast, Evelyn, Prosperi-Porta & Ketcheson (1981) reported that the coelomic fluid of a diseased coho salmon *(O. kisutch)* suffering from bacterial kidney disease (B.K.D.) contained up to 8.0×10^9 cells / ml of *Renibacterium salmoninarum.* Thus, although under half of the fish in this trial revealed signs of low bacterial contamination in their coelomic fluid, the remainder were sterile and it would seem that under normal healthy conditions bacteria are suppressed. Cone (1982) reported the existence of macrophages in the coelomic fluid of rainbow trout, which may in turn be responsible for maintaining the sterility of coelomic fluid, or at least limiting numbers of contaminating bacteria. Even so, results obtained suggest that potentially eggs may be exposed to bacteria from an early stage of development, even within the coelomic cavity and correspondingly a few green (unfertilized) eggs were found to possess bacterial cells *(Staphylococcus epidermis)* adhering to their surfaces. However, as few eggs were contaminated and in such low numbers, it is possible that their presence may only have been the consequence of contamination through contact with the urogenital papilla at stripping or possibly from handling of broodstock by farm staff (Woodroffe & Shaw 1974). Presence of bacteria within milt also suggests that bacterial contamination of eggs could occur at fertilization. However, at the time of egg "laying down", although egg surfaces may not be completely sterile, few bacteria would have been present. During incubation considerable numbers of bacteria were found to accumulate around surfaces of all egg groups. Initially, a few bacteria might

have been added to the recirculatory incubation system along with fertilized eggs but, it is more likely that most bacteria subsequently found contaminating egg surfaces were already present in the hatchery water. Indeed *Cytophaga* sp. were not isolated from coelomic fluid, green eggs or milt but, were frequently found on the surfaces of incubating live eggs, indicative of their presence in the hatchery water prior to the addition of eggs.

Initial bacterial colonization of egg surfaces could have occurred through chemotaxis. In particular dead salmonid eggs have been shown to l ose amino acids, phosphate and potassium (Smith, Armstrong, Springate & Barker 1985), all agents which have the potential to attract microbes. However, in practice under relatively fast flow rates the effectiveness of such a response may be diminished. In addition, bacterial motility may also be important for early egg colonization, for both *P. fluorescens* and *A. hydrophila* are motile, in contrast though, *Cytophaga* sp. only exhibits "gliding motility" (Strohl & Tait 1978) yet was still successful in colonizing live egg surfaces. Initial egg colonization was therefore probably fortuitous, the result of bacteria being swept onto egg surfaces by the flow of water. Once in contact with egg surfaces, bacteria may go through a two stage adhesion process (to be fully described in Chapter 4). In brief, on first contact with a surface bacteria will pass through an instantaneous "reversible" adherence phase, which is dependant on non-specific electrical forces (Marshall, Stout & Mitchell 1971). Generally, all cells carry a net negative electrical charge and tend to repel each other by the electrostatic force. However, electrodynamic or Van der Waals forces between cells are attractive and tend to operate over a greater range than the repulsive electrostatic forces, allowing cells to remain in close contact (Bell 1978). Bacteria remain in contact near a surface under weak attraction for a period of a few hours and then enter a second adherence phase of - "irreversible adherence", achieved by the production of fine extracellular polymer fibrils, glycocalyx fibres, pili or spinae allowing firmer anchorage to the chosen substrate (Marshall 1976). Once established on the surface, aquatic bacteria frequently produce large amounts of exopolysaccharide, termed the glycocalyx (Costerton, Irvin

& Cheng 1981), within which bacterial replication can occur to form an adherent microcolony (Costerton, Geesey & Cheng 1978). Subsequently, cell division within the microcolonies and additional bacteria from the water lead to the production of a "biofilm" overlaying the surface (Costerton, Cheng, Geesey, Ladd, Curtis-Nickel, Dasgupta & Marrie 1987).

In the present trial water flow rates were found to markedly influence numbers of live egg surface colonizing bacteria during the early part of incubation. Flow rate may be an important factor in influencing initial "reversible" bacterial adherence to egg surfaces, for the physical movement of water flowing over eggs may be sufficient to dislodge bacteria held in place solely by a weak electrical attraction. Indeed, under slower flow rates bacteria were recovered from egg surfaces in markedly higher numbers than from eggs maintained under fast flow rates, indicative of more suitable surface conditions for bacterial colonization, growth and proliferation.

Bacteria were also recovered in greater numbers from surfaces of eggs maintained at high density levels compared to low density levels. Poor water circulation (and thus reduced water flow rates) amongst "egg piles" may facilitate "initial reversible" bacterial adherence (for reasons described above). In addition, under conditions of high egg density, bacteria washed from one egg surface are likely to be carried by the flow of water onto another nearby egg surface. Thus "chance" bacterial spread from one egg to the next is likely to be encouraged when egg density is high, compared to when egg density is low and adjacent egg surfaces are further apart.

Frequently, the water supply for salmonid hatcheries is taken from a spring or borehole, guaranteeing both constant temperatures (8 °C - 10 °C) and an absence of fish pathogens (Stevenson 1980). However, such waters contain few dissolved nutrients and in turn only support limited microbial populations (Fletcher 1979), and thus, in most natural waters bacteria are thought to exist under starving conditions (Jannasch 1969). Willoughby, McGrory & Pickering (1983) demonstrated that in hatchery waters sufficient nutrients exist to support limited microbial growth however, attachment and colonization of egg surfaces by bacteria provides them with a micro-environment of

comparative nutrient abundance compared to the surrounding water. Incubating egg surfaces provide an interface to which both organic and inorganic nutrients will be attracted by non-specific electrical forces, similar to those responsible for the initial attraction of bacteria (Costerton *et al* 1981) in turn potentially encouraging greater bacterial growth and proliferation. Furthermore, the development of a "biofilm" (as described) also leads to enriched recovery of nutrients by "nutrient trapping", whereby nutrients become bound to the biofilm matrix and are broken down for use by the component organisms (Costerton *et al* 1987).

Potentially, bacterial adherence to organic (rather than inert) surfaces will also allow additional nutrients to be obtained by utilization of waste products produced by those surfaces, and also from actual digestion of the substrate (Costerton *et al* 1987). However, rainbow trout embryos are extremely efficient at using nutrients available to them and only relatively small quantities of carbon dioxide and ammonia are excreted (Hayes 1949 ; Smith 1947, 1957). Thus, it is unlikely that bacteria on live egg surfaces would greatly benefit from the utilization of waste products derived from developing embryos. Furthermore, it is also unlikely that bacteria on live egg surfaces would obtain nutrients from egg digestion, for trout embryos are protected by a tough ichthulokeratin outer membrane (Young & Smith 1956 ; Bell, Hoskins & Bagshaw 1969), notoriously resistant to enzymatic degradation. In contrast dead egg surfaces support far higher numbers of bacteria than live eggs under identical conditions of incubation, in turn suggesting a far greater availability of nutrients. Increased nutrient availability could be due to the loss of nutrients through the egg shell (Smith *et al* 1985) or colonization by moulds such as *Saprolegnia* sp., degrading the egg, and allowing bacterial access to further nutrients. Water temperature is probably also responsible for increased nutrient availability. As trout embryos are poikilothermic incubation of live eggs at 15 °C compared to 10 °C will increase respiration rates, leading to increased levels of waste by-products over a given period and potentially higher bacterial numbers. Additionally, trout eggs are less well suited to higher incubation temperatures, under such conditions stressed eggs may lo se more nutrient

material leading to still further bacterial proliferation. On dead egg surfaces nutrient availability is potentially less of a critical factor as fewer bacteria were isolated from dead eggs maintained at 10 °C compared to 15 °C. Although temperature may influence rates of nutrient loss from dead eggs the greater numbers of bacteria associated with the higher temperature is probably the consequence of increased bacterial growth rates, in turn a function of increased velocity in cell growth (Jawetz, Melnick & Adelberg 1984). Higher water temperatures would also have further encouraged the growth of moulds such as *Saprolegnia* sp. and thus caused increased dead egg degradation and the availability of further nutrients for bacteria (as discussed).

In this trial, bacteria on dead egg surfaces reached a maximum approaching 400,000 cells / mm^2, on eggs with a mean surface area of 61.9 mm^2. Theoretically, 8 X 10^7 bacterial cells (measuring 1.5 μm X 0.5 μm) would be needed to cover the entire egg surface. In reality, the highest recorded count of 400,000 cells / mm^2 represents a covering of only 30% of the egg surface. However, as egg surface bacterial numbers were calculated using plate count techniques, it is likely that in reality a greater percentage of the egg surface was covered. Even so, although plate count techniques represent only a minimum estimate of the total microflora, such techniques clearly demonstrate significant differences in egg surface bacterial numbers between all egg groups throughout incubation.

Trust (1972) likened the accumulation of bacteria around egg surfaces to the rhizosphere (a region of increased bacterial activity) found in the soil close to leguminous plant roots (Starkey 1929a ; 1929b). Certain similarities do exist, both the rhizosphere and the egg surface are colonized by specific bacterial populations (live egg surfaces mainly *Cytophaga* sp. and *P. fluorescens* ; dead egg surfaces, *P. fluorescens* and *Pseudomonas* sp.). However, major differences also occur for the rhizosphere has been shown to extend many mm's away from the root, to be colonized by a wide range of bacteria and fungi and to involve many root exudates including, carbohydrates, amino acids, nucleotides, flavonones and enzymes (Starkey 1958 ; Parkinson 1967). In contrast in an aquatic environment any gradient of nutrients extending from eggs would

be restricted and readily diluted by currents, colonization is by a limited range of bacteria (mainly *Cytophaga* sp., *P. fluorescens*, *A. hydrophila* and *Pseudomonas* sp.) and unlike plant root exudates, eggs may only release small amounts of ammonia and carbon dioxide (Hayes 1949 ; Smith 1947 ; 1957). Thus, the microbial population of the rhizosphere is stimulated by nutrients exuded from the root, whereas bacterial growth on live egg surfaces is mainly stimulated by increased nutrient concentration in turn accumulated by passive adsorption to surfaces.

Full oxygen saturation of water at 10 °C is 11.10 ppm and at 15 °C is 9.96 ppm (Stevenson 1980). Oxygen levels recorded during this trial revealed that after passing over eggs, oxygen levels were always below these potential maximum figures. As could be expected oxygen levels were always greater at 10 °C and within each temperature (10 °C and 15 °C) water at faster flow rates (compared to slow flow rates) also carried significantly more oxygen. At 15 °C and under a slow water flow rate oxygen levels fell on one occasion to only 4.01 ppm, even so, sufficient oxygen should still have been available for developing embryos, although at high egg density it is possible that poor water circulation could have led to critically low levels. Trust (1972) calculated that if all the bacteria on an egg surface were *Pseudomonads* (dry weight of 10^{-11} g / cell, Brock 1966) then the oxygen demand by bacteria alone on an egg surface could be as much as 15 µl / hour. In comparison, incubating salmon eggs require an estimated 0.2 µl per egg per hour after fertilization rising to 3.4 µl per egg per hour at hatching (Blaxter 1969). Thus, when saturated levels of oxygen are low (higher water temperatures, slow flow rates and high egg density) it is possible that bacterial consumption of oxygen could cause local areas of oxygen shortage in turn causing potential mortality amongst incubating salmonid eggs.

SUMMARY

Bacteria were frequently recovered from samples of both coelomic fluid and milt. In contrast, few bacterial cells were isolated from surfaces of newly stripped eggs. However, during egg incubation considerable numbers of bacteria were found to accumulate around egg surfaces. Furthermore, slow water flow rates, increased egg density and higher water temperatures were all revealed as factors significantly influencing egg surface bacterial populations. Potentially, large numbers of egg surface bacteria coupled with reduced water circulation might in turn lead to insufficient amounts of dissolved oxygen to support further egg development.

CHAPTER THREE

GROWTH RATES AND POTENTIAL NUTRIENT SOURCES FOR BACTERIA ON INCUBATING EGG SURFACES

INTRODUCTION

In common with all living organisms bacteria require certain nutrients for growth. These must provide the elements necessary for increase in cell mass, enzyme activity and transport systems. In addition, nutrients must also provide the organism with a utilizable energy source. All organisms require a carbon source in order to synthesize a wide range of organic compounds that "make-up" the protoplasm. Furthermore, all organisms require a nitrogen source, as many cell constituents (principally the proteins) contain nitrogen. A number of other minerals are also required for growth, for example ; sulphur (like nitrogen) is important in protein structure, phosphorus (assimilated as free inorganic phosphate) is an essential component of adenosine triphosphate (A.T.P.), nucleic acids and many coenzymes. Magnesium (Mg^{2+}) and potassium (K^{+}) are essential for ribosome structure and function. In addition, many other minerals are also frequently essential for cell growth ; calcium (Ca^{2+}), iron (Fe^{2+}), manganese (Mn^{2+}), molybdenum (Mo^{2+}), cobalt (Co^{2+}), copper (Cu^{2+}) and zinc (Zn^{2+}).

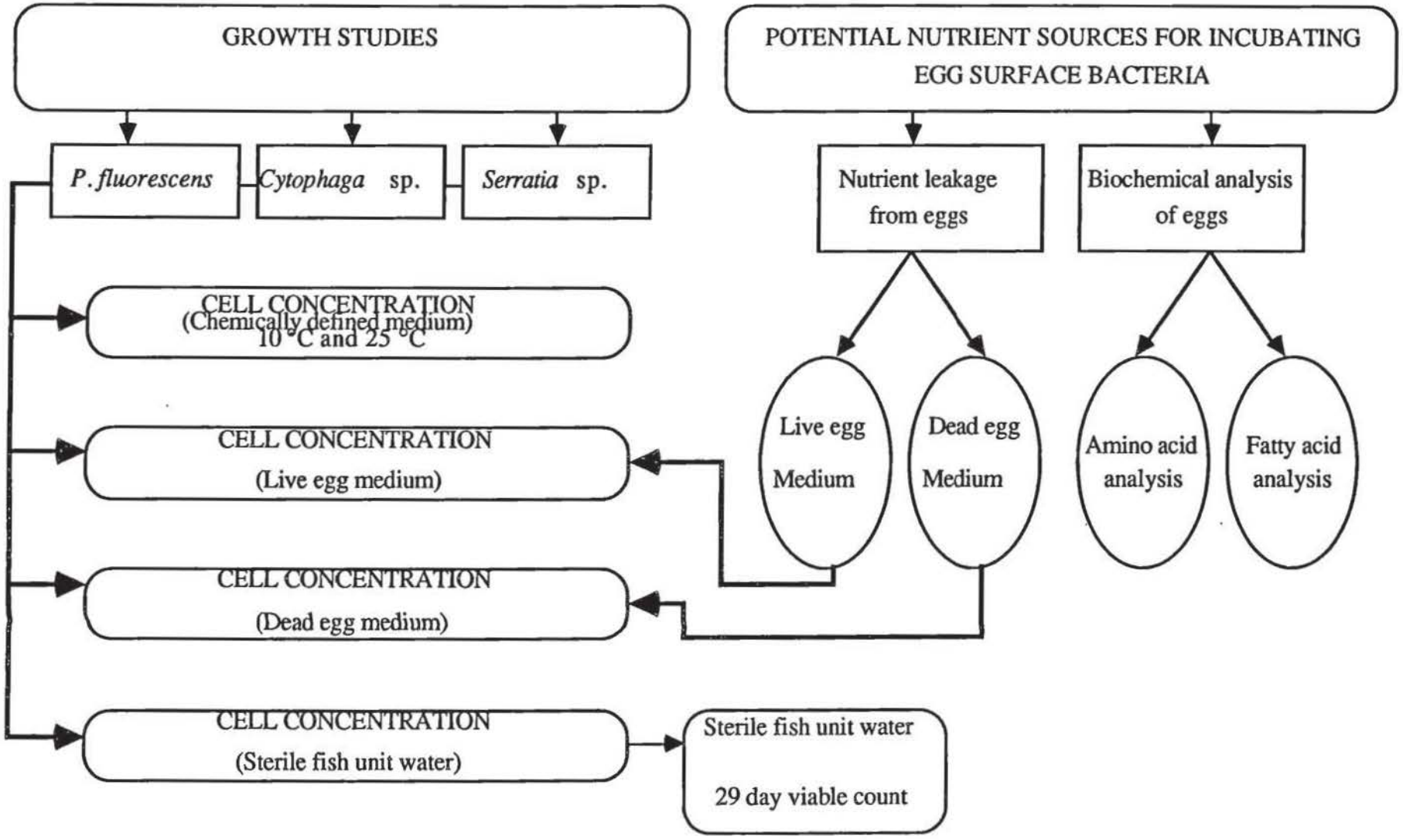
The most suitable water supply for salmonid hatcheries is considered to be from a spring or borehole, guaranteeing constant temperatures (8 °C - 10 °C) and an absence of fish pathogens (Stevenson 1980). Both spring and borehole water (ground waters) collect in permeable rocks which lie below the natural water table, a consequence of water percolating through the overlying soil (termed vadose water). Many dissolved nutrients in such water are filtered out during the process of percolation and thus ground water can only support a limited microbial population (Fletcher 1979). Furthermore, bacteria in such waters are considered to exist under conditions of starvation, with growth limited primarily by low concentrations of a suitable carbon source (Jannasch 1969). Even so, sufficient nutrients may exist to support continuous bacterial growth at extremely low rates or alternatively, bacteria may exist in resting or dormant stages (Stevenson 1978). However, typically most bacteria in natural aquatic

environments do not occur "freely suspended" but attached to surfaces. Surfaces in turn provide an interface to which any nutrients present in water (both organic and inorganic) will be attracted by non specific electrical forces (Marshall, Stout & Mitchell 1971). Zobell & Anderson (1936) first demonstrated that microbial growth on surfaces was probably the result of increased nutrient concentration. Furthermore, bacteria attached to organic substrates may potentially derive nutrients from the substrate, either directly through enzymatic digestion or from utilization of waste products (e.g. ammonia and carbon dioxide) produced by the substrate.

Bacteria frequently associated with surfaces of live, stream incubated salmonid eggs include *P. fluorescens* and *Cytophaga* sp. (Bell, Hoskins & Hodgkiss 1971). In addition, live salmonid eggs incubated under hatchery conditions are also colonized by these same bacterial groups (Trust 1972, Chapter 2). It is likely therefore that *P. fluorescens* and *Cytophaga* sp. possess properties (or fitness traits) that contribute to their presence on egg surfaces. Such properties are likely to include an ability to adhere to egg surfaces (investigated in Chapter 4) and an ability to efficiently utilize any nutrients that might be available within the immediate egg environment. In the present trial an investigation was carried out into the relationship between potential nutrient availability on egg surfaces and and the dominant egg surface bacterial flora. Three bacterial groups were selected for investigation. Two groups, *P. fluorescens* and *Cytophaga* sp. were both frequently recovered from live egg surfaces in Chapter 2. The third bacterial group, *Serratia* sp. was selected as a direct comparison to the above two groups, for despite its common occurrence in many natural environments (Grimont & Grimont 1978), it was rarely recovered from live egg surfaces. Growth rates of all three groups were first determined under conditions of nutrient abundance in a synthetic medium. Subsequently, the ability of bacteria to grow in a medium derived from nutrients "leaked" from live and dead salmonid eggs was also investigated. A biochemical analysis of the nutrients leaked from eggs was undertaken to reveal possible nutrients that bacteria colonizing egg surfaces might utilize to support growth and reproduction. In addition, a biochemical analysis of structural salmonid egg amino

acids and fatty acids was performed to indicate potential carbon and nitrogen sources for bacteria colonizing (in particular) dead egg surfaces. Furthermore, the ability of all three bacterial groups to survive for prolonged periods under conditions of starvation was also examined. A schematic outline of investigative procedures is summarized in Figure 3 : 1.

FIGURE 3 : 1 Flow diagram of experimental protocol.



MATERIALS AND METHODS

Growth Studies

Measurement of bacterial cell concentration. Bacterial cell concentration was determined by measuring optical density (O.D.) of the test bacterial suspension. At low cell concentrations a linear relationship exists between O.D. and cell concentration, summarized by the Beer-Lambert law ;

$$O.D. \propto \frac{I_\infty}{I}$$

where I_∞ = incident light

I = emergent light

At higher cell concentrations the scattering of secondary light invalidates this relationship (Meynell & Meynell 1970). Kenward (1975) established that the Beer Lambert law is obeyed up to an O.D. of 0.3. However, above this value O.D. measurements are lower than expected. Linearity can be restored though by diluting the suspension to an absorbence of less than 0.3 (but above 0.03). A wave length scan of cell free supernatants of stationary phase cultures revealed that absorption by medium constituents and bacterial cell metabolic products was negligible at 470 nm for *P. fluorescens*, 425 nm for *Cytophaga* sp. and 400 nm for *Serratia* sp. These wavelengths were selected for measurements of O.D. for each respective bacterial suspension.

Media. Increase in bacterial cell concentration was measured for each group under varying conditions of nutrient availability. Initially, growth curves were constructed by culturing bacteria in nutritionally rich environment composed of a simple chemically defined medium (C.D.M., Cruickshank, Duguid, Marmron & Swain 1975) (Appendix 2 : 1). In addition bacteria were also cultured in two further media intended to resemble more closely the potential nutrients that might be available on egg surfaces.

The first comprised of nutrients that might be leaked from live eggs. Batches of 10 fertilized rainbow trout eggs were placed in 20 ml of sterile fish unit water maintained at 10 °C on an orbital shaker (40 rpm) for up to 48 hours. Eggs were removed from the water, the resulting medium pooled, filter sterilized and dispensed in 25 ml volumes into sterile 100 ml flasks. (This medium is hereafter referred to as live egg medium). The second medium comprised of nutrients leaked from dead eggs and was obtained in a similar manner to live egg medium. (This medium is hereafter referred to as dead egg medium). In addition, bacterial groups were also incubated in 25 ml volumes of sterile fish unit water with no added nutrients as a control.

Growth measurements. Growth of bacteria was measured by determining changes in O.D. with time. Inocula were prepared from stationary phase cells grown in 25 ml of C.D.M. Bacteria were harvested by centrifugation at 5000 x g for 10 minutes at room temperature, washed twice in double distilled water and inoculated into the appropriate sterile media (e.g . C.D.M. / live egg medium / dead egg medium) at a concentration of 2 X 10^6 cells / ml. Bacteria were incubated at 10 °C (and 25 °C for C.D.M. growth curves). Samples for O.D. measurement were removed at appropriate intervals and diluted when necessary. In addition bacterial cell concentration was determined by viable counts for each bacterial group in sterile fish unit water for a period of 29 days.

Potential nutrients from eggs

Analysis of nutrients leaked from eggs. Nutrient loss from live and dead eggs was determined by placing groups of 10 eggs in 20 ml of sterile fish unit water on an orbital shaker (40 rpm) for up to 50 hours. At suitable intervals five replicate groups of eggs were discarded and the filtrate analysed for :

1) amino acids - by a colourimetric method based on the reaction with ninhydrin (Yemm & Cocking 1955),

2) phosphate - by a colourimetric method based on the reaction with ferrous sulphate molybdate (Taussky & Shorr 1952) and

3) potassium - by flame photometry.

Analysis of structural and free egg amino acids. Structural amino acids were analysed by hydrolysing individual eggs in 2 ml of 6 M HCl in sealed, evacuated tubes at 110 °C for 24 hours. The resulting hydrolysate was almost neutralized with 5.3 ml of 2 M NaOH and diluted with 25 ml of distilled water. After centrifugation aliquots (200 µl) of hydrolysate were "loaded" onto a Locarte amino acid analyser (Locarte, London) and separated using a single column sodium citrate buffer system. Identification and quantification of unknown amino acids were made by comparison to known standards (Appendix 3 : 1).

Free amino acids were analysed by homogenization of 10 rainbow trout eggs in 5 ml of water in a ground glass macerator. After filtering, aliquots of 200 µl were directly loaded onto the amino acid analyser.

Analysis of fatty acids. Egg fatty acids were analysed by conversion to their methyl esters and separated by Gas Liquid Chromatography (G.L.C.) in a similar manner to that outlined by Hammonds & Smith (1986). Eggs were individually extracted in 3 ml of hot isopropanol (80 °C) for 15 minutes to inactivate lipolytic enzymes. Samples were then disrupted separately in a ground glass homogenizer in 3 ml of isopropanol, homogenates were transferred to centrifuge tubes and the homogenizer rinsed with two further 3 ml isopropanol aliquots. Each extract (9 ml) was centrifuged at 1000 x g for 5 minutes and the resulting supernatants decanted through dried pre-weighed filter paper into appropriate 150 ml round bottom evaporating flasks. Individual remaining pellets were further twice extracted with 5 ml of isopropanol : chloroform (1 : 1, $^{v}/_{v}$) and finally 2 ml chloroform. After each extraction the supernatant was again decanted into an appropriate evaporating flask to give a final volume of 21 ml of extract in each flask. Extracts were subsequently reduced in volume to approximately 1 ml *in vacuo* (40 °C) and then taken up in 2 ml of chloroform. Individual extracts were transferred into conical centrifuge tubes and washed three

times with 1 ml 0.88 M KCL (Folch wash), the upper aqueous layer being discarded after each wash. Each extract was then transferred to a 5 ml pear-shaped flask, further reduced in volume (*in vacuo* 40 °C), further transferred to small vials and reduced to dryness at ambient temperature under a stream of nitrogen before temporary storage (-20 °C in the dark). Dried extracts were methylated in the manner outlined by Morrison & Smith (1964) by addition to each vial of 1 ml 25% boron trifluoride (14% in methanol), 20% benzene 55% methanol ($^{v}/_{v}$). Vials were carefully sealed and immersed in boiling water for 45 minutes. Fatty acid methyl esters were recovered by addition of 1 ml distilled water followed by 2 ml pentane. After separation the upper layers were recovered and placed in fresh vials, dried in a stream of nitrogen and stored in the dark at -20 °C prior to G.L.C. analysis. For G.L.C. analysis 250 µl of pentane was added to each vial and subsequently 5 µl aliquots of each extract were injected onto chromatograph columns. Fatty acid methyl esters were separated on a 1.8 M column of 10% DEGS (diethyleneglycol succinate) on chromasorb 101 using a Pye series 304 chromatograph equipped with dual flame ionising detector and computing integrator. Nitrogen (40 ml / minute) was employed as a carrier gas with a column temperature of 190 °C. Methyl esters from samples were identified by comparison of retention times with those of known standards (Ward, Candy & Smith 1982) (Appendix 3 : 2).

Statistical analysis. Amounts of amino acids, phosphate and potassium "leaked" from live and dead eggs was compared by 2 way split -plot analysis of variance. Differences between means were compared by calculating the standard errors and 95% confidence limits appropriate to the split-plot design (Ridgman 1975 ; Snedecor & Cochran 1980).

RESULTS

Growth Studies. Increases in cell concentration of all three bacterial groups in C.D.M. at 10 °C and 25 °C are summarized in Figures 3 : 2 to 3 : 4. From these graphs it can be seen that *Cytophaga* sp. exhibited a slower growth rate compared to the other two genera. However, not surprisingly all three grew markedly faster at 25 °C compared to 10 °C. Increase in cell concentration for *P. fluorescens* is summarized in Figure 3 : 2 (Appendix 3 : 3). From this graph it can be demonstrated that a doubling time of approximately 7 hours was achieved by *P. fluorescens* at 10 °C and 2.5 hours at 25 °C in turn leading to growth rates of 0.14 generations per hour (10 °C) and 0.4 generations per hour (25 °C). Increase in cell concentration for *Serratia* sp. in C.D.M. is summarized in Figure 3 : 3. From this graph it can be shown that a similar growth rate to *P. fluorescens* was obtained. *Serratia* sp. achieved a doubling time of approximately 6 hours at 10 °C and 3 hours at 25 °C, in turn leading to growth rates of 0.17 generations per hour and 0.4 generations per hour respectively (Appendix 3 : 4). Increase in cell concentration for *Cytophaga* sp. in C.D.M. is summarized in Figure 3 : 4, which demonstrates a slower doubling time of 11.9 hours at 10 °C and 8.5 hours at 25 °C, leading in turn to growth rates of 0.08 generations per hour (10 °C) and 0.12 generations per hour (25 °C) (Appendix 3 : 5).

Increases in cell concentration for all three bacterial groups in live egg medium, dead egg medium and sterile fish unit water are summarized in Figures 3 : 5 a-c. In general, dead egg medium supported increased cell concentrations compared to live egg medium which in turn supported greater cell concentrations than sterile fish unit water. Growth of *P. fluorescens* in all 3 media at 10 °C is summarized in Figure 3 : 5a. From this graph it can be seen that initially both live and dead egg media supported bacterial growth. However, neither media could provide sufficient nutrients for sustained growth although dead egg medium supported greater bacterial growth than live egg medium. In sterile fish unit water numbers of *P. fluorescens* did not increase

FIGURE 3:2 Increase in cell concentration in chemically defined medium

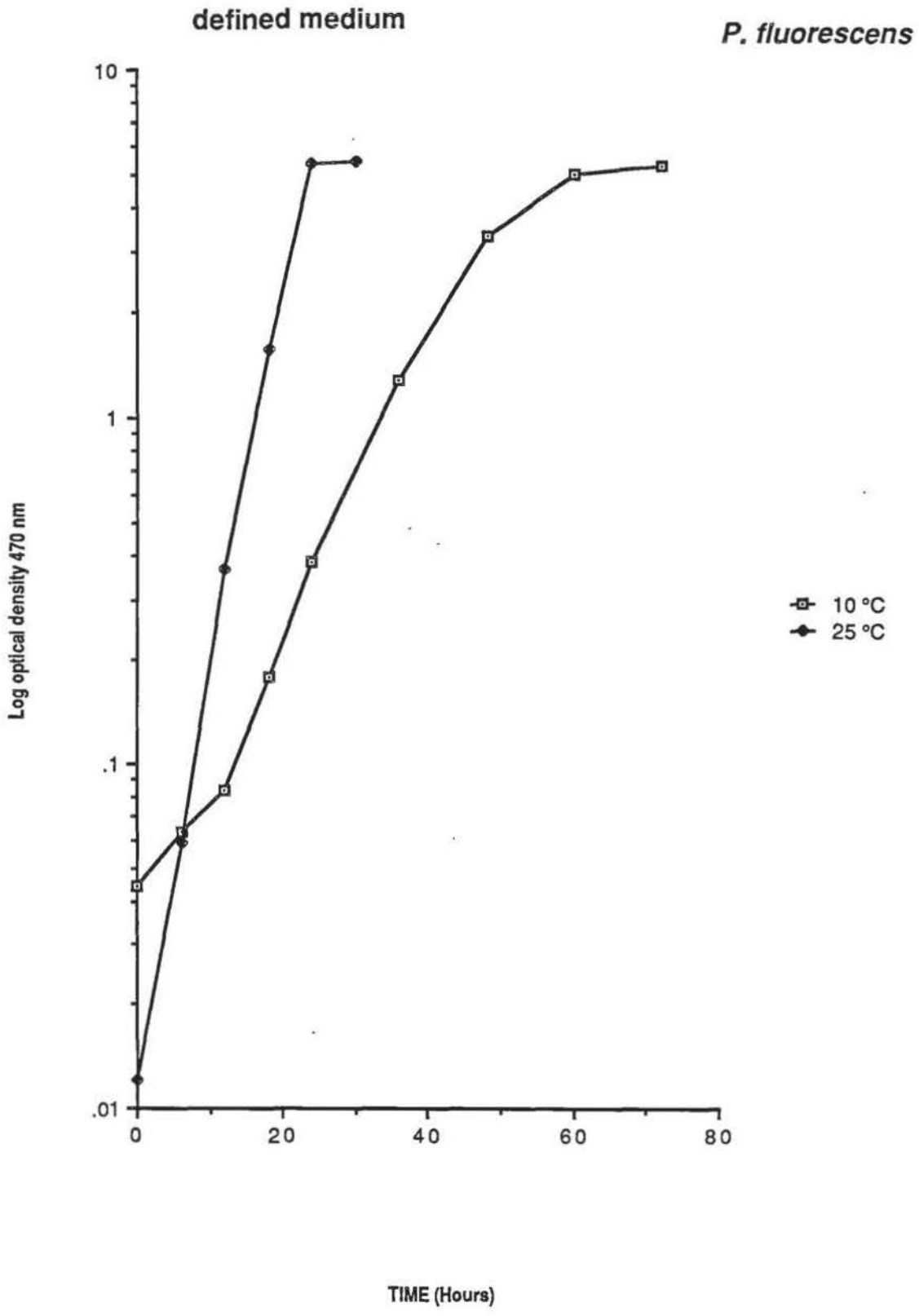


FIGURE 3:3 Increase in cell concentration in chemically defined medium *Serratia* sp.

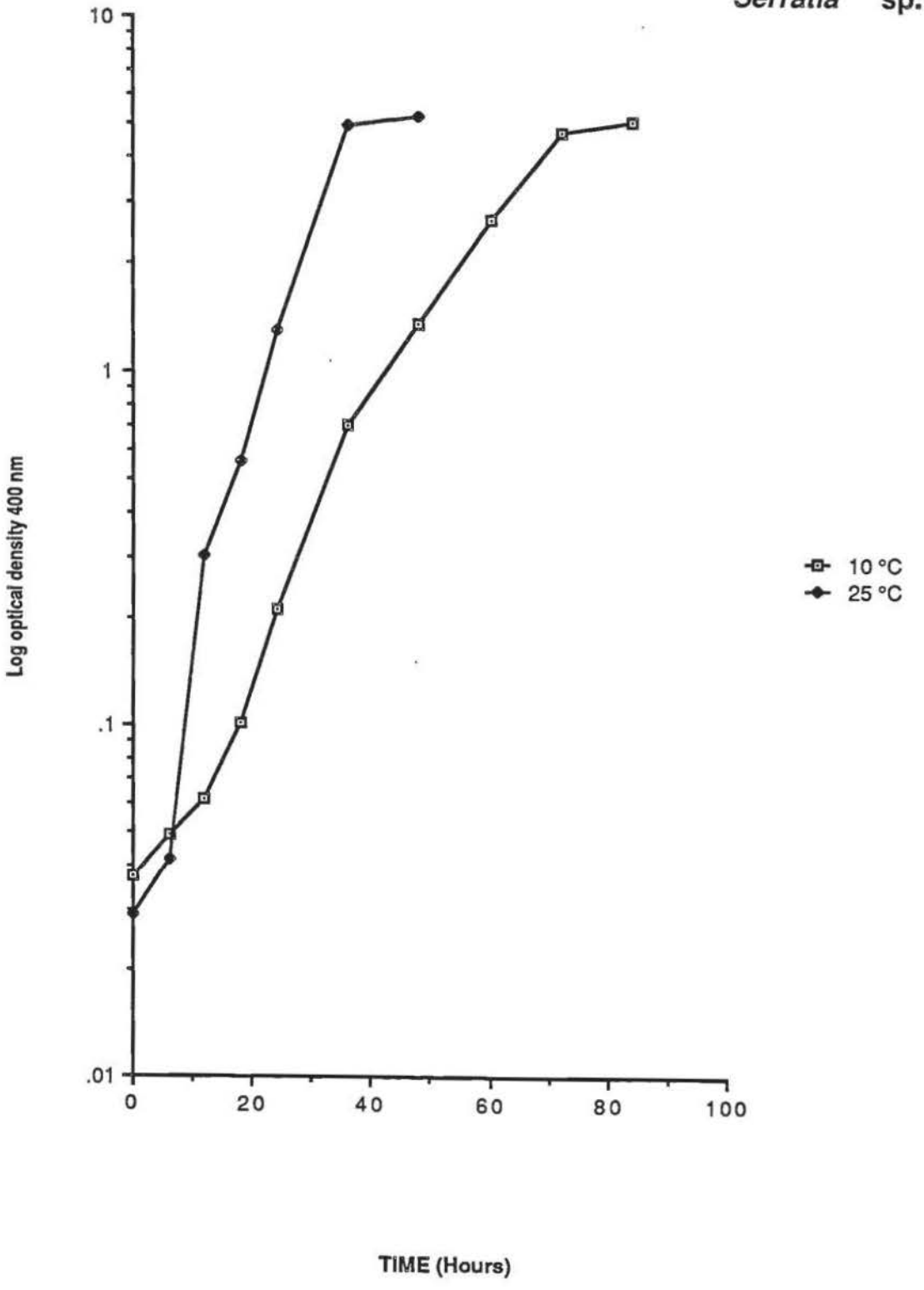


FIGURE 3:4 Increase in cell concentration in chemically defined medium

***Cytophaga* sp.**

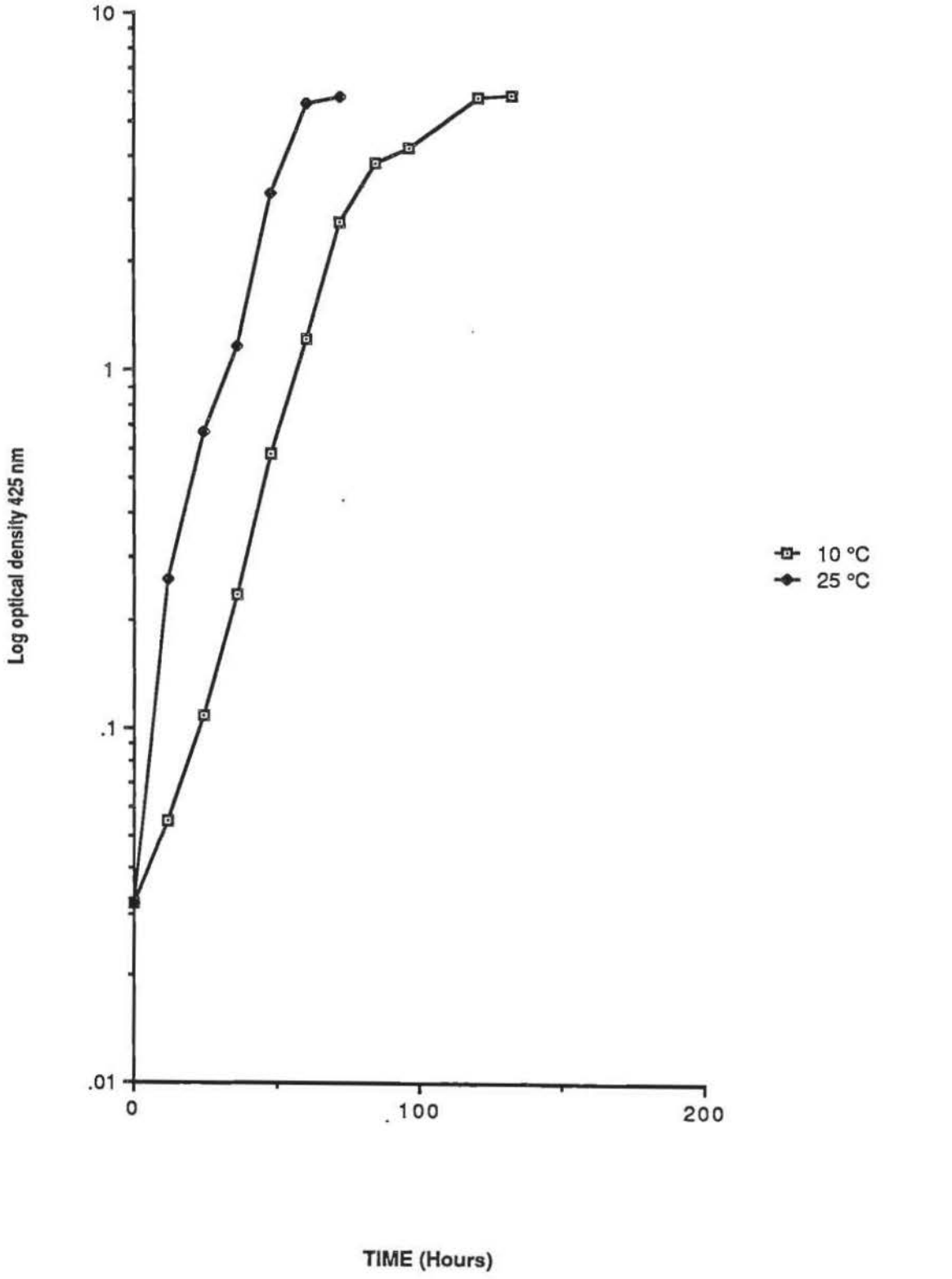


FIGURE 3:5 (a) Increase in cell concentration in medium made up with nutrients from live eggs, dead eggs and sterile fish unit water. *P. fluorescens*

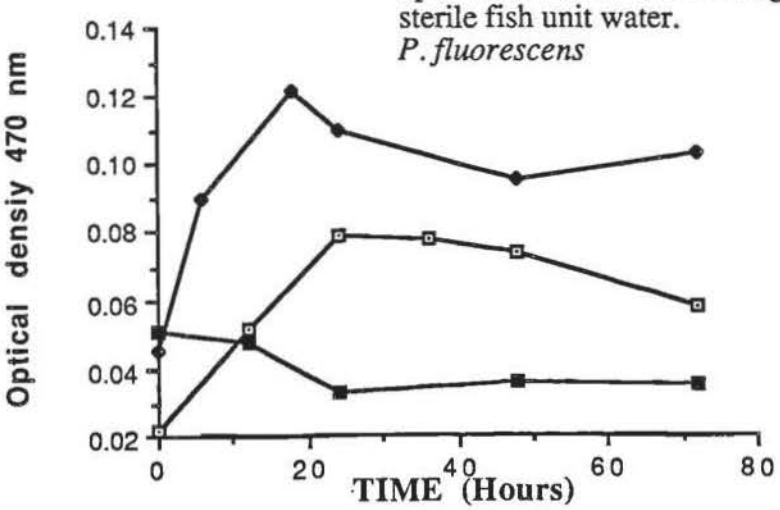


FIGURE 3:5 (b) Increase in cell concentration in medium made up with nutrients from live eggs, dead eggs and sterile fish unit water. *Serratia* sp.

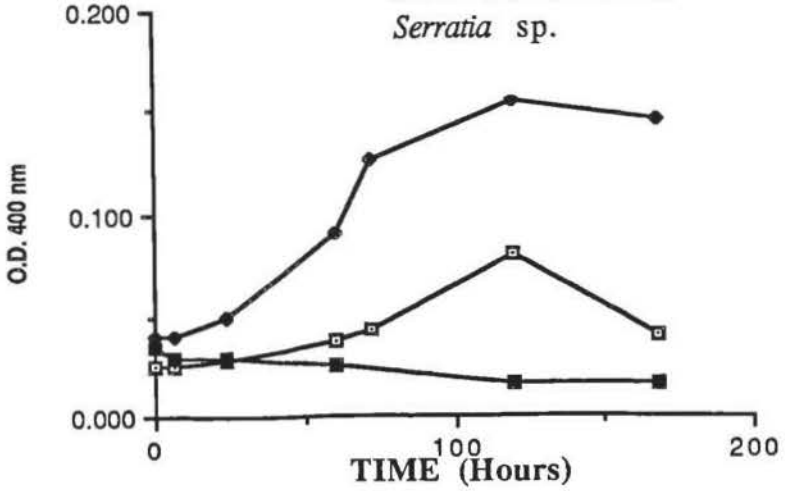
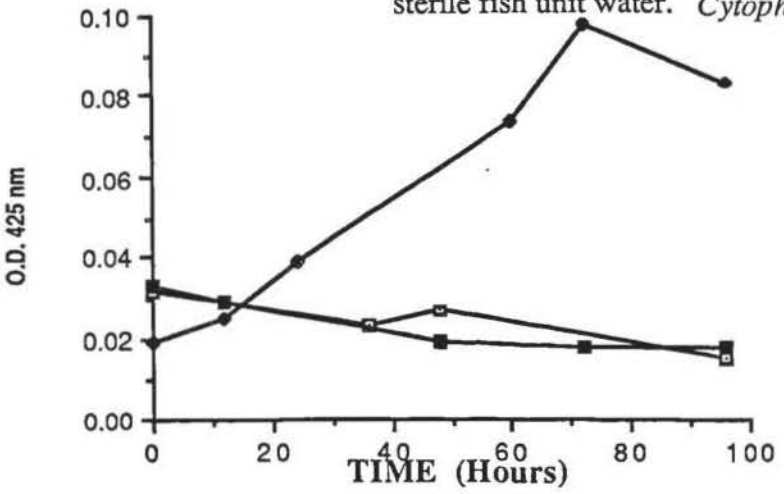


FIGURE 3:5 (c) Increase in cell concentration in medium made up with nutrients from live eggs, dead eggs and sterile fish unit water. *Cytophaga* sp.



Live egg medium Dead egg medium Sterile fish unit water

as measured by O.D. (Appendix 3 : 6). Growth of *Serratia* sp. in live egg medium, dead egg medium and sterile fish unit water is summarized in Figure 3 : 5b. From this graph it can be seen that both live and dead egg media supported increased numbers of bacterial cells. In common with *P. fluorescens* sterile fish unit water did not provide an adequate environment for increased numbers of *Serratia* sp. (Appendix 3 : 7). Growth of *Cytophaga* sp. in live egg medium, dead egg medium and sterile fish unit water is summarized in Figure 3 : 5c. From this graph it can be seen that in contrast to *P. fluorescens* and *Serratia* sp. live egg medium did not support growth of *Cytophaga* sp. However, *Cytophaga* sp. did increase in cell concentration when maintained in dead egg medium. In common with *P. fluorescens* and *Serratia* sp., sterile fish unit water did not mediate increased cell concentrations of *Cytophaga* sp. (Appendix 3 : 8).

Bacterial growth in sterile fish unit water was also monitored by viable count techniques over a 29 day period at 10 °C. Results obtained for all 3 bacterial groups are summarized in Figure 3 : 6 (a-c). From this figure it can be seen that viable colonies of all 3 bacterial groups can be recovered on agar over the complete 29 day period. Furthermore, in contrast to results measuring cell concentration by optical density both *P. fluorescens* and *Cytophaga* sp. demonstrated an increase in numbers of recovered colony forming units (cfu's) compared to the original inoculum, before a general reduction in numbers was obtained towards the end of the sampling period (Appendix 3 : 9).

Potential nutrients from eggs. The levels of amino acids leaked from live and dead eggs are summarized in Figure 3 : 7. Statistical analysis by two way split-plot analysis of variance revealed that the loss of amino acids between these groups was highly significantly different ($P < 0.001$). A highly significant effect of time was also seen ($P < 0.001$) and a highly significant interaction between time and state of egg was found ($P < 0.001$) (Appendix 3 : 10). Levels of phosphate leaked between live and dead eggs are summarized in Figure 3 : 8 and were found to be highly significantly different ($P < 0.001$). A highly significant increase in amounts of phosphate leaked from both live and dead eggs with time was also revealed ($P < 0.001$). In addition, a

FIGURE 3:6 (a) Recovery of cells maintained in sterile fish unit water over a 29 day period

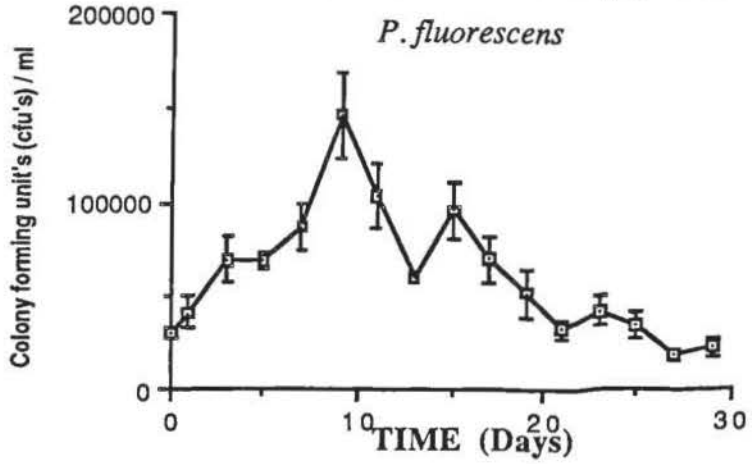


FIGURE 3:6 (b) Recovery of cells maintained in sterile fish unit water over a 29 day period

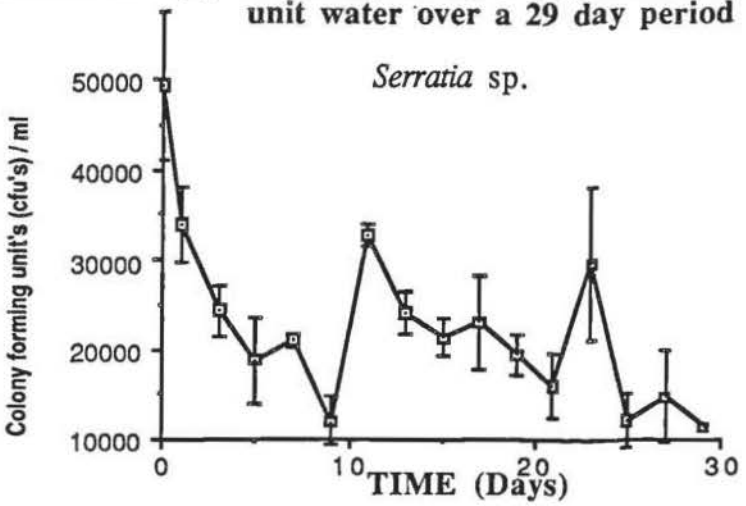
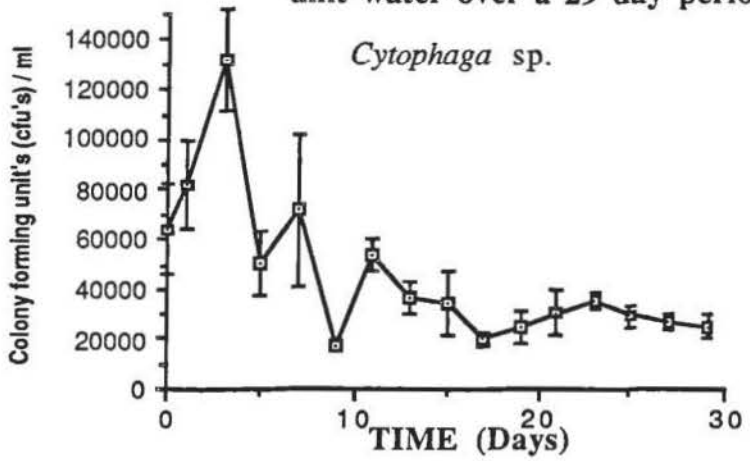
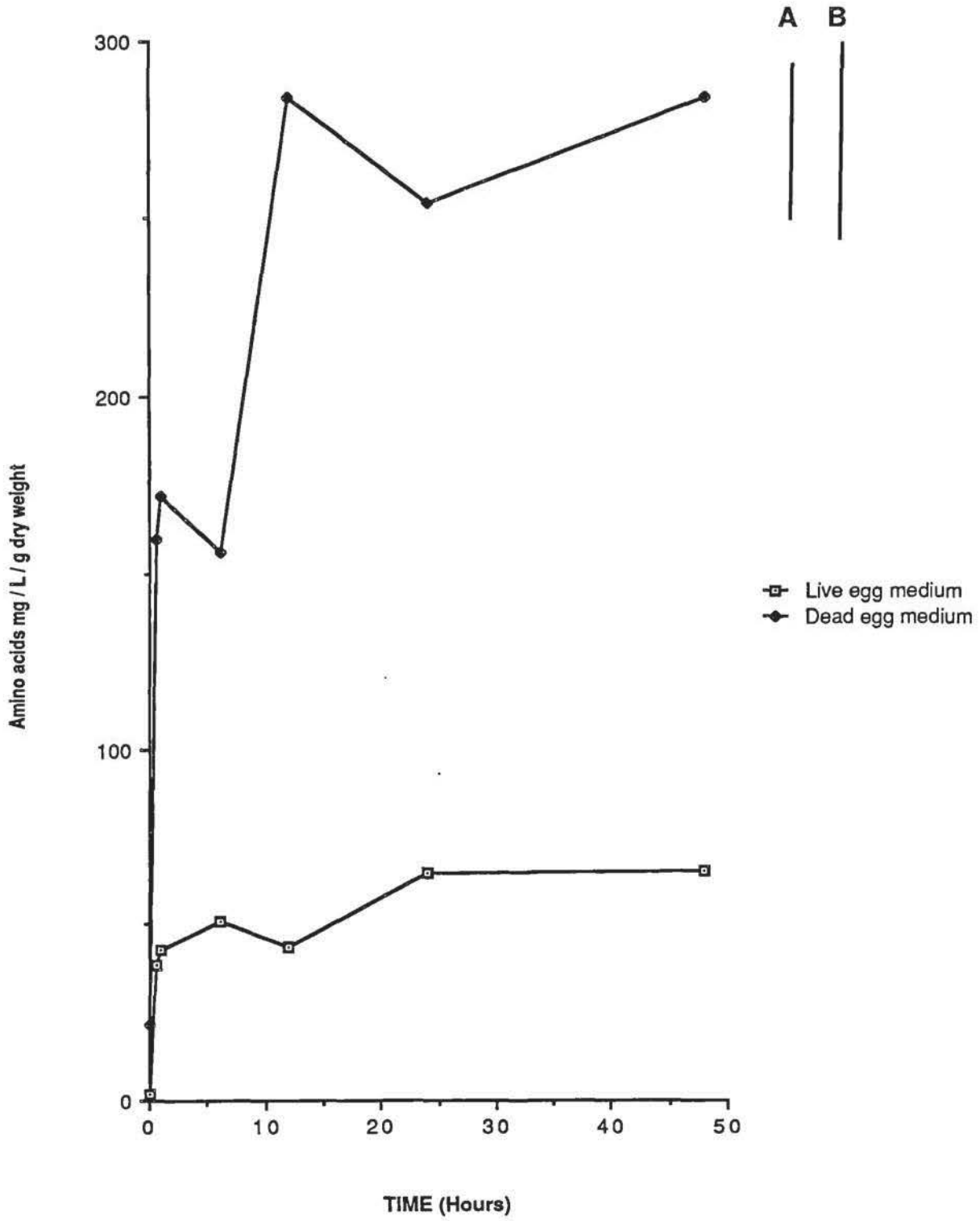


FIGURE 3:6 (c) Recovery of cells maintained in sterile fish unit water over a 29 day period



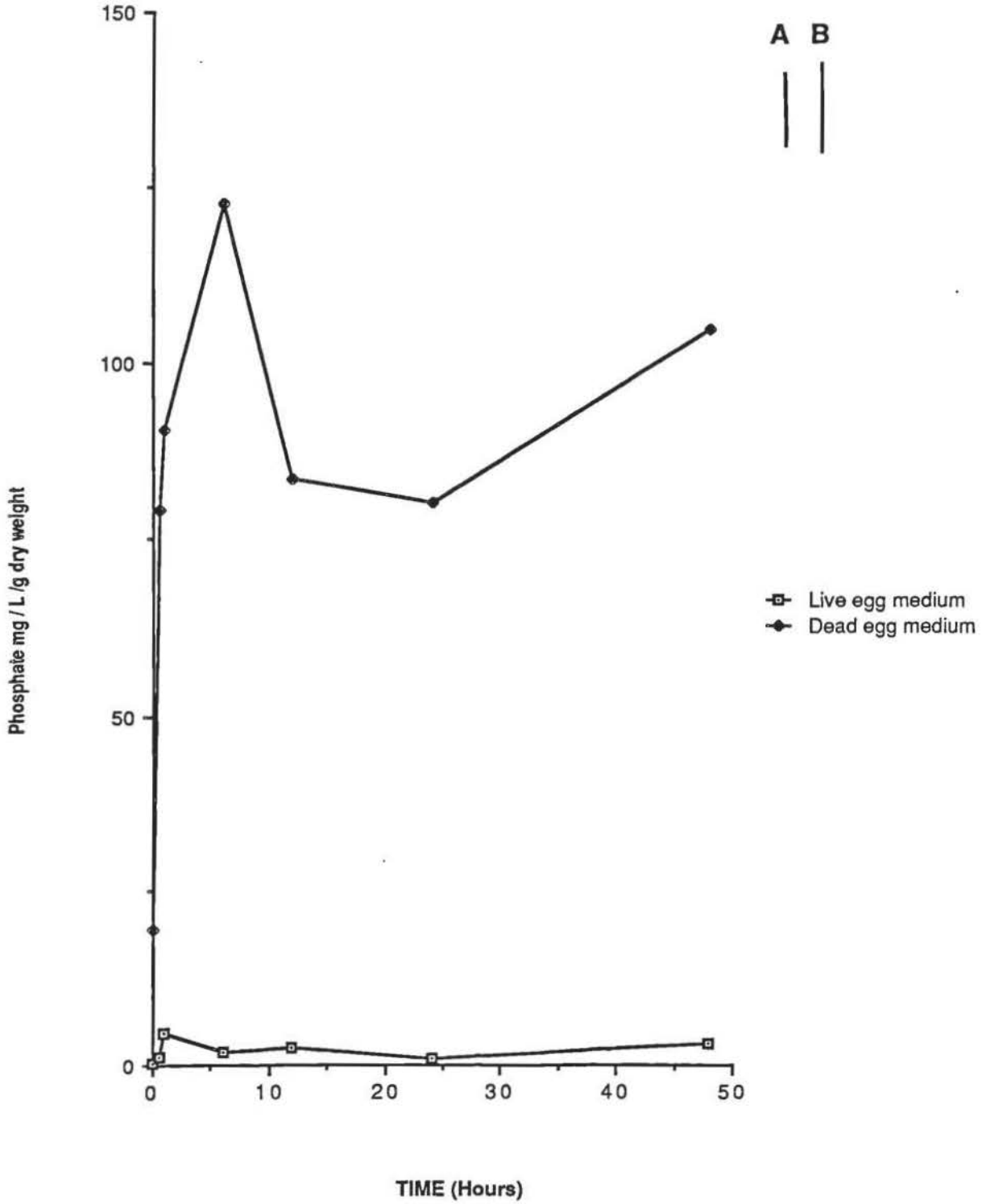
Mean figures from 3 replicates ± standard error of the mean

FIGURE 3:7 Amino acids "leaked" from live and dead eggs



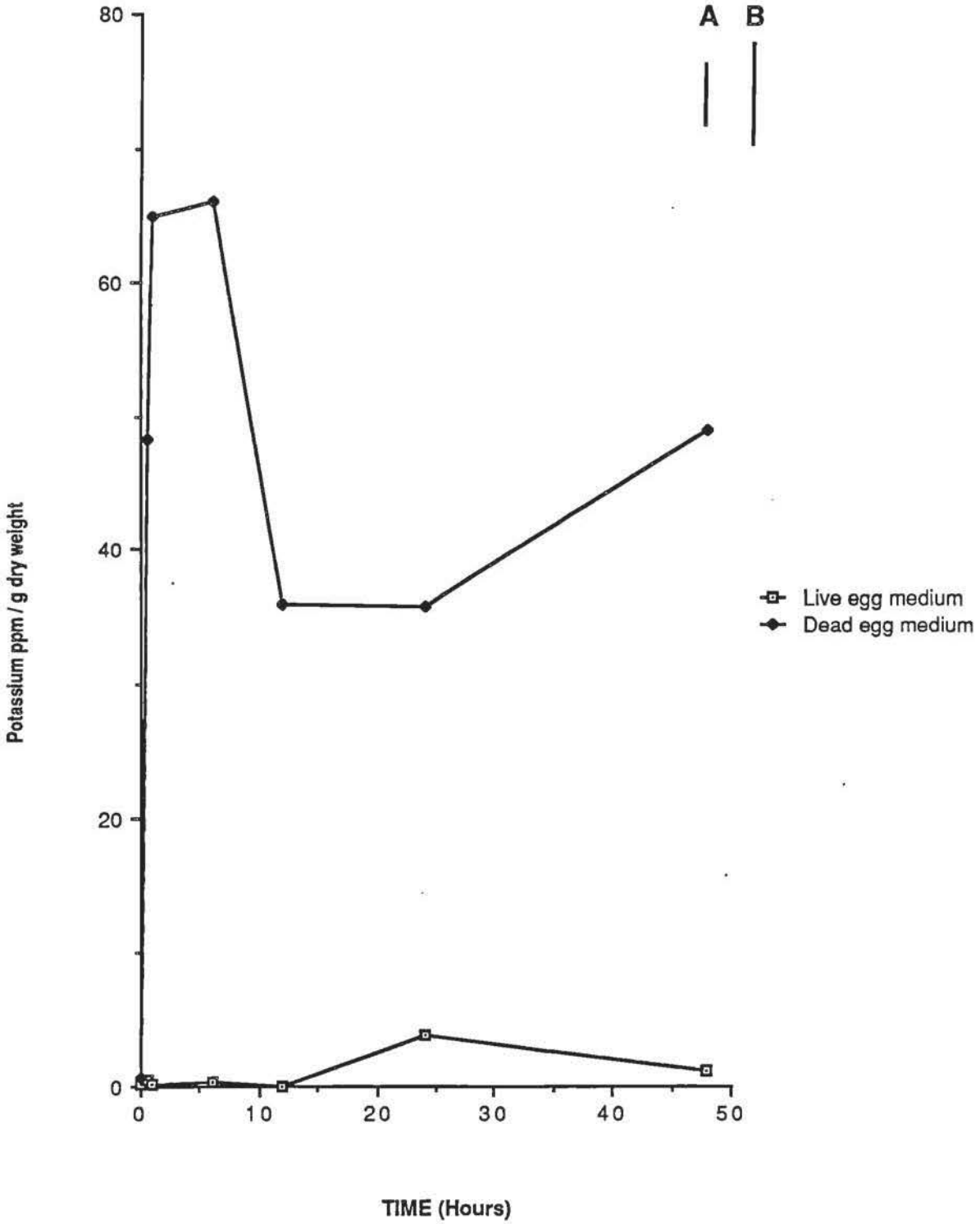
A = 95% confidence limits for differences within a treatment and B = 95% confidence limits between 2 treatments at the same or different times.

FIGURE 3:8 Phosphate "leaked" from live and dead eggs



A = 95% confidence limits for differences within a treatment and B = 95% confidence limits between 2 treatments at the same or different times.

FIGURE 3:9 Potassium "leaked" from live and dead eggs



A = 95% confidence limits for differences within a treatment and B = 95% confidence limits between 2 treatments at the same or different times.

TABLE 3 : 1 Free and structural amino acids of rainbow trout eggs

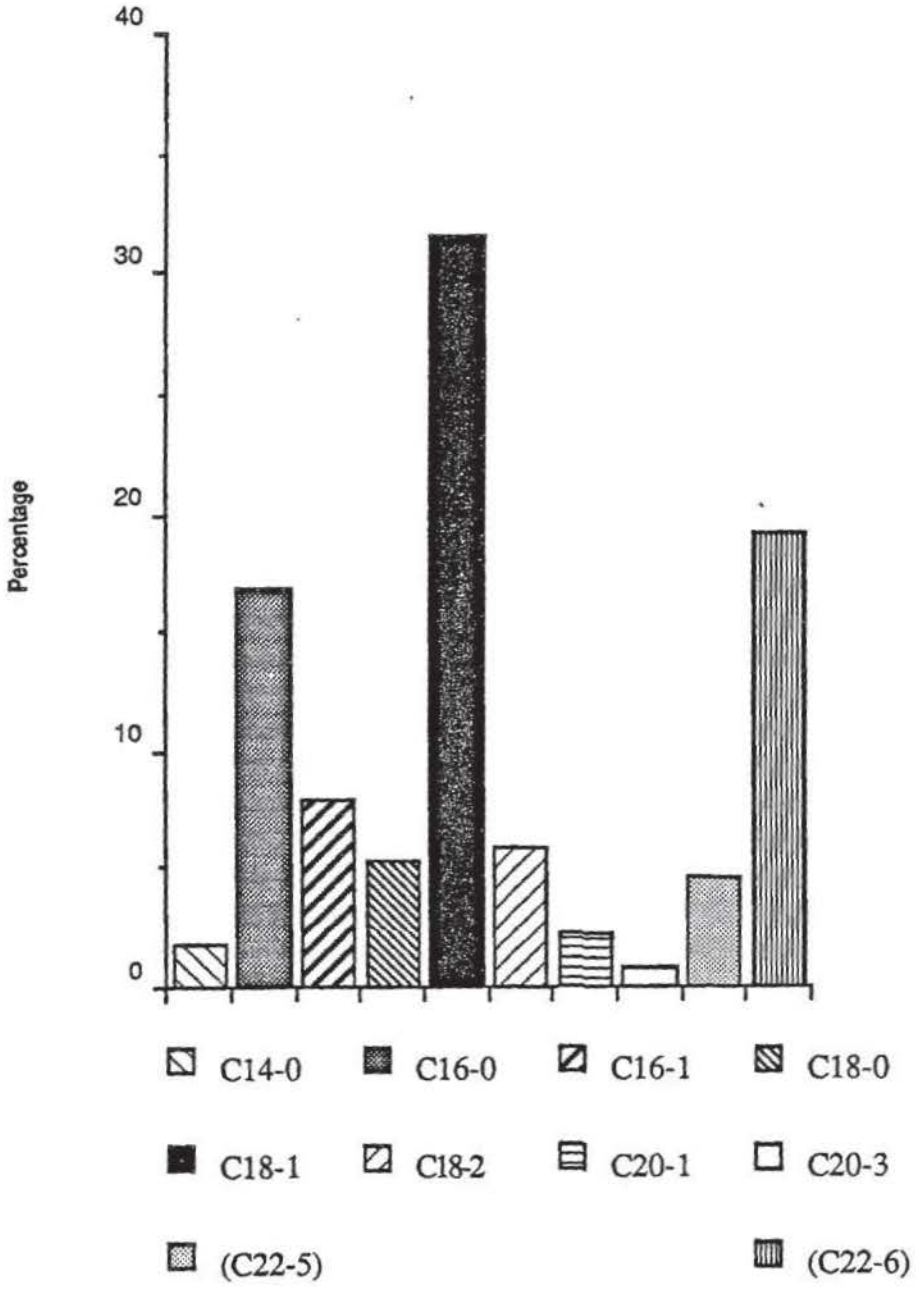
Amino acid	Free	Structual
L-Aspartate	39.6	29.9
L-Threonine	15.3	27.3
L-Serine	30.0	39.8
L-Glutamine	25.5	41.4
L-Proline	9.6	38.6
Glycine	8.9	24.1
L-Alanine	10.6	43.5
L-Cystine	trace	8.6
L-Valine	5.2	26.6
L-Methionine	5.1	10.6
L-Isoleucine	6.0	27.4
L-Leucine	6.1	34.5
L-Tyrosine	4.7	12.6
L-Phenylalanine	4.8	15.6
L-Histidine	6.3	8.7
L-Lysine	5.4	24.0
L-Arginine	48.1	42.2

Average of three samples

Free amino acids (n mol / mg dry weight per 10 eggs)

Structual amino acids (n mol / mg dry weight per egg)

FIGURE 3 : 10 Fatty acid analysis of trout eggs



highly significant interaction between time and state of egg was also shown to have occurred ($P < 0.001$) (Appendix 3 : 11). Levels of potassium leaked from live and dead eggs are summarized in Figure 3 : 9. From this graph it can be seen that a highly significant difference ($P < 0.001$) exists between the amounts of potassium leaked from live and from dead eggs. A highly significant effect of time was seen ($P < 0.001$) and a highly significant interaction between time and state of egg was found ($P < 0.001$) (Appendix 3 : 12).

Analyses of free and structural amino acids from rainbow trout eggs are summarized in Table 3 : 1. From this table it can be seen that arginine, aspartate, serine and glutamine comprised the bulk of free amino acids. In comparison, the most abundant structural amino acids were alanine, arginine, glutamine and serine (Appendix 3 : 13).

Analyses of structural fatty acids in rainbow trout eggs are summarized in Figure 3 : 10. From this graph it can be seen that the fatty acids recovered in the highest amounts were oleic acid (18 : 1) 31.6 %, an unknown (probably, 22 : 6) 19.2% and palmitic acid (16 : 0) 16.8% (Appendix 3 : 14).

DISCUSSION

In natural aquatic environments bacteria are considered to exist under conditions of starvation (Jannasch 1969). Thus, initially it might seem "artificial" to measure bacterial growth rates in chemically defined medium and conditions of nutrient abundance. However, such conditions of nutrient excess may still provide a realistic comparison of growth rate between groups. Growth rate measurements were mainly taken at a temperature of 10 °C to reflect the the prevailing water temperature in most U.K. hatcheries. At 10 °C, *P. fluorescens* and *Serratia* sp. exhibited similar growth rates, but *Cytophaga* sp. was markedly slower. Differential growth rates are often considered to be responsible for displacement of one organism by another (Alexander 1971). Even so, few well defined examples of such a simple type of interaction exist although, Jannasch (1968) demonstrated that slow growth rates of *Enterobacteriaceae* are the main cause for their displacement from sea-water by other microorganisms. In this trial *Cytophaga* sp. proved to have the slowest growth rate of all three groups in C.D.M. but, even so, is frequently found dominating live egg surfaces (Bell, Hoskins & Hodgkiss 1971 ; Trust 1972). Thus, fast growth rate alone would not seem to be the only significant factor responsible for successful live egg colonization.

A potentially more appropriate nutrient environment for present bacterial growth studies was obtained by gently rocking live and dead eggs on a shaker in sterile fish unit water. Analysis of the water surrounding eggs at appropriate intervals revealed that amino acids, phosphates and potassium were leaked from eggs. In turn, certain bacteria when added to nutrient material derived from living eggs increased in cell concentration compared to those inoculated into sterile fish unit water alone. Thus, nutrients are lost from live eggs in sufficient quantity and variety to support bacterial growth albeit limited. In addition to nutrients leaked from live eggs, bacteria colonizing live egg surfaces might also utilize waste products excreted by developing embryos as although salmonid embryos are extremely efficient at using nutrients available to them

small quantities of ammonia and carbon dioxide are excreted (Hayes 1949 ; Smith 1947, 1957). However, bacteria colonizing live egg surfaces probably obtain the bulk of their nutrients from the surrounding hatchery water. Frequently bacteria on surfaces in natural aquatic systems form adherent microcolonies and subsequently a "biofilm" (Costerton, Cheng, Geesey, Ladd, Curtis-Nickel, Dasgupta & Marrie 1987). Such biofilms greatly assist the process of nutrient trapping, and thus their establishment on live egg surfaces may prove to be more important in successful bacterial colonization of living egg surfaces than growth rates alone.

The yolk of salmonid eggs is composed of salts and vitellogenin and is contained in the yolk sac membrane. Damage to this membrane allows salt to pass from the yolk and out of the egg, leaving the larger globulin molecule behind. Vitellogenin is a protein that is soluble in salt solutions but when salt leaves the egg it precipitates out as a white solid. Precipitated vitellogenin therefore gives dead eggs their characteristic white / opaque appearance (Stevenson 1980). Biochemical analysis of sterile fish unit water containing dead eggs revealed that significantly higher levels of amino acids, phosphate and potassium were lost compared to live eggs. Thus, bacteria colonizing dead egg surfaces would potentially have access to increased levels of nutrients compared to those colonizing living eggs. The markedly greater bacterial cell concentration supported by nutrients released from dead eggs indicates that the the increased nutrient loss from dead eggs is likely to support greater bacterial colonization. Furthermore, it is also likely that bacteria colonizing dead egg surfaces obtain nutrients from the substrate (i.e. the egg) itself. Dead eggs are frequently colonized by considerably greater numbers of bacteria than live eggs (Bell *et al* 1971, Chapter 2), and as a consequence, a general physical softening of the egg shell is often observed. Biochemical analysis of egg structural amino acids and fatty acids revealed many possible sources of carbon and nitrogen that in turn could potentially support bacterial growth and proliferation. Structural amino acids present in relative abundance were alanine, arginine, glutamine and serine, closely agreeing with previous work by Suyama & Suzuki (1978). In agreement with Atchinson (1975) and Nakagawa &

Tsuchiya (1976) analysis of egg fatty acids demonstrated oleic acid (18 : 1), an unknown polyunsaturated fatty acid (probably, 22 : 6) and palmitic acid (16 : 0) to be the most abundant.

The predominant bacterial flora of dead eggs tends to be fluorescent *Pseudomonads* and not *Cytophaga* sp. (Bell *et al* 1971, Trust 1972 and Chapter 2). The relative abundance of *P. fluorescens* on dead egg surfaces could be due to a variety of factors, including perhaps tolerance of a wide range of fatty acids which can potentially be toxic to some bacteria (Atlas & Bartha 1981). Furthermore, *P. fluorescens* is known to produce a wide range of extracellular lipases (Sugiura & Isobe 1975 ; Macrae 1983) and in addition can utilize alanine and serine as sole carbon sources (Palleroni 1984). Thus, on dead egg surfaces *P. fluorescens* may readily derive sufficient nutrients to support rapid growth and proliferation, which in turn coupled with its' markedly greater potential growth rate compared to *Cytophaga* sp., would further promote its' establishment at the expense of *Cytophaga* sp. Moreover, *P. fluorescens* has clearly demonstrated the ability to inhibit other competing microorganisms (Cornick, Chudyk & McDermot 1969 ; Dubois-Darnaudpeys 1977 ; Gurusiddaiah, Weller, Sarkar & Cook 1986 ; Hatai & Willoughby 1988) and thus may inhibit establishment of *Cytophaga* sp. under conditions of greater nutrient availability.

The *Serratia* sp. used during this trial was initially isolated from a live egg surface during a previous trial (Chapter 2). However, *Serratia* sp. are rarely recovered from egg surfaces despite their frequent presence in many natural ecosystems (Grimont & Grimont 1978). In this trial *Serratia* sp. demonstrated an equal ability to utilize nutrients from live and dead egg media and a growth rate equal to that of *P. fluorescens*. In addition, *Serratia* sp. (as well as *P. fluorescens* and *Cytophaga* sp.) were recoverable on agar after 29 days in sterile fish unit water. Thus, *Serratia* sp. certainly possess the ability to exist under limited nutrient conditions for the complete period of egg incubation at 10 °C and to utilize any nutrients that might become available from incubating eggs. Thus, the failure of *Serratia* sp. to colonize egg surfaces is unlikely to be due to either physiological unsuitability or an inability to use

available nutrients but, may be due to other factors, perhaps the initial ability to adhere to egg surfaces.

SUMMARY

Bacterial populations on live egg surfaces are probably largely supported by nutrients derived from the surrounding hatchery water. In addition, low amounts of potassium, phosphate and amino acids might be obtained, "leaked" from live eggs. In contrast, bacteria on dead egg surfaces have access to significantly greater amounts of the same (above) nutrients. Furthermore, biochemical analysis of eggs reveals both amino acids and fatty acids that might additionally be utilized by bacteria colonizing dead egg surfaces.

Under conditions of limited nutrient availability, such as occur around surfaces of live eggs, *Cytophaga* sp. is frequently found to predominate. However, on dead egg surfaces, fluorescent *Pseudomonas* sp. comprise the bulk of the population, a possible consequence of greater nutrient availability, faster growth rate, tolerance of toxic fatty acids and an ability to inhibit other competing microorganisms.

CHAPTER FOUR

ADHESION PROPERTIES OF BACTERIA COLONIZING INCUBATING SALMONID EGG SURFACES

INTRODUCTION

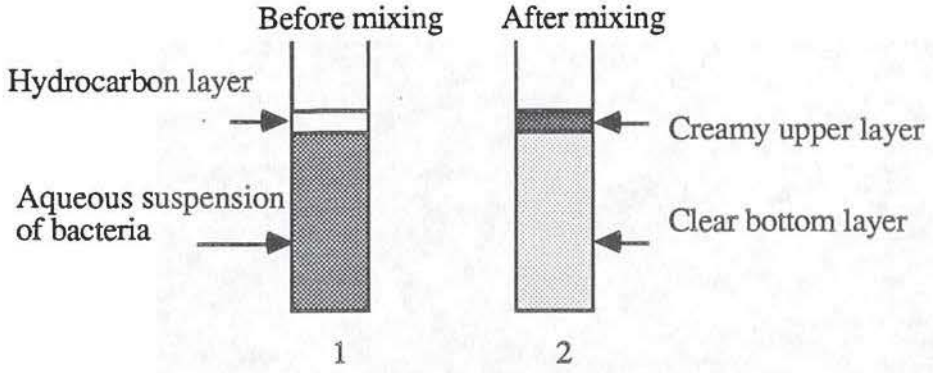
Spring or borehole water is frequently used to supply salmonid hatcheries. These waters typically contain few nutrients and thus in turn support few bacteria (Fletcher 1979). Indeed, in such waters bacteria are considered to exist under conditions of starvation (Jannasch 1969). However, once diverted into a hatchery any remaining nutrients in the water will tend to accumulate on available surfaces (Zobell & Anderson 1936), including hatchery troughs, trays and incubating eggs, in turn supporting bacterial growth and reproduction (Chapter 3). Initially, both bacteria and nutrients will be attracted to surfaces by similar mechanisms as explained by Derjaguin & Landav and Verivey & Oberbeck (the D.L.V.O. theory) (Rutter & Vincent 1980). Their concept states that interaction energy (V_T) of two smooth particles is determined solely by the sum of Van de Waals attractive energy (V_A) and repulsive electrostatic energy (V_R) : $V_T = V_A + V_R$. When a particle approaches a surface it experiences a weak Van de Waals attraction induced by the fluctuation of dipoles within the molecules of the two approaching surfaces. Attraction between the surfaces increases as the distance between them decreases. However, as both particle and surface are negatively charged there will also be a repulsion, the magnitude of which is dependant on surface potentials, ionic strength and the dielectric constant of the surrounding medium. Attractive Van de Waals forces tend to operate over a greater range than repulsive electrostatic forces thus allowing surfaces to remain in contact. Bacteria in contact with a surface proceed through this phase of adherence termed "reversible" adherence to a further phase of "irreversible" adherence (to be described later). One important factor in determining both phases of adhesion is the hydrophobic nature of the cell surface (Pethica 1980 ; Klotz, Drutz & Zajic 1985), directly influencing the "stickiness" of the cell. Bacteria in low nutrient environments (such as hatchery water) exhibit a range of different characteristics including increased cell surface hydrophobicity (Kjelleberg &

Hermansson 1984). A variety of techniques exist to measure bacterial cell surface hydrophobicity including, contact angle measurements of dried cell layers, hydrophobic interaction chromatography, partitioning of bacteria in aqueous polymer two phase systems and adherence to hydrocarbons. No single test can adequately describe cell surface hydrophobicity (Rosenberg, Gutnick & Rosenberg 1980) and thus frequently a series of tests is selected. In this study, an examination was made of the adherence properties of two bacterial genera frequently recovered from surfaces of live incubating salmonid eggs, *Pseudomonas fluorescens* and *Cytophaga* sp. In addition, a third bacterial group (*Serratia* sp.) rarely isolated from live egg surfaces was also used for comparison. The cell surface hydrophobicity of all three organisms was studied by the following series of selected techniques ;

1) *Bacterial Adherence To Hydrocarbons* (B.A.T.H.). Rosenberg *et al* (1980) demonstrated that bacterial hydrophobicity could be indirectly measured through relative cell surface affinity for liquid hydrocarbons. The above authors added three hydrocarbons, octane, xylene and hexadecane at three different volumes to turbid aqueous suspensions of test bacteria. After a brief period of mixing, suspensions were left to settle and the original turbid solution was found to separate into a clear bottom layer and a "creamy" upper layer (Figure 4 : 1a). Microscopic examination of the upper layer revealed an oil-in-water emulsion consisting of droplets of the respective hydrocarbon covered with adhering bacterial cells. Initially, during this forthcoming trial, adherence to all three hydrocarbons (octane, xylene and hexadecane) was examined. However, microscopic examination of bacteria adhered to xylene suggested the possibility that some cells had suffered damage and thus further tests with this particular hydrocarbon ceased. In contrast cells adhered to both octane and hexadecane revealed no indication of such damage. Further trials demonstrated that most cells adhered slightly better to hexadecane than octane and thus hexadecane was finally selected for the forthcoming trials.

2) *Hydrophobic Interaction Chromatography* (H.I.C.). H.I.C. is a technique used to assay the degree of hydrophobicity of bacterial cells by comparing the

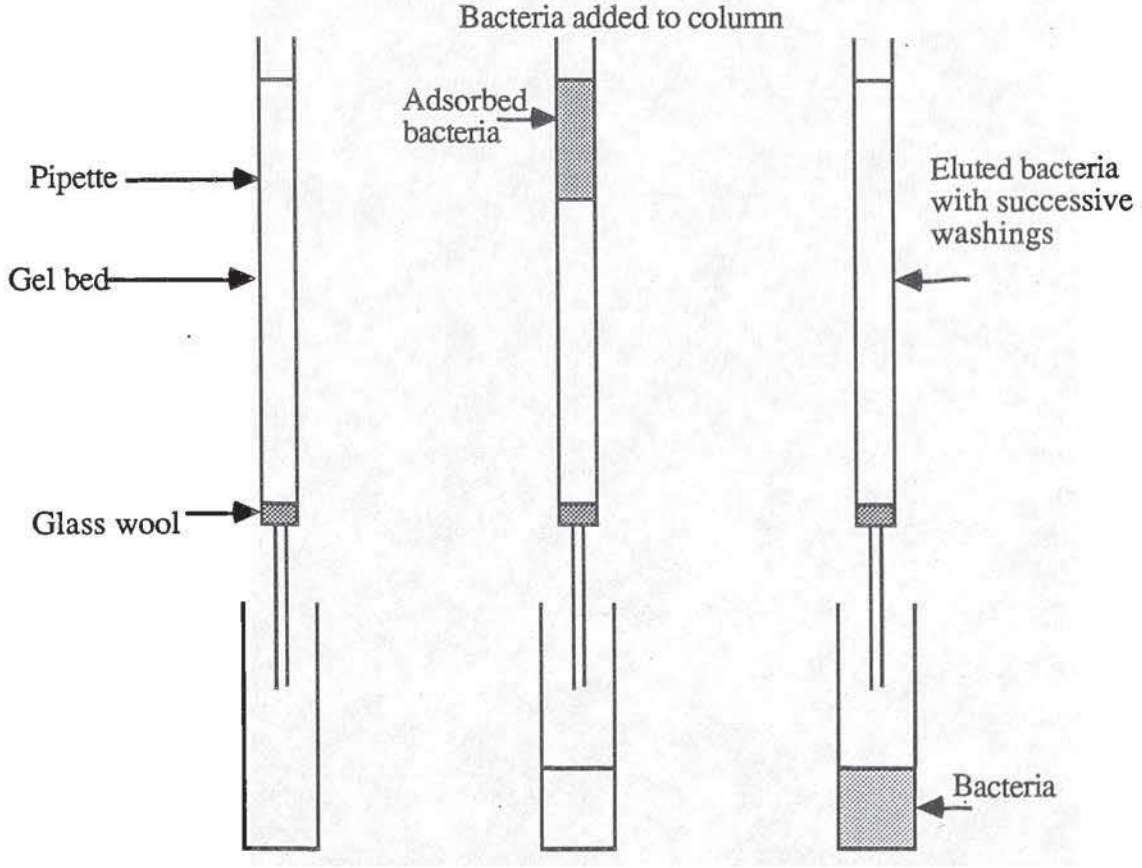
FIGURE 4:1(a) Bacterial Adherence to Hydrocarbon (B.A.T.H.)



1 Hydrocarbon added to aqueous suspension of bacteria

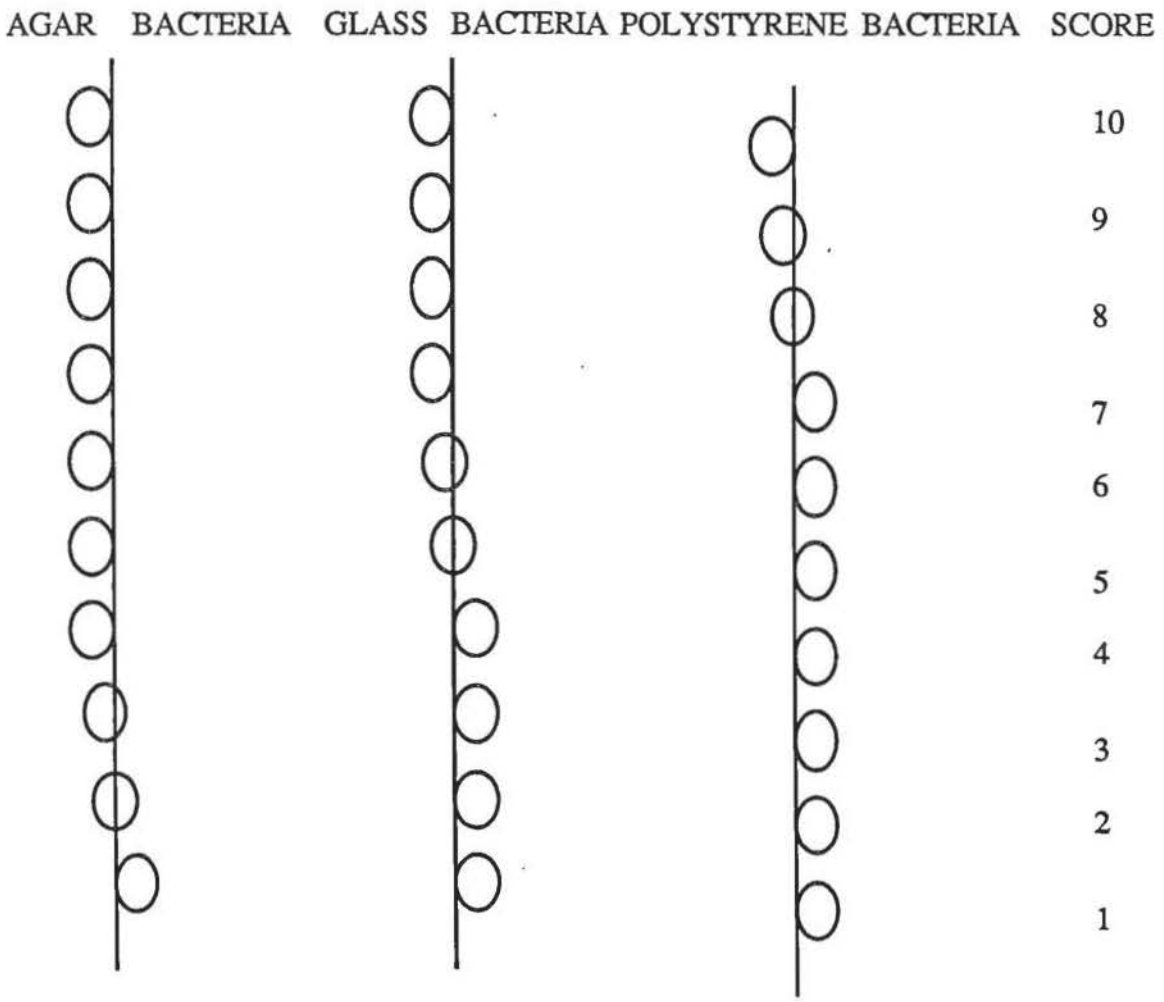
2 After mixing and allowing to stand, adherant bacterial cells rise with the hydrocarbon to form a creamy upper layer and a clear aqueous phase
from Rosenberg, Gutnick & Rosenberg (1980)

FIGURE 4:1(b) Hydrophobic Interaction Chromatography (H.I.C.)



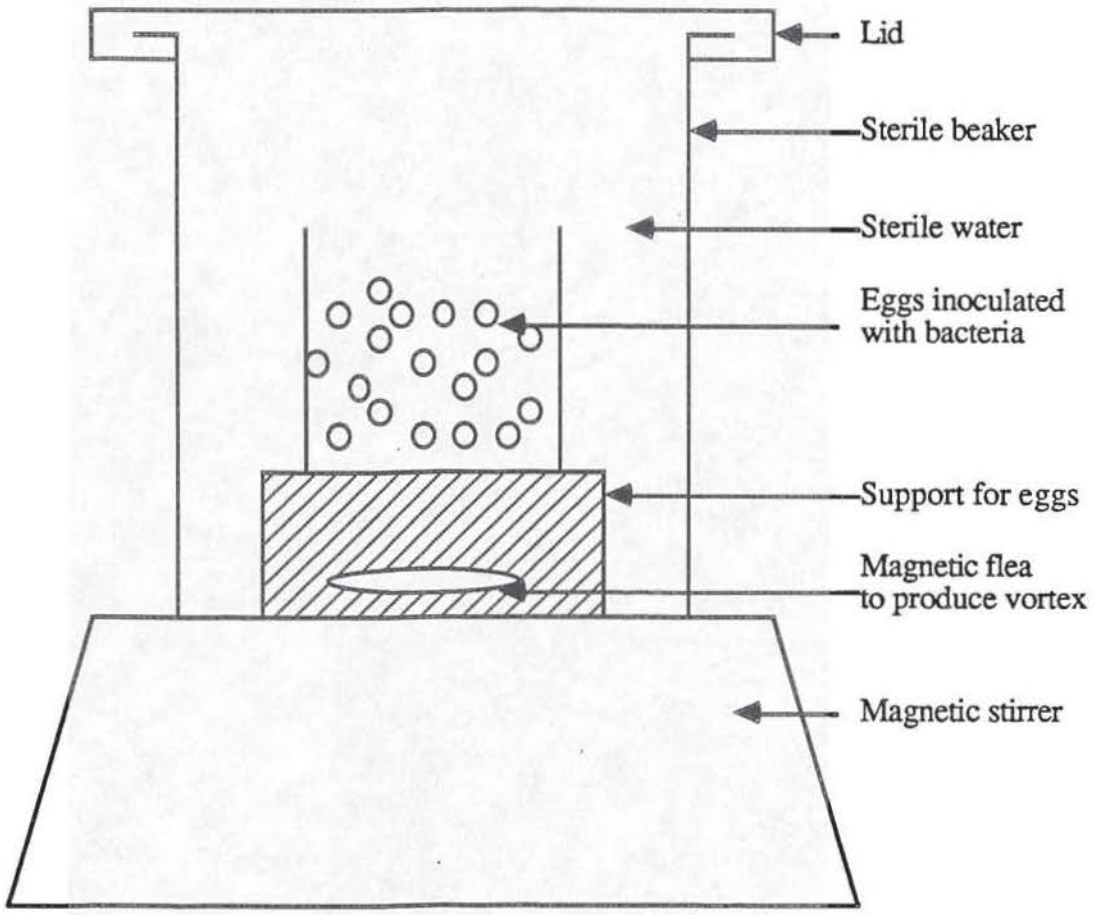
Bacteria of known Optical Density (O.D.) added to column and eluted with 3 successive washings. O.D. of eluate recorded and results expressed as percent of bacteria adhering to column.

FIGURE 4:1(c) Scoring for measuring the surface hydrophobicity of microbial lawns by the Direction of Spreading (D.O.S.) method from Sar & Rosenberg (1987)



A small drop of water is placed at the interface between a bacterial lawn and agar, a bacterial lawn and a glass cover slip and a bacterial lawn and a polystyrene cover slip. The direction of spread of the drop determines the score ; the most hydrophobic is 10 and the least is 1. For example a score of 7 is achieved when the bacteria are more hydrophobic than agar and glass (drop spreading away from bacteria) and less hydrophobic than polystyrene. A score of 9 is achieved when bacteria have the same hydrophobicity as polystyrene.

FIGURE 4:1(d) *In Situ* Detachment From Eggs



After inoculation with bacteria, eggs placed in well circulated sterile water. Numbers of bacteria removed from surfaces recorded every 2 hours.

adsorption to hydrophobic groups on uncharged bed material (Figure 4 : 1b) (Smyth, Jonsson, Olsson, Söderlind, Rosengren, Hjertén & Wadström 1978 ; Xiu, Magnusson, Stendahl & Edebo 1983). In the following trial the ability of bacterial groups to adhere to two sepharose derivatives, octyl and phenyl sepharose was tested.

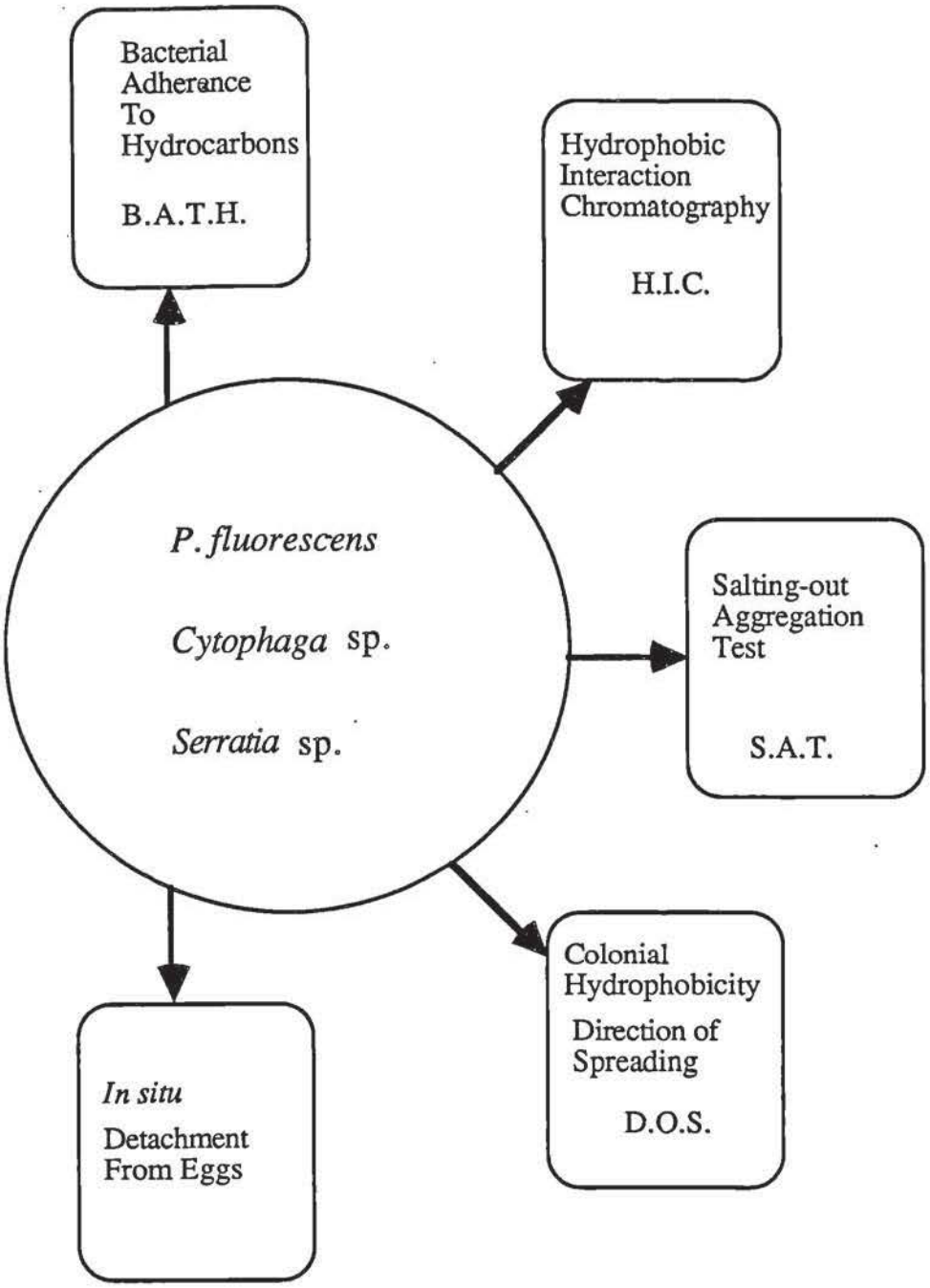
3) *Salting-out Aggregation Test* (S.A.T.) Salting out is a simple technique to measure the hydrophobicity of bacterial cell surfaces based on precipitation by salts (e.g. $(NH4)_2SO_4$), and was first demonstrated by Lindahl, Faris, Wadström & Hjertén (1981) in characterizing surface protein antigens of different strains of enterotoxigenic *Escherichia coli*. The greater the cell surface hydrophobicity the lower the corresponding salt concentration required to cause cell aggregation.

4) *Colonial Hydrophobicity* (Direction of Spreading - D.O.S.). D.O.S. is a technique to measure the surface hydrophobicity of bacterial colonies rather than individual cells. The technique consists of placing a single drop of water at the interface between bacterial lawns and other "test" surfaces and subsequently recording the direction to which the water drop spreads (Figure 4 : 1c). Sar & Rosenberg (1987) first introduced the technique to analyse the hydrophobicity of bacterial colonies isolated from fish mucus against three surfaces ; agar, glass cover slip and a polystyrene coverslip. In contrast to the above work, during the forthcoming trial the hydrophobicity of bacterial groups was tested against four surfaces ; agar, glass coverslip, polystyrene (bacteriological) and polystyrene (tissue culture).

5) *"In situ" Detachment From Eggs.* A final unique trial was devised to directly compare the ability of the three bacterial groups to adhere to egg surfaces. Egg surfaces were inoculated with test bacteria and exposed to a fast flow of water. All bacterial cells removed from egg surfaces were carefully monitored over a 24 hour period (Figure 4 : 1d).

A schematic outline of all tests employed during this study is summarized in Figure 4 : 2.

FIGURE 4 : 2 Flow diagram of experimental protocol.



MATERIALS AND METHODS

Bacterial Adherence To Hydrocarbons (B.A.T.H.). Cell surface hydrophobicity was determined by a method based on that of Rosenberg *et al* (1980). Bacterial cells were harvested at early logarithmic and early stationary phases of growth, washed and resuspended in phosphate-urea-magnesium buffer (P.U.M. buffer) pH 7.1, containing 22.2 g K_2 HPO_4. $3H_2O$, 7.26 g KH_2PO_4, 1.8 g urea, 0.2 g Mg $SO_4.7H_2O$ and distilled water to 1000 ml. Cells were adjusted to an absorbence of (optical density - O.D.) 0.3. 1.2 ml of each bacterial suspension was then placed in acid washed glass tubes (12 X 75 mm) and three volumes of hexadecane ; 0.05 ml, 0.1 ml and 0.2 ml were added to separate tubes containing the bacteria. After preincubation at 25 °C for 15 minutes the two phases were uniformly mixed on a vortex stirrer for 2 minutes and allowed to stand for 15 minutes to allow the hydrocarbon phase to completely "rise". After separation had occurred the aqueous phase was carefully removed with a pasteur pipette and transferred to a 1 ml cuvette and the O.D. determined.

Hydrophobic Interaction Chromatography (H.I.C.). H.I.C. was carried out according to the method of Smyth *et al* (1978) and Xiu *et al* (1983). Cultures were grown in 100 ml volumes of Tryptone Soya Broth (T.S.B., Oxoid, Basingstoke) in 1 litre flasks (120 rpm at 25 °C) corresponding to about 2 X 10^8 colony forming units (cfu's) / ml. Cells were harvested, washed twice and resuspended in 1M $(NH_4)_2$ SO_4 (pH 6.8) at a final concentration of 1 X 10^9 cfu's / ml, buffered with 10 mM $Na_2HPO_4.2H_2O$ to reduce the tendency of cells to aggregate. Hydrophobic derivatives of sepharose (octyl and phenyl) (Pharmacia, Milton Keynes) were washed with distilled water to remove fine particles and ethanol was added to the gel suspension as a preservative. Pasteur pipettes (internal diameter 5 mm ; length 85 mm) were plugged with glass wool, filled with gel to a height of 40 mm by gravity feed and washed with

10 ml of buffered 1 M $(NH_4)_2 SO_4$. Bacterial suspensions 100 µl of 1 x 10^9 cells / ml were added to columns and allowed to drain into gel beds. Gel beds were washed with 5 ml of buffered 1 M $(NH_4)_2 SO_4$ (flow rate 1 - 2 ml / minute), the eluate collected and the O.D. measured. A further 5 ml of 0.5 M $(NH_4)_2 SO_4$ were added to columns and the O.D. of the resulting eluate measured. Finally, 5 ml of 95% ethanol was added to the columns and again the O.D. of the eluate measured. As a control a similar test was run on a sepharose column to test for non-specific adsorption to the column. In addition, 100 µl of the inoculum was added to 5 ml of the eluting medium and after mixing the O.D. recorded. The difference between this measurement and that of the eluate from the sepharose column gave the value of non-specific adsorption to the sepharose column. Relative surface hydrophobicity of the cell suspension was expressed as the percentage of cells remaining on the column after each of the three washings.

Salting-out Aggregation Test (S.A.T.) Bacterial cell surface hydrophobicity as measured by "salting out" was performed according to the method of Lindahl *et al* (1981). Cells were grown in 100 ml volumes of T.S.B. media in 1 l flasks to stationary growth phase (120 rpm at 25 °C). Cells were harvested, washed twice and resuspended in 2 mM $Na_2HPO_4.2H_2O$ (pH 6.8) at a concentration of 1 x 10^9 cells / ml. A solution of 4 M $(NH_4)_2 SO_4$ dissolved in 2 mM $Na_2HPO_4.2H_2O$ (pH 6.8) was further serially diluted with 2 mM $Na_2HPO_4.2H_2O$ (pH adjusted with NH_4OH). The serial dilution of $(NH_4)_2 SO_4$ was constructed ranging from 4.0 to 0.5 M differing by 0.5 M per dilution and in addition, a second serial dilution ranging from 0.5 M to 0.05 M differing by 0.05 M was also constructed. The pH of each dilution was corrected to pH 6.8 using NH_4OH as required. Bacterial suspensions (25 µl of 1 X 10^9 cells / ml) were mixed with an equal volume of salt solution on ground glass cavity slides. The bacterial and salt solution mixture was gently rocked for 2 minutes at room temperature and the result recorded. A positive result was achieved when most bacteria aggregated (white aggregates of approximately 0.1 mm diameter) surrounded by a clear medium. In

contrast a reaction with relatively few or no aggregates was recorded as negative. All readings were compared to a positive control - the highest molarity of salt plus bacteria and a negative control - bacterial suspensions mixed with 2 mM $Na_2HPO_4.2H_2O$ without addition of salt.

Colonial Hydrophobicity. (Direction of Spreading). Cells were cultured in 25 ml volumes of T.S.B. in 100 ml flasks and stationary phase cells were heavily streaked onto half of an agar plate (diameter 90 mm - tryptone soya agar, T.S.A., Oxoid). Onto the other half of the agar the following surfaces were placed ; sterile glass coverslip, polystyrene (bacteriological) and polystyrene (tissue culture). Cells were left to grow for 48 hours at 25 °C. A small drop of water (0.01 ml) was placed at the interface between the bacterial lawn and each of the above three surfaces, and between the bacterial lawn and the agar. The direction to which the drop of water spread was recorded.

In situ Detachment From Eggs. Bacteria were cultured in 100 ml volumes of T.S.B. in 1 litre flasks to stationary phase, washed and resuspended in 100 ml volumes of sterile water taken from the Aston Fish Culture Unit at a final concentration of 1 x 10^6 cells / ml. Rainbow trout eggs, after stripping were sub-divided into groups of 100 eggs. Each group of eggs was added to the above bacterial suspensions (*P. fluorescens, Cytophaga* sp. and *Serratia* sp.) and water hardened for 1 hour at 5 °C. 50 eggs were carefully removed (5 batches of 10 eggs), added to 5 ml of diluent (peptone, 0.1% / saline, 0.85% - Chapter 2) and shaken on a vortex stirrer for 2 minutes, allowing the number of cells adhering to each egg to be estimated. The remaining 50 eggs in each group were carefully transferred to 500 ml of sterile water, maintained at 5 °C, and placed in a vortex strong enough to ensure slight egg movement. The number of bacteria released from egg surfaces into the surrounding water was recorded immediately and thereafter, every 2 hours for a 24 hour period.

Results were expressed as the percentage of bacteria released from egg surfaces into the surrounding water.

Statistical analysis. The numbers of cells adhering to hexadecane (B.A.T.H.) and to phenyl and octyl sepharose (H.I.C.) were compared by 2 way analysis of variance. The numbers of cells removed from egg surfaces (*in situ*) were analysed by 2 way spilt-plot analysis of variance. The differences between means were compared by calculating the standard errors and 95% confidence limits appropriate to the split-plot design (Ridgman 1975 ; Snedecor & Cochran 1980).

RESULTS

Bacterial Adherence To Hydrocarbons. Adherence of the three bacterial groups to n-hexadecane is summarized in Figure 4 : 3 (a-c). Overall, all three bacterial groups demonstrated relatively high cell surface hydrophobicity by B.A.T.H. techniques. However, stationary phase cells adhered markedly better to hexadecane than log growth phase cells. Figure 4 : 3 (a) compares the affinity of early log growth *P. fluorescens* cells to early stationary phase cells after the addition of 3 different volumes of the test hydrocarbon. Stationary phase cells adhered in significantly greater amounts ($P < 0.05$) to the hydrocarbon at all 3 volumes compared to log phase cells. However, as volume of added hydrocarbon increased, from 0.05 ml to 0.2 ml, a corresponding increase in numbers of adhered cells (both growth stages) was also recorded (Appendix 4 : 1). Figure 4 : 3 (b) compares the affinity of early log growth phase cells of *Cytophaga* sp. to early stationary phase cells at 3 different volumes of added hydrocarbon. Again, early stationary phase cells adhered in significantly greater amounts ($P < 0.05$) to the test hydrocarbon compared to early log growth cells but, the difference in adherence between the two phase of cell growth was less marked compared to the *P. fluorescens* cells (above). Indeed, in comparison to *P. fluorescens* cells, those of *Cytophaga* sp. adhered slightly less well to the test hydrocarbon at all 3 volumes and at both stages of cell growth (Appendix 4 : 2). Figure 4 : 3 (c) compares the affinity of early log growth phase cells of *Serratia* sp. to early stationary phase cells at 3 different volumes of added test hydrocarbon. Stationary phase cells adhered in very significantly greater amounts compared to log growth phase cells ($P < 0.01$). Overall, a significant increase in numbers of adhering cells occurred with addition of increased volumes of hydrocarbon ($P < 0.05$), due possibly to the unique hydrophobicity characteristics exhibited mainly by stationary phase cells. Furthermore, overall,

FIGURE 4:3 (a) Bacterial adherance to hexadecane *(P. fluorescens)*

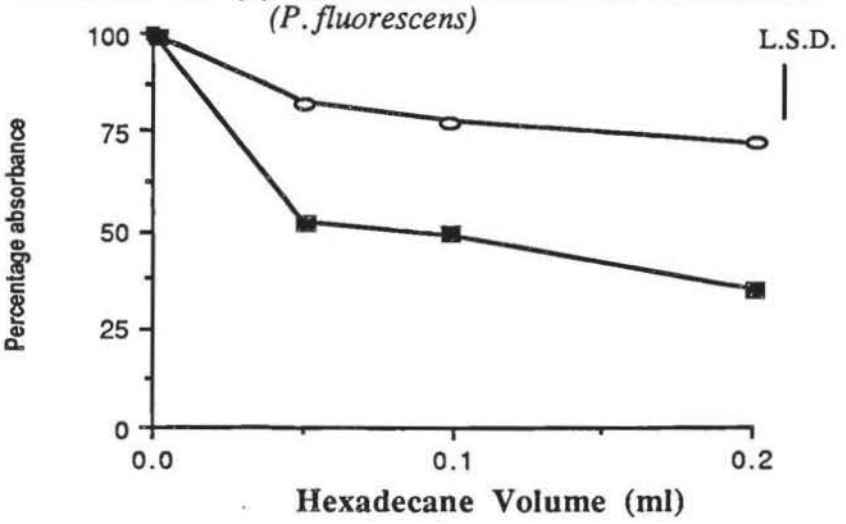


FIGURE 4:3 (b) Bacterial adherance to hexadecane *(Cytophaga sp.)*

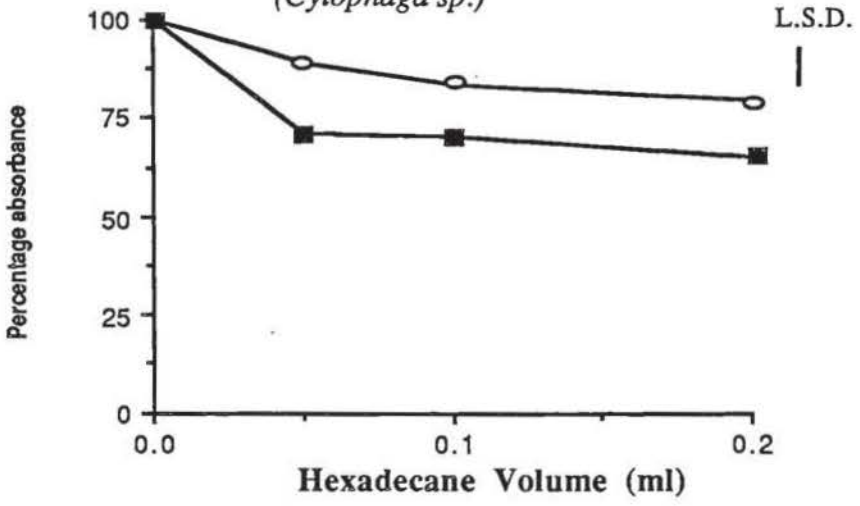
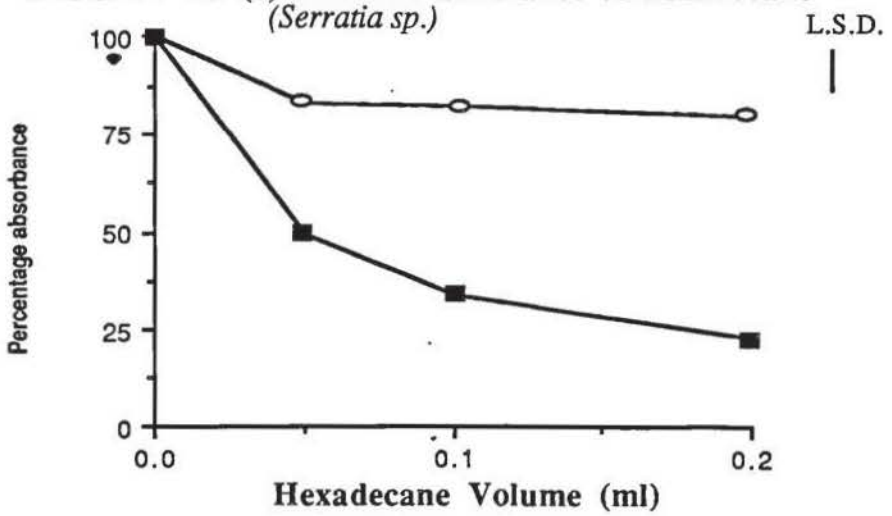


FIGURE 4:3 (c) Bacterial adherance to hexadecane *(Serratia sp.)*



○ Early log growth phase cells ■ Early stationary phase cells

L.S.D. = Least significant difference between means at the 95% level

FIGURE 4 : 4 (a) Hydrophobic Interaction Chromatography Adherance to Phenyl Sepharose

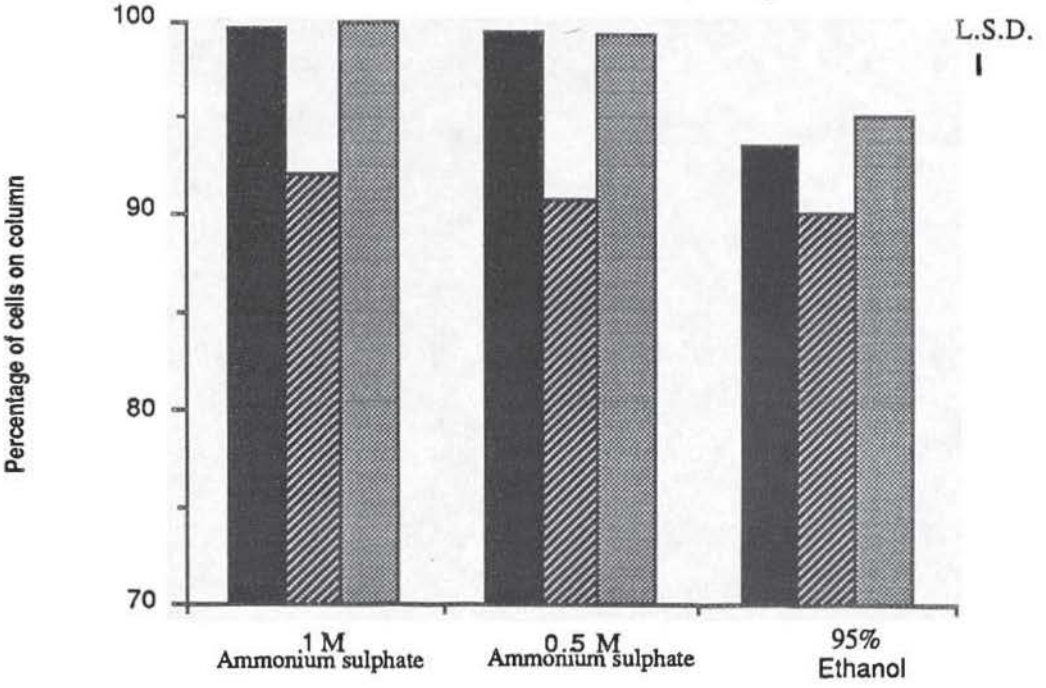
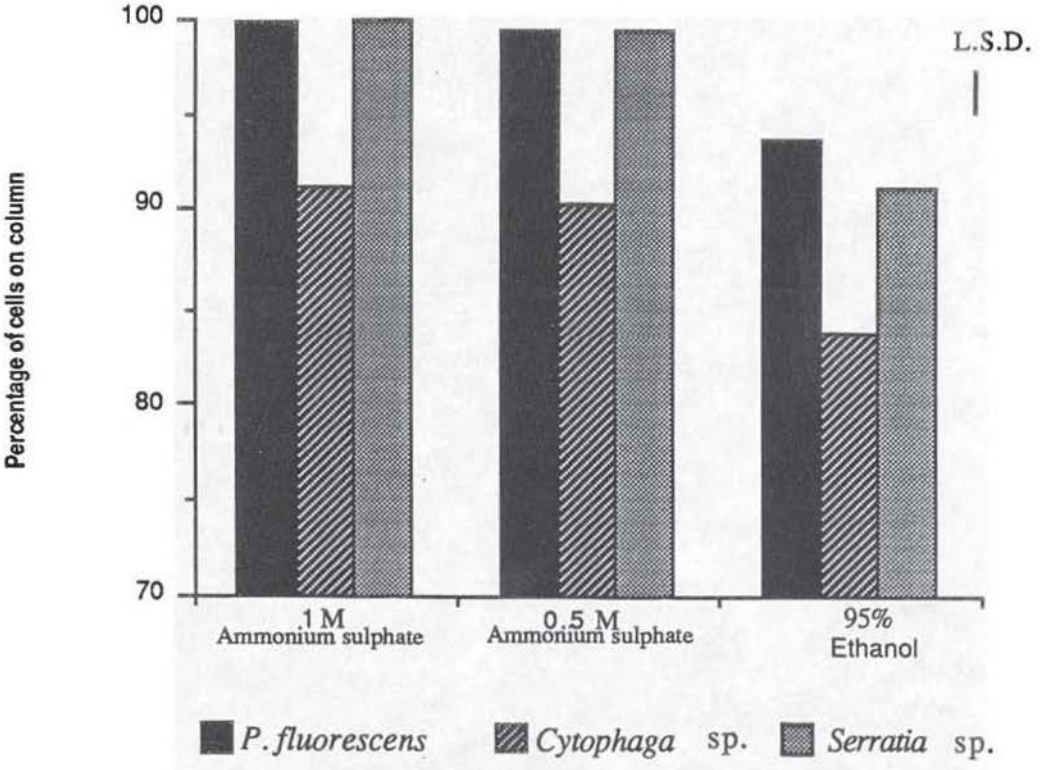


FIGURE 4 : 4 (b) Hydrophobic Interaction Chromatography Adherance to Octyl Sepharose



L.S.D. = Least significant difference between means at the 95 % level.

stationary phase cells of *Serratia* sp. adhered in far greater numbers to the test hydrocarbon than cells of either *P. fluorescens* or *Cytophaga* sp. (Appendix 4 : 3).

Hydrophobic Interaction Chromatography. Adherence of bacterial groups to phenyl and octyl sepharose are summarized in Figure 4 : 4 (a-b). All 3 bacterial groups adhered strongly to both gels in the presence of $(NH_4)_2\ SO_4$, although phenyl sepharose appeared to have a slightly higher adsorptive capacity than octyl sepharose. Statistical analysis revealed highly significant differences in numbers of each bacterial species adhering to both gels ($P < 0.001$ and $P < 0.001$). In addition, a highly significant difference in numbers of adhering cells for both gels was revealed after each elution step ($P < 0.001$ and $P < 0.001$). However, overall relatively few bacterial cells were removed from columns during the first two elution steps and even after addition of 95% ethanol, the majority of cells still remained adhering to columns. Addition of *P. fluorescens* to both phenyl and octyl sepharose resulted in adherence of bacteria as tight bands at the top of gel beds. Wash eluates contained very few bacterial cells and over 90% of cells remained attached to columns even after addition of ethanol. Addition of *Serratia* sp. to columns resulted in adherence throughout the columns. Wash eluates contained few bacteria and most cells adsorbed tightly to both gels. Relatively similar numbers of *Serratia* sp. adsorbed to gels as *P. fluorescens*, although slightly more *Serratia* sp. were removed by ethanol from octyl sepharose than from phenyl sepharose. In contrast, *Cytophaga* sp. did not adhere as tightly to columns as the other two bacterial groups. Addition of *Cytophaga* sp. to gels resulted in cells moving down the column and wash eluates (especially 1 M $(NH_4)_2\ SO_4$) contained markedly more cells than were obtained from *P. fluorescens* or *Serratia* sp. After elution with ethanol more *Cytophaga* sp. cells remained attached to phenyl rather than octyl sepharose but, even so, despite addition of ethanol over 80 % of cells still remained attached to both columns (Appendix 4 : 4 to 4 : 5). In comparison, during control experiments in which bacteria were passed through uncharged sepharose columns most of the bacteria (65% to 70%) were removed after the first two elution steps.

Salting-out Aggregation Test. A summary of the results obtained to measure cell surface hydrophobicity by "salting out" is shown in Table 4 : 1. All three bacterial groups demonstrated high cell surface hydrophobicity, for only low concentrations of salt ($(NH_4)_2\ SO_4$) were required to mediate cell aggregation. *Serratia* sp. and *P. fluorescens* revealed similar cell surface hydrophobicity by this technique with a concentration of 0.15 M to 0.2 M $(NH_4)_2\ SO_4$ required to cause cell aggregation. In contrast to other earlier trials (B.A.T.H. and H.I.C.) *Cytophaga* sp. proved to have the most hydrophobic cell surface with only 0.05 M to 0.1 M $(NH_4)_2\ SO_4$ required to mediate cell aggregation.

Direction Of Spreading. Bacterial lawns were compared to four surfaces of increasing hydrophobicity values ; polystyrene (tissue culture), 2% agar, glass and polystyrene (bacteriological). All 3 bacterial groups exhibited high colonial hydrophobicity. *P. fluorescens* was more hydrophobic than polystyrene (tissue culture), 2% agar and glass but, less hydrophobic than polystyrene (bacteriological) and thus was allocated a score of 7 points (scale 1 to 10). *Serratia* sp. proved to be slightly more hydrophobic than *P. fluorescens* and was allocated a score of 8. *Cytophaga* sp. proved to possess the highest colonial hydrophobicity, equal to that of polystyrene (bacteriological), and was thus awarded a score of 9 points.

In situ Detachment From Eggs. Statistical analysis of this trial revealed overall a significant difference between numbers of cells washed off eggs between bacterial groups ($P < 0.05$). A highly significant increase in numbers of cells removed from egg surfaces with time was demonstrated ($P < 0.001$) and a highly significant interaction between bacterial species and time was also revealed ($P < 0.001$). During this trial, eggs inoculated with bacteria were carefully placed into the 500 ml volumes of sterile water. However, despite such care many bacteria were detached from egg surfaces as soon as they entered the water, possibly due to surface tension effects

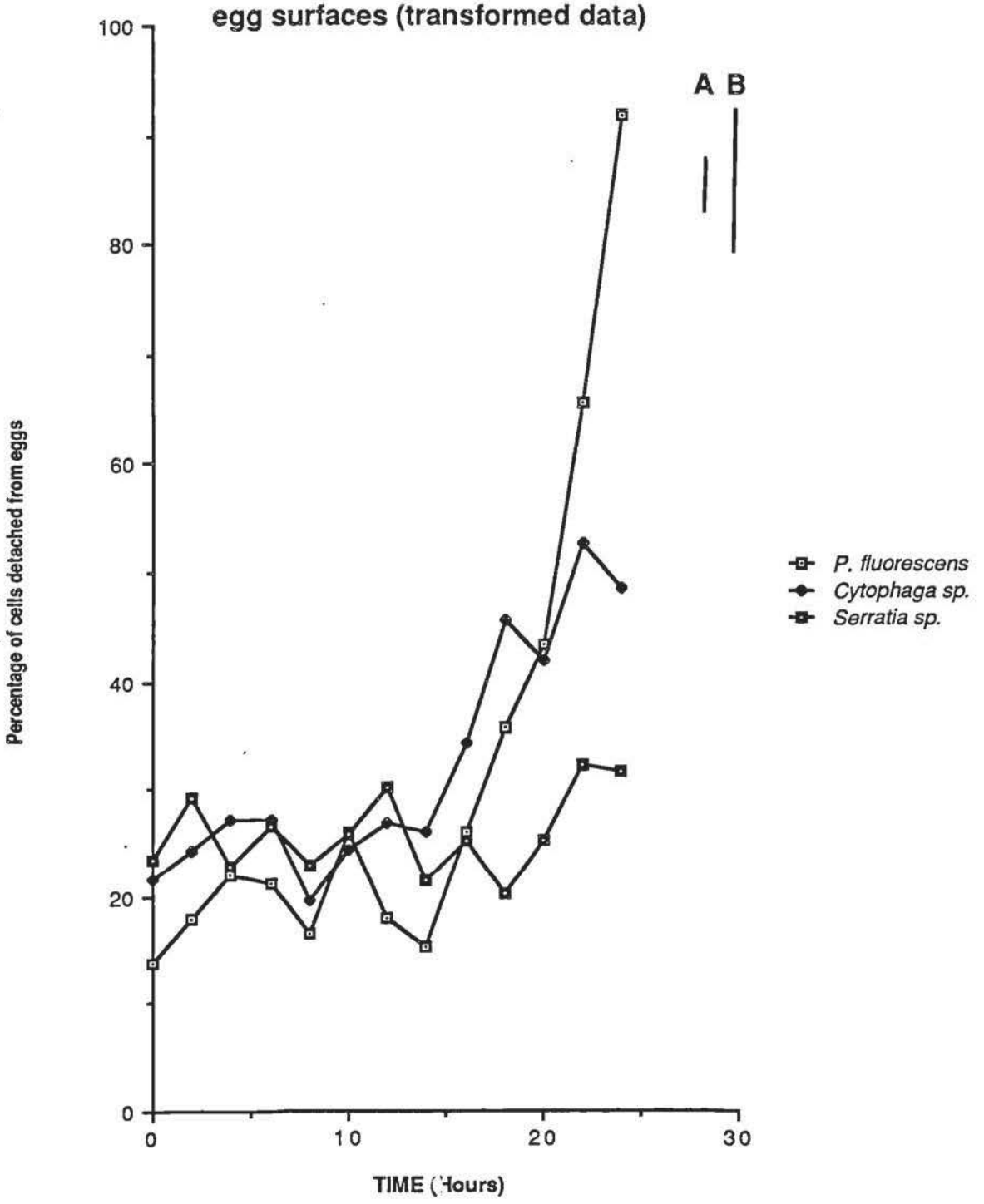
Table 4 : 1 Salt Aggregation Test - concentration of $(NH_4)_2 SO_4$ required to precipitate bacterial suspensions

Salt concentration		*P. fluorescens*	*Serratia* sp.	*Cytophaga* sp.
4 M	1)	+	+	+
	2)	+	+	+
	3)	+	+	+
3.5 M	1)	+	+	+
	2)	+	+	+
	3)	+	+	+
3 M	1)	+	+	+
	2)	+	+	+
	3)	+	+	+
2.5 M	1)	+	+	+
	2)	+	+	+
	3)	+	+	+
2 M	1)	+	+	+
	2)	+	+	+
	3)	+	+	+
1.5 M	1)	+	+	+
	2)	+	+	+
	3)	+	+	+
1M	1)	+	+	+
	2)	+	+	+
	3)	+	+	+
0.5 M	1)	+	+	+
	2)	+	+	+
	3)	+	+	+
0.45 M	1)	+	+	+
	2)	+	+	+
	3)	+	+	+
0.4 M	1)	+	+	+
	2)	+	+	+
	3)	+	+	+
0.35 M	1)	+	+	+
	2)	+	+	+
	3)	+	+	+
0.3 M	1)	+	+	+
	2)	+	+	+
	3)	+	+	+
0.25 M	1)	+	+	+
	2)	+	+	+
	3)	+	+	+
0.2 M	1)	+	+	+
	2)	-	+	+
	3)	+	-	+
0.15 M	1)	-	-	+
	2)	-	-	+
	3)	-	-	+
0.1 M	1)	-	-	+
	2)	-	-	+
	3)	-	-	-
0.05 M	1)	-	-	-
	2)	-	-	-
	3)	-	-	-

+ = precipitated, - = not precipitated

FIGURE 4:5 In Situ Detachment From Eggs

Percentage of bacteria removed from inoculated egg surfaces (transformed data)



A = 95% confidence limits for differences within a treatment and B = 95% confidence limits between 2 treatments at the same or different times

(Figure 4 : 5). An estimated 13.8% of total *P. fluorescens* cells originally attached to egg surfaces were immediately recovered from the surrounding water. Similarly, 21.7% of total *Cytophaga* sp. cells and 23.5% of *Serratia* sp. cells were immediately recovered from the surrounding water. During the trial little marked difference in numbers of bacteria detached from egg surfaces was recorded until after 16 hours. Generally, from 16 hours until the end of the trial (24 hours) *P. fluorescens* cells (compared to *Cytophaga* sp. and *Serratia* sp.) were recovered in increasing numbers from the surrounding water. Indeed by 24 hours an estimated 91.7% of *P. fluorescens* cells originally attached to eggs had been "dislodged" from egg surfaces. In contrast, 48.3% of *Cytophaga* sp. cells were removed by 24 hours and only 31.4% of all *Serratia* sp. cells had been removed by the end of the trial (Appendix 4 : 6).

DISCUSSION

Successful bacterial colonization of live incubating salmonid egg surfaces is potentially dependant on initial ability to adhere to eggs. The frequent recovery of *Cytophaga* sp. and *P. fluorescens* from the surfaces of live eggs (Bell, Hodgkiss & Hoskins 1971 ; Trust 1972) indicates that these two groups possess such abilities. In contrast, *Serratia* sp. are only rarely recovered from egg surfaces, despite their common occurrence in many natural environments ; soil, water, plant surfaces and even as opportunistic human pathogens (Grimont & Grimont 1978). It is therefore possible that *Serratia* sp. do not possess the initial ability to adhere to egg surfaces. However, a range of tests to determine adherence properties of all three (above) bacterial groups revealed little significant difference in cell surface hydrophobicity between them. Indeed, adherence to hexadecane by B.A.T.H. techniques revealed *Serratia* sp. to be the most hydrophobic followed by *P. fluorescens* and *Cytophaga* sp. Similar results have also been reported by Rosenberg *et al* (1980) and Kjelleberg, Lagercrantz & Larsson (1980) who have clearly shown the high cell surface hydrophobicity of *Serratia* sp. using B.A.T.H. techniques.

During B.A.T.H. trials, removal of cells from the aqueous phase depends on their ability to adhere to the hydrocarbon phase. Thus, the surface area created during the mixing of the two liquid phases is of great importance and directly dependant on size and number of hexadecane droplets present in the aqueous phase (Van Loosdrecht, Lyklema, Norde, Schraa & Zehnder 1987). Addition of increased volumes of hexadecane should therefore in turn increase the number of adhering bacterial cells, which was subsequently shown by all three bacterial groups in the present trials.

Culture age was also found to influence results obtained, as early stationary phase cells invariably exhibited higher levels of cell surface hydrophobicity compared to early log growth phase cells, a phenomenon demonstrated during studies by other authors (Rosenberg *et al* 1980 ; Rosenberg, Perry, Bayer, Gutnick, Rosenberg & Ofek

1981). Such a close correlation between increasing cell age and increasing cell surface hydrophobicity might be a significant factor in the process of bacterial dispersal. Older cells (and therefore more hydrophobic) within colonies may produce daughter cells of a relatively lower hydrophobic nature, which in turn may not effectively adhere to the colony but pass "freely-suspended" into water. In this state bacterial cells could be dispersed to new environments where, with increasing cell age and therefore hydrophobicity, colonization of new surfaces would be promoted.

Measurements of cell surface hydrophobicity by H.I.C. also revealed that all three bacterial groups were highly hydrophobic, agreeing with Olsson & Westergren (1982) that a close correlation between B.A.T.H. and H.I.C. results can be obtained. H.I.C. revealed that *Serratia* sp. and *P. fluorescens* had the highest hydrophobicity, followed by *Cytophaga* sp. Pretreatment of gel columns and first elution with 1 M $(NH_4)_2\ SO_4$ decreases the charge on the gel surface, suppressing electrostatic interactions, and in turn encouraging adhesion by accentuating hydrophobicity (Smyth *et al* 1978). Decreasing salt concentrations, as in the second elution with 0.5 M $(NH_4)_2\ SO_4$, increases gel surface charge and cells expressing weak hydrophobicity are eluted. In this trial few cells (including *Cytophaga* sp.) were eluted with 0.5 M $(NH_4)_2\ SO_4$, a reflection of high cell surface hydrophobicity of all 3 groups. Great emphasis should not be placed on the slightly poorer performance of *Cytophaga* sp. compared to *P. fluorescens* and *Serratia* sp. for this might simply be a consequence of limited cell damage as Griffin (1981) considered Gram -ve bacteria to be extremely sensitive to reduced osmotic potentials as generated in this trial by high salt concentrations.

A close relationship exists between H.I.C. and S.A.T. techniques (Lindahl *et al* 1981) and which was further emphasized in this trial by the common use of $(NH_4)_2\ SO_4$ in both trials. In H.I.C. techniques, the lower the salt concentration needed to elute the bacteria, the more hydrophobic the cell surface. Similarly during "salting out" the lower the concentration of salt needed to cause aggregation the more hydrophobic the cells. All three bacterial groups required low concentrations of salt to cause aggregation and were thus highly hydrophobic. Little difference in cell surface

hydrophobicity existed between *Serratia* sp and *P. fluorescens* but, in contrast to H.I.C., *Cytophaga* sp. were considered to be most hydrophobic by S.A.T. However, the improved performance of *Cytophaga* sp. in this trial should not be over stressed for complete agreement between H.I.C. and "salting out" cannot be expected (Lindahl *et al* 1981) and may simply be due to experimental variation, procedures carried out on different occasions and use of different batches of bacteria.

Measurement of colonial hydrophobicity by D.O.S. can only ever produce semi-quantitative data which may be subject to severe variation between individuals undertaking the technique. However, careful control of experimental conditions can reveal sufficient correlation between replicates to maintain confidence in the chosen test. Previous trials (B.A.T.H., H.I.C. and S.A.T.) all revealed information on surface hydrophobicity of individual suspended cells, which though may not necessarily indicate the hydrophobicity of the colony surface from which these cells originated (Sar & Rosenberg 1987). Furthermore, all previous techniques have necessitated a number of washings and resuspensions of cells, removing at best partially any coatings that might have been produced by cells in turn potentially altering their true hydrophobic nature. However, in common with suspended cells, bacterial colonies exhibited marked hydrophobicity, ranging from a score of 7 (*Serratia* sp.) to the most hydrophobic, 9 (*Cytophaga* sp.). The high score obtained by *Cytophaga* sp. during this trial could be due to spatial differentiation of cells within the colony, deliberately placing the most hydrophobic cells near the colony edge or, might be a result of certain characteristics (such as slime) suffering less damage during the performance of this technique.

Desorption of bacteria from egg surfaces was designed to assess adherence *in situ* by placing eggs exposed to bacteria under a fast flow of water, and recording the relative numbers of bacteria "washed off" over a 24 hour period. Initially, eggs were exposed to bacteria for a period of 1 hour at 5 °C, potentially sufficient time for most bacterial cells to adhere "reversibly" to egg surfaces. However, the removal of many bacterial cells from eggs at the start of the trial (possibly by surface tension effects) demonstrates that bacteria are only weakly held in contact with surfaces during the

phase of "reversible" adherence. Throughout the trial fluctuations in bacterial numbers were recorded for all 3 groups, possibly due to the effect of inherent error involved in plate count techniques (Buck 1979), or possibly the result of a continual cycle of bacterial attachment and desorption from both egg and glass surfaces, a consequence of the relatively weak attraction of cells to surfaces during "reversible" adherence (as described). All equipment and water used during the trial were maintained at 5 °C to preclude the possibility that increased bacterial growth alone could be responsible for the higher numbers of bacteria recovered from the surrounding water. However, it is possible that marked increases in numbers of *P. fluorescens* recovered towards the end of the trial may be complicated by effects of additional growth, and not simply a result of cells being washed off egg surfaces. However, such a large increase in numbers of recovered bacteria towards the end of the trial was not shown with either *Serratia* sp. or *Cytophaga* sp. (despite similar growth rates between *P. fluorescens* and *Serratia* sp., Chapter 3).

Bacterial cell surface hydrophobicity is not only important in encouraging initial colonization of surfaces by "reversible" adherence but, is also important during the second stage of adherence - "irreversible" adherence. Indeed during "irreversible" adherence, properties of cell surface hydrophobicity can be altered by cells to further facilitate adherence to the selected substrate (Marshall 1976). Bacterial adherence can be further sub-divided into two sections ; 1) specific adhesion and 2) non-specific adhesion. Specific adhesion is frequently mediated by surface structures such as simple proteins (lectins) which often provide a link between bacterial cells and the substrate by binding to specific sugar moieties. Lectins are extremely selective, tending to bind to particular sugar molecules or groups of molecules (Sharon 1977). However, in natural aquatic environments bacterial adherence is more likely to be achieved by the production of a range of cell surface components that together contribute to increased cell surface hydrophobicity. Such examples include the production of fimbriae (or surface fibrils) (Stenström & Kjelleberg 1985), glycocalyx fibres, pili or spinae, all providing firmer anchorage to the substrate (Marshall 1976 ; Costerton, Irvin & Cheng

1981). In some instances (e.g. *Aeromonas salmonicida*) Trust, Kay & Ishiguro (1983) considered that cell hydrophobicity is due to a single "adhesin" factor which dominates the outer cell surface. In common, the cell surface hydrophobicity of *Serratia* sp. may also be due to a single factor. Rosenberg *et al* (1980) and Kjelleberg *et al* (1980) reported a positive correlation between increased pigmentation of *Serratia* sp. cells and increased cell surface hydrophobicity. Pigmentation in *Serratia* sp. is a function of pyrrol containing pigments (termed prodigiosins) which are localized on the cell surface and thus may in turn also provide increased cell surface hydrophobicity.

Once established on surfaces, bacteria produce a mass of tangled fibres, composed of polysaccharide (termed the glycocalyx), which in turn surrounds the bacterial cell wall (Costerton, Geesey & Cheng 1978). Within the glycocalyx bacterial replication can occur to form an adherent microcolony (Costerton *et al* 1981) and it is likely that the predominant bacterial populations in aquatic ecosystems are composed of such glycocalyx enclosed microcolonies (Costerton & Geesey 1979 ; Geesey, Richardson, Yeoman, Irvin & Costerton 1977). Subsequent cell division within microcolonies and additional bacteria from surrounding water leads to the production of a "biofilm" overlaying the surface, increasing both nutrient availability and firmer adherence (Costerton, Cheng, Geesey, Ladd, Curtis-Nickel, Dasgupta & Marrie 1987).

Overall, all three bacterial groups during this study exhibited high levels of cell surface hydrophobicity. However, it must be remembered that the four standard adherence tests all involve the use of *in vitro* techniques but, bacteria propagated *in vitro* are known to loose several of their original characteristics, including their glycocalyx (Costerton *et al* 1981). Therefore, data obtained with cultured laboratory strains may only offer a poor prediction of bacterial behaviour under natural conditions (Olsson & Westergren 1982). Thus, a unique *in vivo* test involving direct attachment to egg surfaces was constructed to offer a slightly more realistic measurement of adherence properties to live egg surfaces. After initial losses, despite a strong water flow rate to remove cells from egg surfaces all 3 bacterial groups adhered strongly to eggs for the majority of the sampling period. Therefore, *P. fluorescens*, *Cytophaga* sp

and *Serratia* sp. all revealed high cell surface hydrophobicity *in vitro*, and *in vivo* with an ability to firmly adhere to egg surfaces. However as stated above, under natural conditions, few *Serratia* sp. cells are actually recovered from incubating egg surfaces, despite their undoubted presence in the environment. Thus, although *Serratia* sp. have a hydrophobic nature and a proven ability to adhere to salmonid eggs, successful colonization of egg surfaces would seem to involve other crucial factors, possibly including resistance to physical / chemical factors, or specific nutritional requirements. Therefore, although the ability to adhere to eggs may not necessarily result in successful colonization, it must certainly be a prerequisite of great importance.

SUMMARY

Investigations into adherence properties of *Pseudomonas fluorescens* and *Cytophaga* sp.(both frequently isolated from incubating egg surfaces) and *Serratia* sp. (rarely recovered from incubating egg surfaces) indicates that all three bacterial genera possess high cell surface hydrophobicity. It is likely that such a property is advantageous for both initial colonization and subsequent domination of egg surfaces. However, as a range of varying tests revealed little difference in adherence properties between all three bacterial genera, it must be assumed that a combination of other factors are responsible for "inhibiting" the establishment of *Serratia* sp. on incubating salmonid egg surfaces.

CHAPTER FIVE

THE BACTERIAL FLORA OF RAINBOW AND BROWN TROUT EGGS RELATED TO INCUBATING EGG MORTALITY

INTRODUCTION

Incubation of rainbow trout eggs often meets with varying degrees of success. Many diverse factors are probably responsible for such varying success ranging from sperm motility (Moccia & Munkittrick 1987 ; Munkittrick & Moccia 1987) to time of egg stripping (Springate, Bromage, Elliott & Hudson 1984). Water quality is also of equal importance for, flow rate, dissolved oxygen, pH and water temperature will all effect egg hatching success. In addition, quality of husbandry practice will not only influence the bacterial population of the incubating water and of egg surfaces (Chapter 2) but, will also directly mediate egg survival. Excessive illumination of eggs (Leitritz & Lewis 1976), physical disturbance of eggs before eyeing resulting in damage to developing embryo's (Smirnov 1975 ; Laird & Wilson 1979) and failure to prevent fungal colonization through malachite green treatment (Cline & Post 1972) will all result in egg loss. Besides environmental conditions and husbandry practices - factors which are under the control of the farmer, it is likely that some further losses will be due to failures in egg physiology whether this be genetic or embryological. Furthermore, tradition amongst aquaculturists suggests that eggs of other salmonids are better suited to commercial hatchery environments and often have higher hatching success rates than rainbow trout eggs. Little direct evidence for this notion exists in the scientific literature (Bromage & Cumaranatunga 1988) but, through disparate studies associated in the main with egg quality and nutrition, the substance of such a notion becomes visible. For example, from 10 separate batches of rainbow trout eggs, Hirao, Yamada & Kikuchi (1954) obtained eyeing figures ranging from 37.5 % to 94.0 % with a mean of 72.8 %. In comparison, survival to eyeing for other salmonids is often greater ; chinook salmon 56% (Johnson 1984) and 76% (Erdahl, Erdahl & Graham 1984) ; pink salmon 96% (Wertheimer 1984) ; Atlantic salmon 77% and 85% (Crim & Glebe 1984) ; and brown trout 76% (Erdahl *et al* 1984).

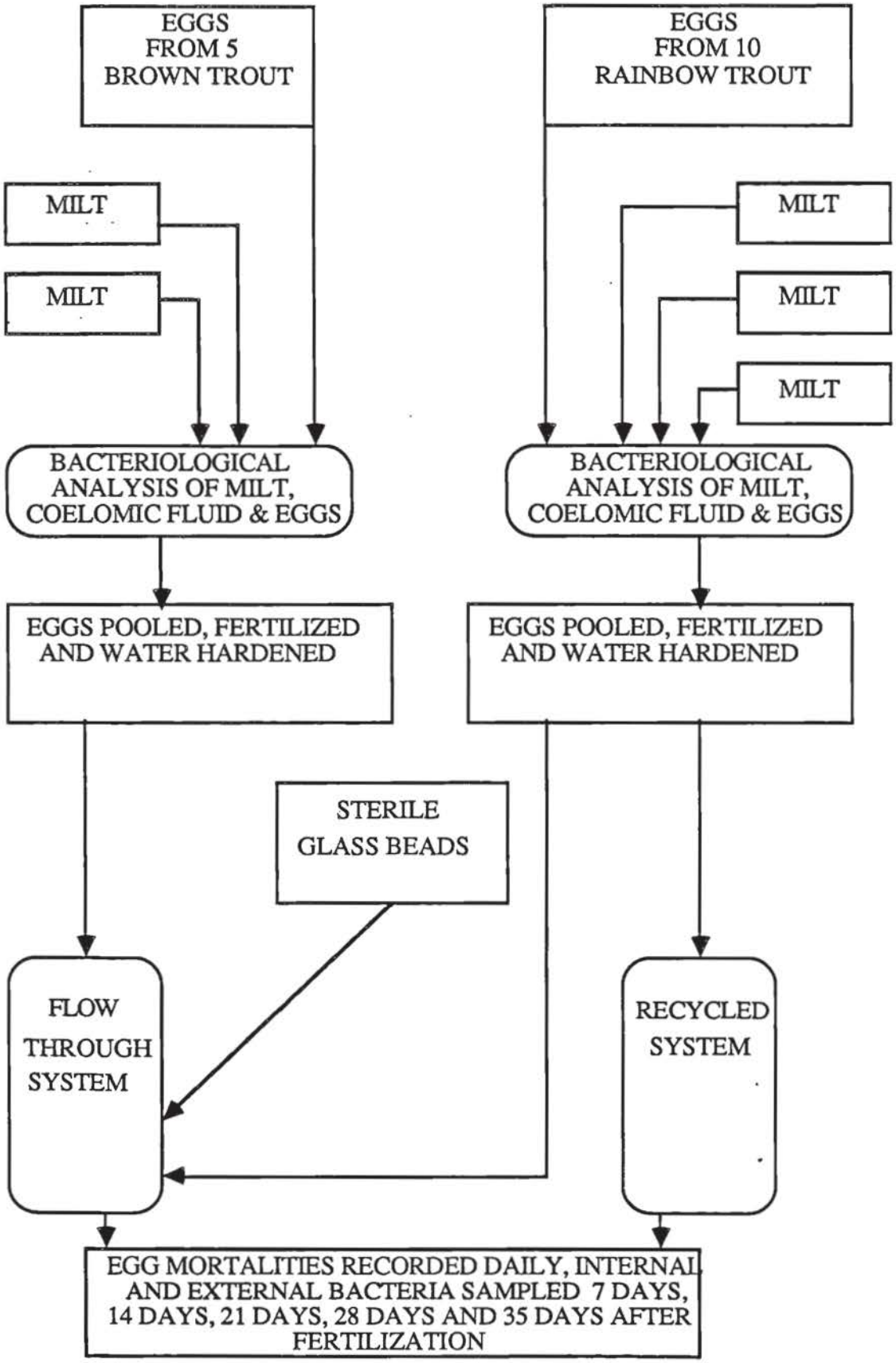
It is now clear from previous studies (Chapter 2) and from the scientific literature that large numbers of bacteria accumulate around surfaces of incubating salmonid eggs (Bell 1966 ; Bell, Hoskins & Hodgkiss 1971 ; Trust 1972 ; Yoshimizu, Kimura & Sakai 1980). However, little is understood about the effects these bacteria might have on egg development and, inturn, premature egg mortality. Bell *et al* (1971) considered it unlikely that bacteria on egg surfaces would be able to produce enzymes or toxins that would be capable of penetrating the egg, for the salmonid embryo is protected by a tough outer shell composed of icthulokeratin (Young & Smith 1956 ; Bell, Hoskins & Bagshaw 1969). Trust (1972) also considered it unlikely that bacterial produced enzymes or toxins could be responsible for egg death but, on the other hand, considered it possible that under certain circumstances (such as high egg density, coupled with poor water circulation) dissolved oxygen might be reduced to detrimental levels by large numbers of egg surface bacteria (Chapter 2).

Few reports exist in the literature of bacteria present inside salmonid eggs. Cone (1982) discovered an unidentified *Lactobacillus* sp. within 33 of 35 eggs taken from diseased rainbow trout suffering from a mixed bacterial infection and in addition *Renibacterium salmoninarum* (responsible for Bacterial Kidney Disease B.K.D.) is also considered to exist inside eggs (Evelyn, Ketcheson & Prosperi-Porta 1984). However, neither of the two (above) bacteria have ever been implicated in premature mortality of incubating salmonid eggs. Recently, work on chinook salmon *(O. tshawytscha)* Walbaum, by Sauter, Williams, Meyer, Celnik, Banks & Leith (1987) has revealed a wide range of bacteria within eggs including both Gram positive and negative organisms. In addition, Sauter *et al* (1987) considered whether specific bacteria within eggs could influence "early life stage death" of eggs and fry and although, no definite conclusions could be drawn the following bacterial genera were implicated ; *Vibrio, Listeria, Corynebacterium* and *Staphylococcus.*

In this present trial it was decided to investigate the external and internal bacterial flora of incubating salmonid eggs. Both brown trout (*Salmo trutta* L.) and rainbow trout eggs were incubated under identical conditions placed under a flow-

through system of water (to be described in full later). Sterile glass beads were also incubated to allow the bacterial flora of an inert surface to be compared to that of incubating salmonid eggs. Further rainbow trout eggs were also incubated under a separate pumped recirculatory system. Daily egg mortalities were carefully recorded to investigate any possible correlation between bacterial presence and egg death. A schematic outline of procedures followed during the trial is summarized in Figure 5 : 1.

FIGURE 5:1 Flow diagram of experimental protocol.



MATERIALS AND METHODS

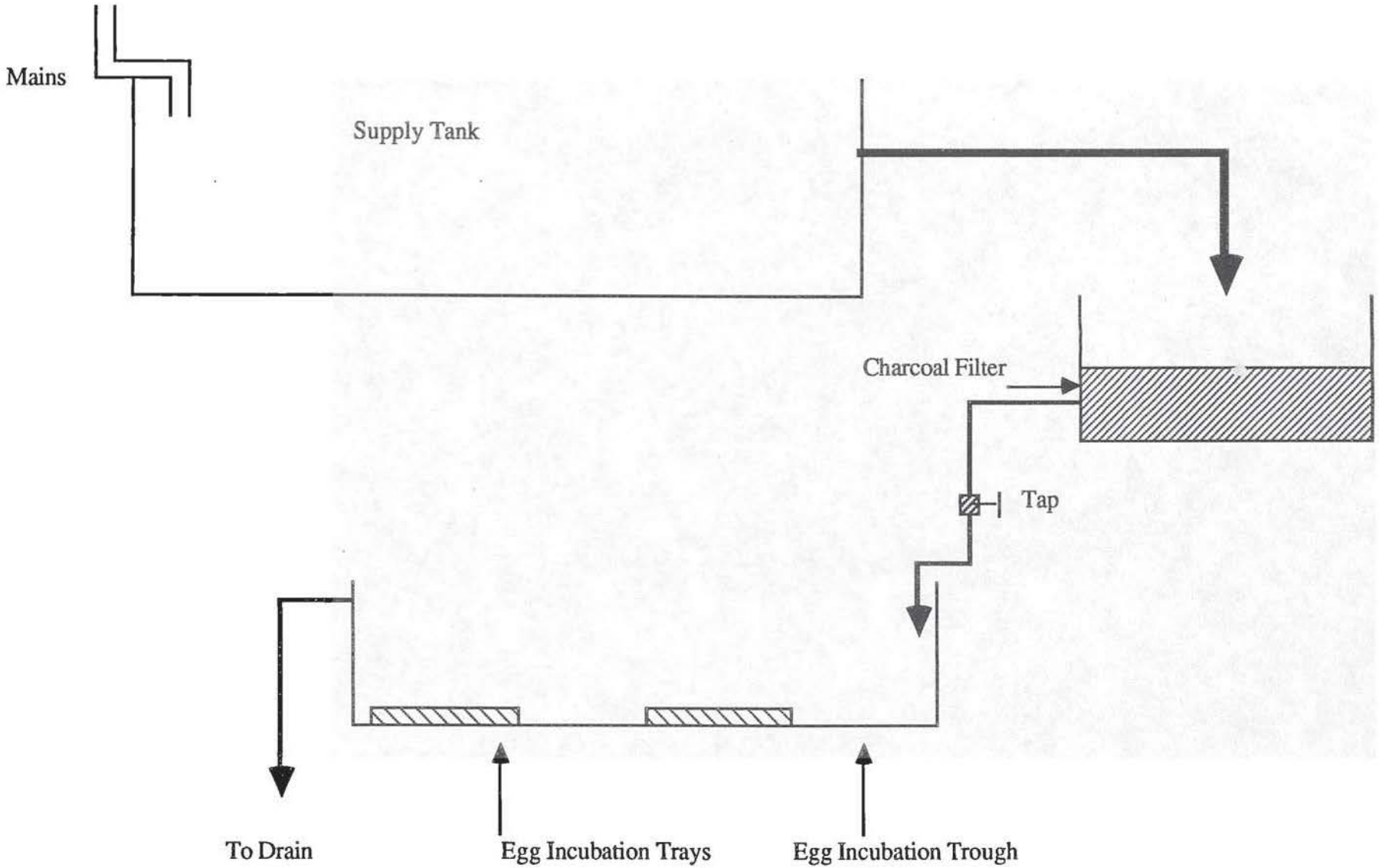
Experimental Design

Incubation systems. Two separate incubation systems were used during this trial. One system comprised of a single trough containing two aluminium trays (to support eggs) each divided into 6 sections. Water at 10 °C ± 1 °C passed over eggs at a flow rate of 3600 l / day and then went to drain (Figure 5 : 2). (This incubation system is hereafter referred to as source 1). The second incubation system comprised of a single trough containing one aluminium tray (to support eggs). Water at 10 °C ± 1 °C after passing over eggs at the same (above) flow rate, was directed through a gravel filter bed (as a biological filter) before being pumped and recycled. A small volume of fresh "make-up" water (approximately 5% per day) was added to allow for evaporation and spillage (see Figure 2 : 3). (This incubation system is hereafter referred to as source 2).

Collection of Eggs and Milt.

Stripping and egg incubation. Eggs were obtained from 10 ripe rainbow trout and 5 ripe brown trout from a single commercial fish farm. Eggs of each fish were stripped into individual sterile containers. The first eggs from each fish were discarded to avoid any contamination from the surrounding water or ventrolateral surface of the female. Milt, also collected in sterile containers, was obtained from 3 sex-reversed females for rainbow trout and 2 normal males for brown trout. Milt and eggs were immediately put on ice and returned to the laboratory. Eggs, coelomic fluid and milt

FIGURE 5 : 2 Flow-through incubation system



from each container were removed for bacteriological examination. The remaining eggs for each species were pooled and fertilized. After 5 minutes excess milt was washed off and the eggs water hardened for 45 minutes. Eggs from each species were assigned randomly to two sections of each aluminium tray (source 1). The final two sections of each tray contained sterile glass beads with a similar diameter to that of eggs. Each section was filled with enough eggs or glass beads to form a monolayer. Rainbow trout eggs were also laid down at the same density in the pumped recirculatory system (source 2).

Egg fertilization rates were determined after 7 days at 10 °C by placing 100 eggs for each species into clearing solution (acetic acid : methanol : water, 1 : 1: 1 ^{v}/ $_{v}$) (Springate & Bromage 1984). Fertilized eggs could be clearly distinguished by the presence of a developing neural tube. At eyeing, eggs were "shocked" by siphoning from a height of 1m into a sterile beaker and any unfertilized eggs removed. Eyed eggs were replaced into their relevant sections in the incubators. Eggs were treated with malachite green oxalate (Difco, Surrey) twice a day at a concentration of 2 ppm.

Bacteriological Examination

Coelomic fluid and milt. Aliquots of coelomic fluid from each fish were serially diluted and inoculated in 0.02 ml amounts (drop inoculation method) onto Tryptone soya agar (T.S.A., Oxoid, Basingstoke), peptone beef extract glycogen agar (P.B.G., McCoy & Pilcher 1974) and low nutrient *Cytophaga* agar (Anacker & Ordal 1959). Plates were incubated at 20°C for up to 10 days and enumeration performed only from drops where total separation of colonies occurred.

Eggs. Eggs were sampled at the green stage (unfertilized) and during incubation after 7 days, 14 days, 21 days and 28 days. Due to the slower development of brown trout eggs a further sample at 35 days was possible.

External sampling. For, each sampling time / treatment 4 separate batches of 10 eggs (or glass beads) were removed at random from appropriate sections. Subsequently, samples were rinsed in 4 changes of sterile water to remove any detritus or loosely adhered bacteria and added to 5 ml of sterile diluent (peptone - 0.1%, saline - 0.85%) before shaking vigorously for 2 minutes on a vortex stirrer, in a manner adapted from Evelyn *et al* (1984). Aliquots of diluent were serially diluted and inoculated in 0.02 ml amounts (drop inoculation method) onto *Cytophaga* agar, P.B.G. and T.S.A. The number of viable cells transferred to each of the 3 different media was determined by plate count. Dividing by 10 gave the average count per egg and by taking into account dilution factor, volume of diluent and egg surface area, the number of viable colony forming units (cfu's) per mm^2 was estimated (as described in Chapter 2).

Internal sampling. At each sampling time 25 eggs randomly chosen for each species were removed and surface sterilized by placing in iodophor disinfectant (Buffodine - Evans Vanodine International Ltd, Preston) for 15 minutes at an available iodine concentration of 200 ppm. Such a process constitutes a longer treatment time and twice the manufacturers recommended concentration of iodine, as egg surface sterility was considered more important in an experimental context than any damage that might have occurred to the embryo. However, an even greater concentration of iodine (and thus guaranteeing surface sterility) could not be employed as there is evidence to suggest that substances of low molecular weight (such as iodine) may pass across the chorion into the perivitelline space (Hayes 1949 ; Potts & Rudy 1969 ; Rudy & Potts 1969) potentially influencing numbers of bacteria isolated. After treatment eggs were removed and rinsed in four changes of sterile water to remove any traces of disinfectant. Eggs were placed individually into small sterile tubes (LP3- Luckhams, Burgess Hill) containing 1ml of tryptone soya broth (T.S.B.) and incubated at 20 °C for 10 days. Surface sterilization was only considered to have been successful when the surrounding media was still clear and no growth occurred after a further 10 days when samples of the media were dropped onto the three agars. Surface sterilized eggs were

lanced in the tube (still containing sterile T.S.B. media) with a 1ml syringe and needle (0.5 x 16 mm) and the egg contents removed. Drops were inoculated directly onto agar media and into further tryptone soya broth.

Identification of bacteria. Bacteria were isolated by a variety of techniques, procedures and schemes outlined by Krieg and Holt (1984), Cowan (1974), the tables of Allen, Austin & Colwell (1983) and Stanier, Palleroni & Doudoroff (1966). Some Gram -ve bacteria, especially *Aeromonas hydrophila*, were more easily identified using API 20E and 20NE identification strips (API laboratory products -Basingstoke).

Statistical analysis. Numbers of colony forming units per mm^2 for each species of egg were compared using a two way split-plot analysis of variance (Ridgman 1975; Snedecor & Cochran 1980). Differences between means were compared by calculating the standard errors and 95% confidence limits appropriate to the split-plot design (Snedecor & Cochran 1980). Numbers of hatched eggs against eggs that failed to hatch were compared using a chi square (χ^2) test. Effect of bacterial presence on egg surfaces was analysed by multiple regression chosen to compare numbers of surface bacteria with egg deaths by relating mortality to time (age) and bacterial numbers.

RESULTS

Egg development

Hatching Success. Data obtained for eyeing and hatching during this trial is summarized in Table 5 : 1. From this table it can be seen that exceptionally high estimated fertilization rates were obtained ; 1336 and 2689 eggs (100%) for rainbow trout and 883 eggs (96%) for brown trout, in turn, leading to high hatching rates of 1268 and 2467 eggs (94.9% and 91.7%) for rainbow trout and 882 (95.9%) for brown trout. It can also be seen from the table that "estimated fertilized egg losses" (calculated by subtracting numbers of "estimated unfertilized eggs" from the total number of eggs that failed to hatch) ranged from 68 to 222 (5.1% to 8.3%) for rainbow trout eggs and only 1 (0.1%) for brown trout eggs. Analysis of overall hatching success between egg groups revealed no significant difference between brown trout eggs and rainbow trout eggs incubated under the same flow-through system (source 1). However, a highly significant difference was found between rainbow trout eggs (source 1) and rainbow trout eggs (source 2) ($\chi^2 = 13.38$, $P < 0.001$). Similarly, a highly significant difference in hatching success was found between brown trout eggs (source 1) and rainbow trout eggs (source 2) ($\chi^2 = 17.45$, $P < 0.001$) (Appendix 5 : 1).

Daily mortalities of eggs are recorded in Figure 5 : 3, from which it can be seen that the pattern of egg mortality between brown trout eggs and rainbow trout eggs (source 1), both incubated under the same flow-through system, is similar. Both egg species (although more exaggerated in brown trout eggs), show periods of stability, followed by periods of additional egg mortality throughout incubation, leading to a levelling off in mortality prior to hatching. In contrast, rainbow trout eggs (source 2) exhibit a virtual constant and accumulative egg mortality with no indication of mortality levelling off until the very last days of incubation. Analysis of egg mortality data at 7 day intervals throughout incubation reveals significant differences in "rate" of egg death

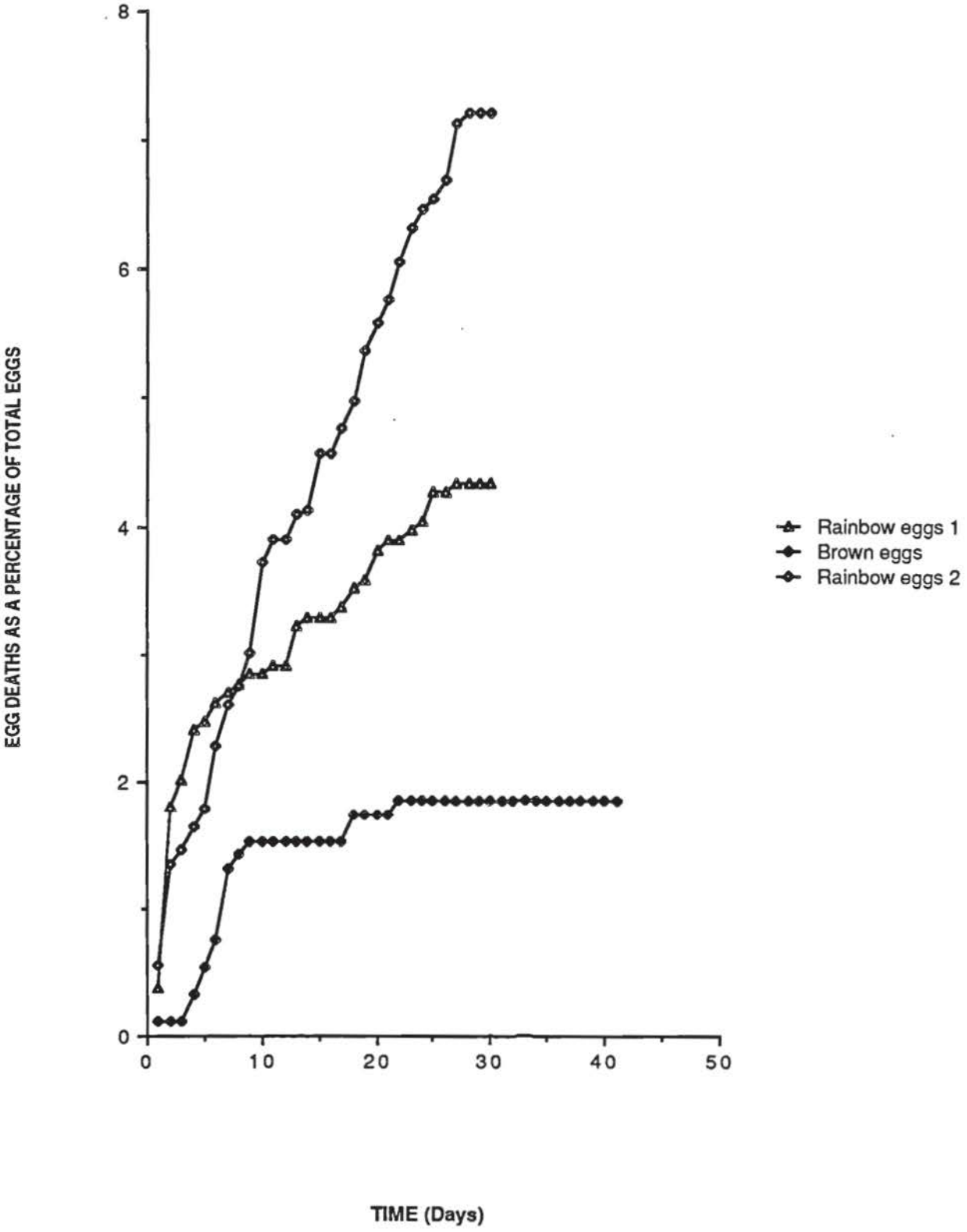
TABLE 5 : 1 Eyeing and hatching rates for rainbow trout and brown trout eggs.

EGG SURVIVAL :	Rainbow trout (Source 1)	Brown trout (Source 1)	Rainbow trout (Source 2)
Total number of eggs	1336	920	2689
Estimated fertilized eggs	1336	883	2689
Actual eyed eggs	1274	883	2506
Actual hatched eggs	1268	882	2467
EGG LOSS :			
Deaths to eyeing	52	16	155
Deaths at shocking	10	21	28
Deaths from eyeing to hatching	6	1	39
Eggs that failed to hatch	68	38	222
UNEXPLAINED LOSSES :			
Estimated unfertilized eggs	0	37	0
Estimated fertilized egg losses	68	1	222
Estimated fertilized egg losses (%)	(5.1)	(0.1)	(8.3)

Figures in brackets = percentages.

FIGURE 5:3 Accumulative total of dead eggs

(recorded daily)



between rainbow trout eggs (source 1) and brown trout eggs (source 1) between 7-14 days and 14-21 days incubation ($\chi^2 = 6.76$, $P < 0.05$ and $\chi^2 = 8.55$, $P < 0.05$). After 21-28 days incubation a very significant difference was seen in egg mortality between these two above groups ($\chi^2 = 10.94$, $P < 0.01$). Rainbow trout eggs (source 1) and (source 2) suffered a similar "rate" of egg death for the first half of incubation (1-14 days) but, from 14-21 days rainbow trout eggs (source 2) exhibited significantly greater egg mortalities ($\chi^2 = 6.1$, $P < 0.05$) and, over the last part of incubation (21-28 days) suffered highly significantly greater mortalities ($\chi^2 = 12.49$, $P < 0.01$) than rainbow trout eggs under the flow-through system. Throughout the whole of incubation (compared at 7 day intervals) rainbow trout eggs (source 2) suffered significantly greater mortalities than brown trout eggs ; 1-7 days ($\chi^2 = 5.01$, $P < 0.05$), 7-14 days ($\chi^2 = 13.53$, $P < 0.001$), 14-21 days ($\chi^2 = 23.95$, $P < 0.001$) and 21-28 days ($\chi^2 = 36.71$, $P < 0.001$) (Appendix 5 : 2).

Bacteriological examination

Coelomic fluid. Bacteria were isolated from coelomic fluid of 6 rainbow trout and 2 brown trout (Table 5 : 2). Genera isolated included *Pseudomonas, Aeromonas, Staphylococcus, Corynebacterium* and *Flavobacterium.*

Milt. Bacteria were found in milt of all 5 samples. Rainbow trout milt contained higher numbers and a greater range of bacterial species (Table 5 : 3), probably, due to the removal of the testis and subsequent handling by farm staff (Woodroffe & Shaw 1974), a process necessary with sex-reversed fish. Species isolated were similar to those in coelomic fluid but were present in slightly higher numbers.

External sampling. Few bacteria were found attached to surfaces of green (unfertilized) eggs. However, bacteria isolated included *Staphylococcus epidermis, Pseudomonas* sp. and *Bacillus* sp. During incubation large numbers of bacteria accumulated around egg surfaces (Figure 5: 4). From this graph it can be seen that

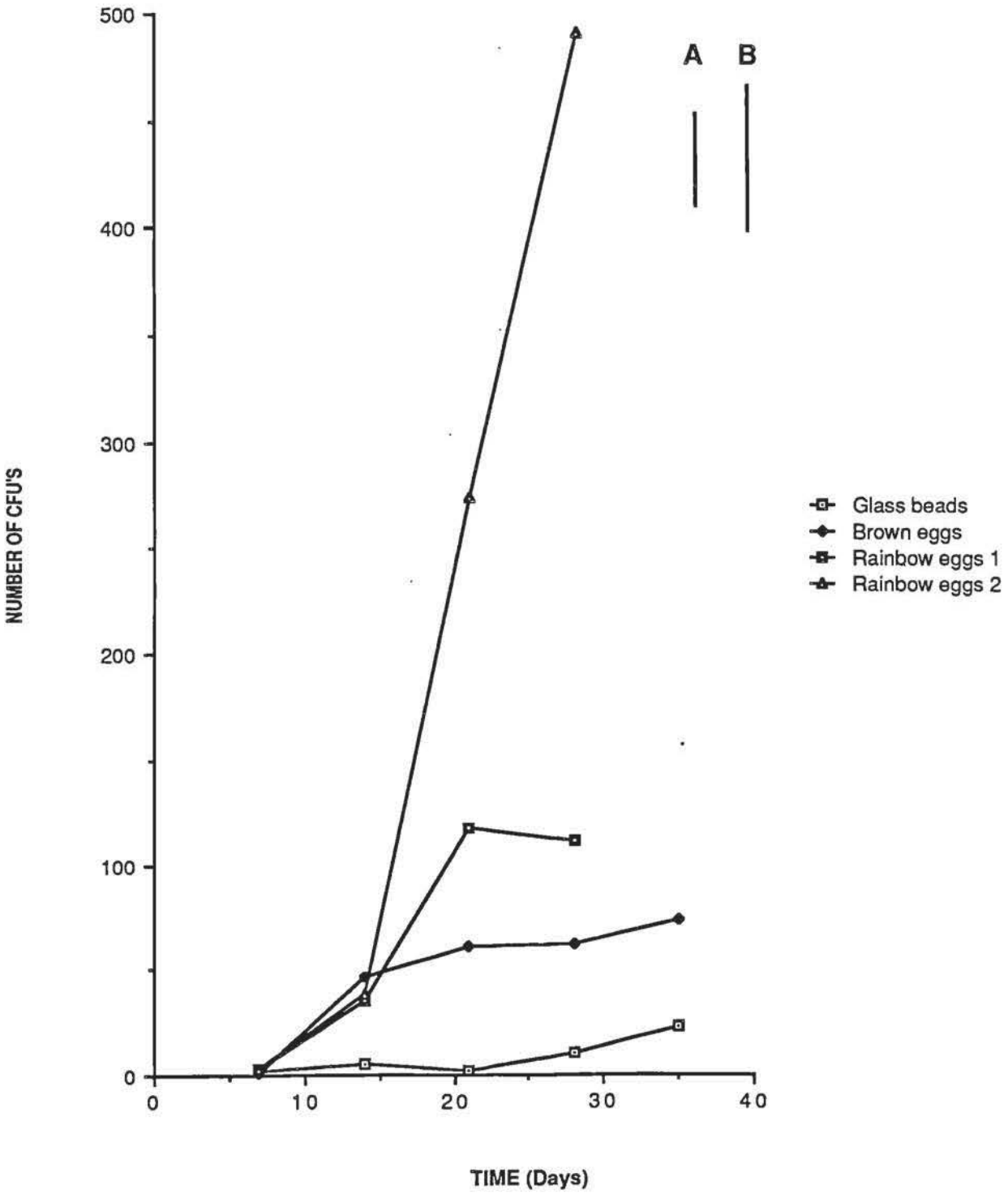
Table 5 : 2. Bacteria present in the coelomic fluid of five brown and ten rainbow trout.

Rainbow trout (Fish)	Bacteria	Range cfu's/ml	Brown trout (Fish)	Bacteria	Range cfu's/ml
(1)	*Aeromonas hydrophila*	10^1	(1)	- -	
	Pseudomonas fluorescens	10^1			
(2)	- -		(2)	- -	
(3)	*Pseudomonas maltophila*	10^1	(3)	*A. hydrophila*	10^1
	P. fluorescens	10^1			
(4)	*A. hydrophila*	10^2	(4)	*P. fluorescens*	10^1
	P. fluorescens	10^1		*Pseudomonas* sp.	10^1
(5)	*Pseudomonas* sp.	10^1	(5)	- -	
	A. hydrophila	10^1			
	P fluorescens	10^1			
(6)	- -				
(7)	- -				
(8)	*Staphylococcus epidermis*	10^2			
	Corynebacterium sp.	10^1			
(9)	*Pseudomonas* sp.	10^1			
	A. hydrophila	10^1			
	Flavobacterium sp.	10^1			
(10)	- -				

Table 5 : 3. Bacteria present in the milt of two brown and three rainbow trout.

(Fish)	Rainbow trout Bacteria	Range cfu's/ml	(Fish)	Brown trout Bacteria	Range cfu's/ml
(1)	*S. epidermis*	10^2	(1)	*A. hydrophila*	10^2
	P. fluorescens	10^1		*Pseudomonas* sp.	10^1
	A. hydrophila	10^1			
	Pseudomonas sp.	10^2			
(2)	*A. hydrophila*	10^2	(2)	*Pseudomonas* sp.	10^1
	Flavobacterium sp.	10^1		*A. hydrophila*	10^1
	Pseudomonas sp.	10^2			
	S. epidermis	10^2			
(3)	*A hydrophila*	10^1			
	S. epidermis	10^2			
	Flavobacterium sp.	10^1			

FIGURE 5:4 Estimated colony forming units for all bacteria per sq mm egg surface



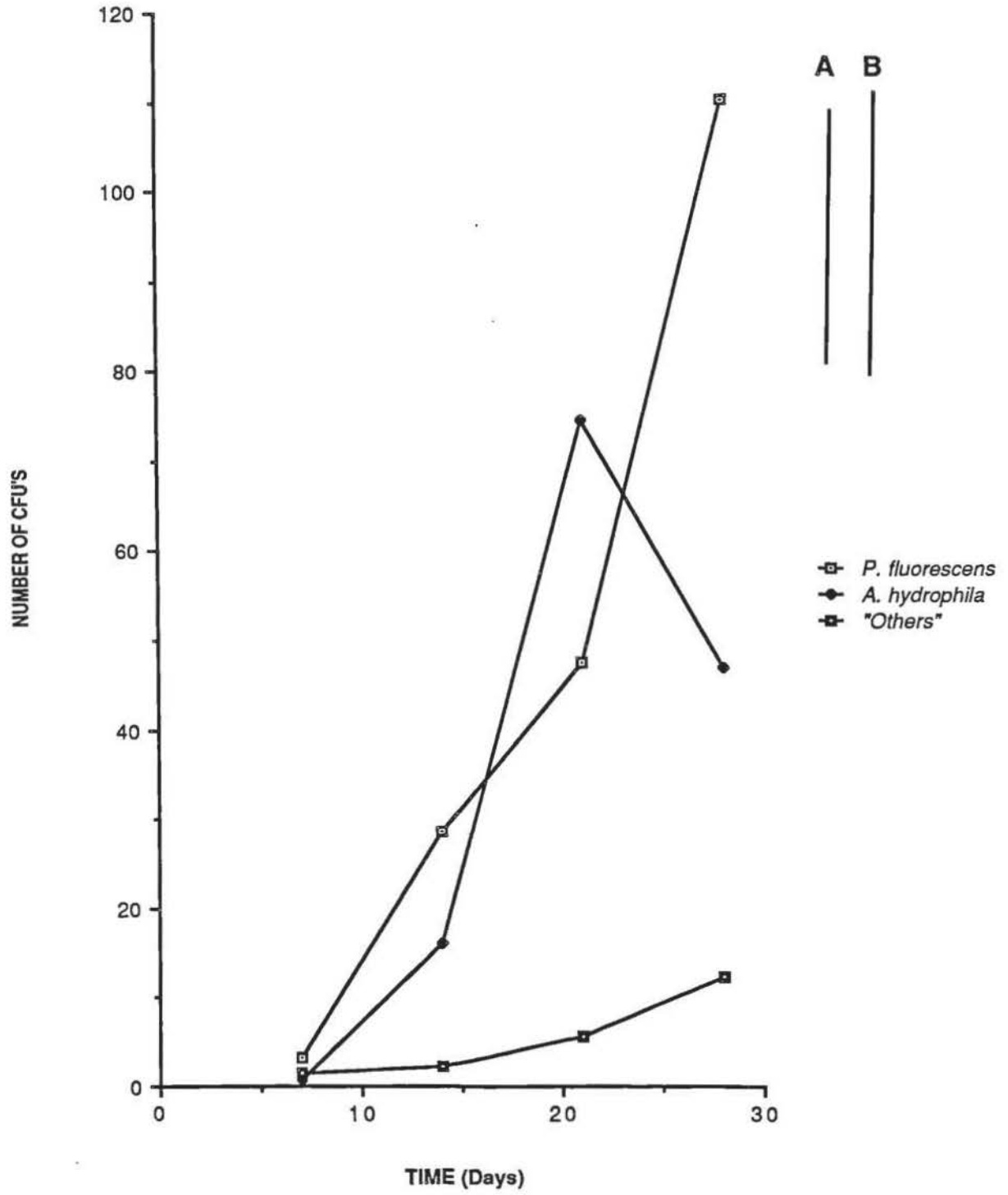
A = 95% confidence limits for differences within a treatment and B = 95% confidence limits between 2 treatments at the same or different times.

glass beads supported the least numbers of bacteria. Rainbow trout eggs (source 1) and brown trout eggs (source 1) were colonized by equal numbers of bacteria up until 14 days of incubation but, from 14-28 days, rainbow trout eggs supported slightly more bacteria. Rainbow trout eggs (source 2) were colonized with equal numbers of surface bacteria as the other egg groups (source 1) until 14 days of incubation. However, after 14 days numbers of bacteria on rainbow trout eggs (source 2) proliferated greatly and a marked difference was seen between this group of eggs under the recycled water system and groups under the flow-through system. Analysis of cfu's recovered for all 4 groups (by 2 way split-plot analysis of variance) revealed overall a highly significant difference in numbers of egg surface bacteria ($P < 0.001$). A highly significant effect of time was seen ($P < 0.001$) and a highly significant interaction between time and numbers was found ($P < 0.001$) (Appendix 5 : 3).

Examination of species of bacteria found on egg surfaces revealed a similar pattern of colonization on all egg group surfaces under the flow-through system. Generally, both rainbow and brown trout egg surfaces (source 1) were dominated by *Pseudomonas fluorescens*, followed by *Aeromonas hydrophila* and low numbers of "other" bacteria (frequently, *Cytophaga* sp. and *Pseudomonas* sp.) (Figures 5 : 5a and 5 : 5b). Surfaces of glass beads revealed a similar pattern of colonization to the above. However, proportionally "other" bacteria were represented in higher numbers including the following genera ; *Cytophaga* sp., *Flavobacterium* sp., *Pseudomonas* sp. and *Achromobacter* (Figure 5 : 5c). In contrast, rainbow trout eggs (source 2) were dominated by *Cytophaga* sp. (only present in low numbers on surfaces under the flow-through system), and *P. fluorescens* was also frequently isolated. In addition, *A. hydrophila* and "other" bacteria, comprising mainly of *Pseudomonas* sp. were recovered (Figure 5 : 5d).

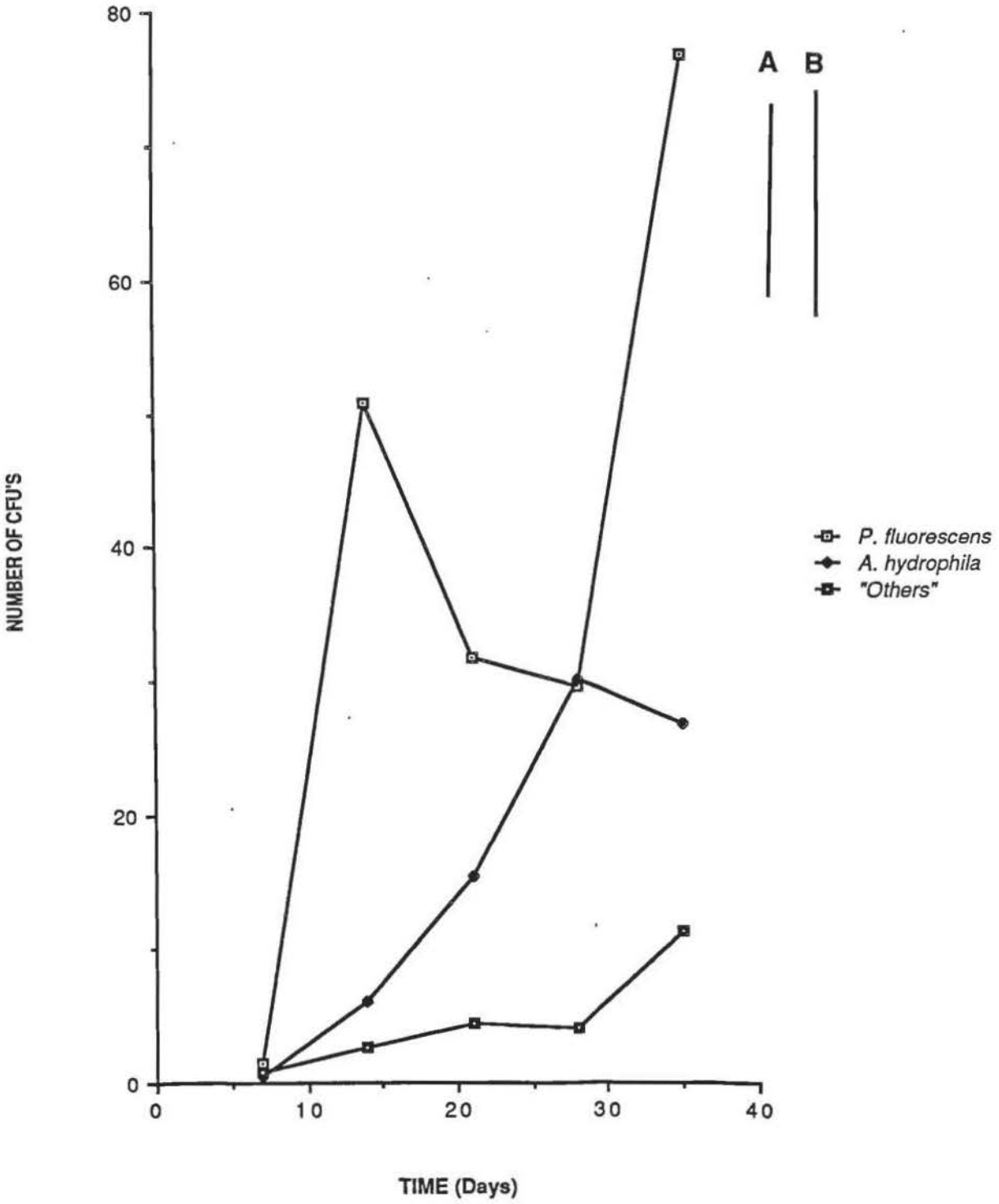
Internal sampling. Few individual eggs were considered to contain bacteria. Over the entire sampling period of 7-28 days, a total of 300 eggs were examined (75 eggs at each sampling time). Only 10 eggs were found to contain bacteria ;

FIGURE 5:5(a) Estimated colony forming units per sq mm egg surface (rainbow trout eggs - source 1)



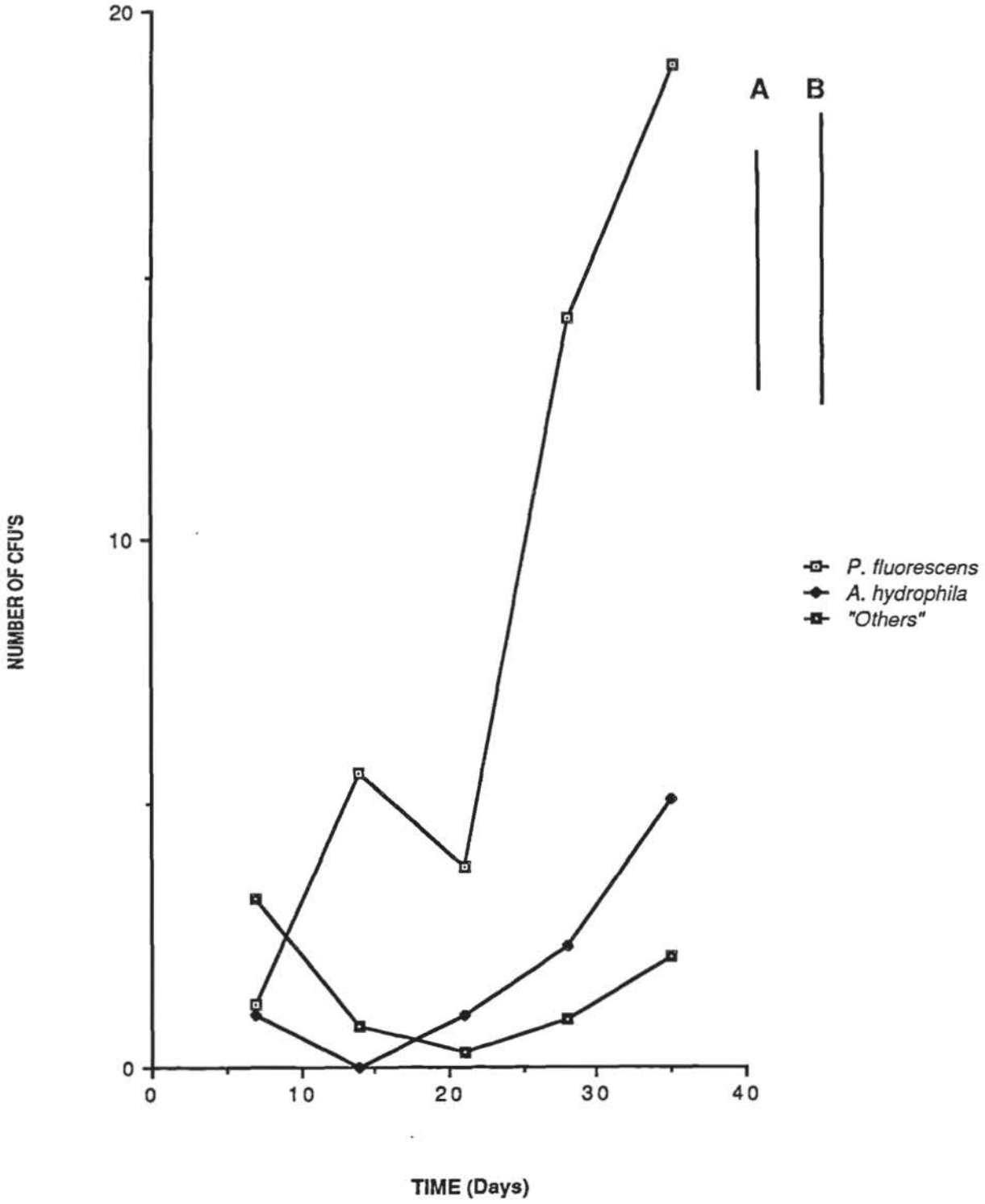
A = 95% confidence limits for differences within a treatment and B = 95% confidence limits between 2 treatments at the same or different times

FIGURE 5:5(b) Estimated colony forming units per sq mm egg surface (brown trout eggs - source 1)



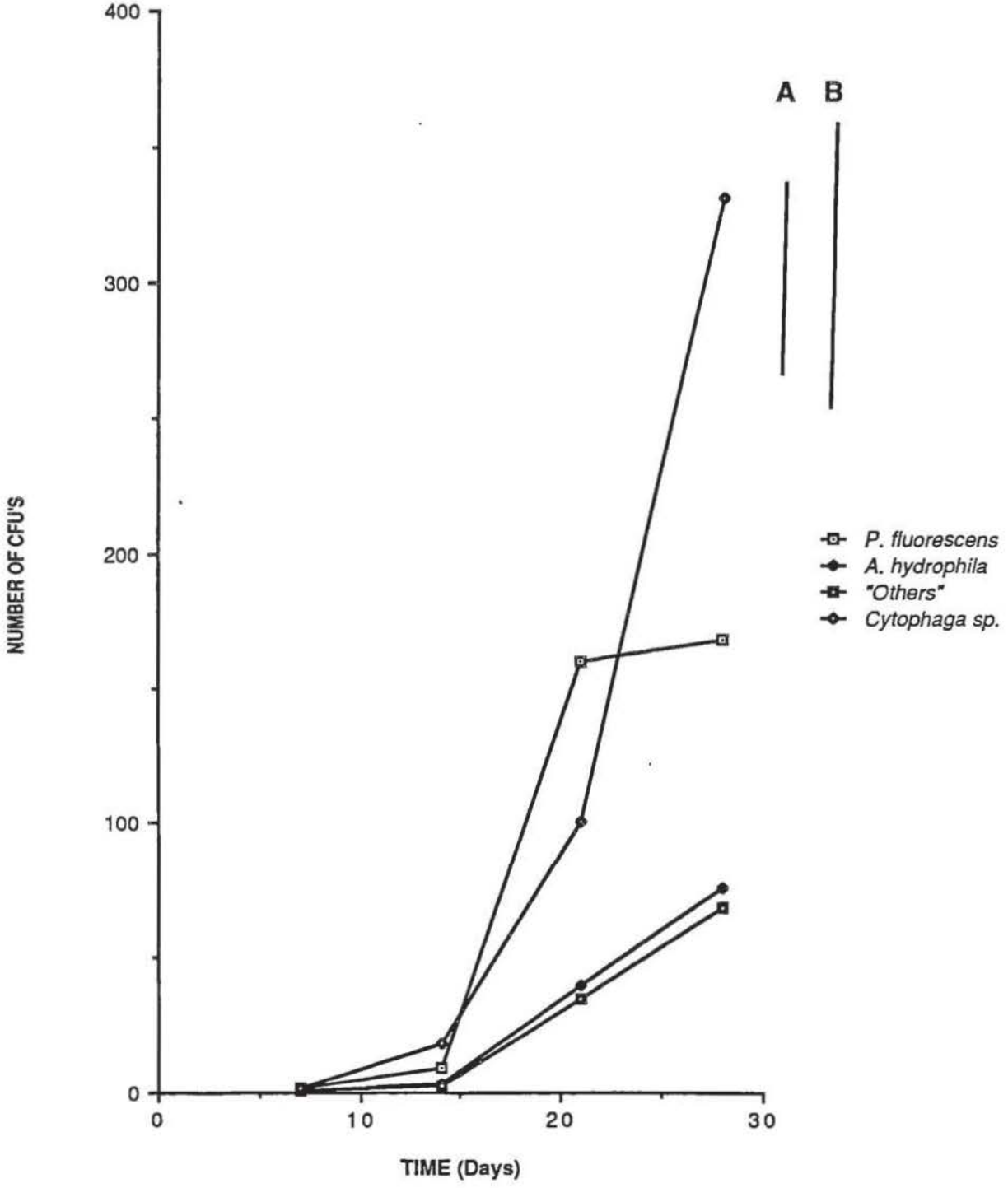
A = 95% confidence limits for differences within a treatment and B = 95% confidence limits between 2 treatments at the same or different times

FIGURE 5:5(c) Estimated colony forming units per sq mm glass bead surface (source1)



A = 95% confidence limits for differences within a treatment and B = 95% confidence limits between 2 treatments at the same or different times

FIGURE 5:5(d) Estimated colony forming units per sq mm egg surface (rainbow trout eggs - source 2)



A = 95% confidence limits for differences within a treatment and B = 95% confidence limits between 2 treatments at the same or different times

Pseudomonas sp. were isolated from 8 eggs and *A. hydrophila* from two. *Cytophaga* sp. common place on surfaces of eggs (source 2) were not found within.

Egg death. As so few eggs were colonized internally by bacteria it was decided to disregard any effect they might have on incubating egg death. However, the effect of bacterial presence on egg surfaces and their survival was analysed by multiple regression which demonstrated a correlation between bacterial numbers and egg deaths ($r = 0.74$) (Appendix 5 : 4).

DISCUSSION

During the course of this trial eggs were only taken from a total of 15 fish in order that great care could be taken with stripping, fertilization and with the provision of optimum incubation conditions. Exceptionally high fertilization rates of 100% for rainbow trout eggs provide sufficient evidence that this was achieved. Similarly, brown trout egg fertility, estimated at 96% , although slightly lower than that of rainbow trout eggs, was still extremely high. Under the flow-through system of incubation provided, direct comparison of the performance of rainbow trout eggs and brown trout eggs is completely valid. At first sight, (due to the fertilization figures above), it would seem that rainbow trout eggs performed better than brown trout eggs, contradicting the traditional views that suggest rainbow trout eggs perform less well than those of other salmonids (as previously described). However, closer examination indicates that this is not truly the case because, as a consequence of the 100% fertilization, all the rainbow trout eggs should potentially go on to hatch but, 5.1% failed to do so even under the optimum incubation conditions provided. On the other hand, of the estimated 96% of brown trout eggs fertilized only 0.1% failed to hatch.

Numbers of egg surface bacteria were estimated by plate counts and although, only a conservative technique (as fully discussed in Chapter 2), differences in numbers of bacteria per mm^2 were found between groups of eggs and glass beads. Under the flow-through system (source 1) glass beads supported the least surface bacteria, although after 35 days as many as 23 cfu's per mm^2 were estimated to be present drawn from a wide range of bacterial genera. Their presence on glass beads (as an inert surface) is not unexpected as nutrients sufficient for limited microbial growth are known to exist in hatchery waters (Willoughby, McGrory & Pickering 1983), and will tend to accumulate around solid surfaces (Zobell & Anderson 1936) in turn supporting microbial growth and reproduction. In comparison, brown trout eggs were colonized by higher numbers of bacteria per mm^2 than glass beads, while maintained under

identical conditions. Salmonid embryo's are extremely efficient at using nutrients available to them and only relatively small quantities of carbon dioxide and ammonia are excreted (Hayes 1949 ; Smith 1947 ; 1957). Therefore, although higher numbers of bacteria are associated with surfaces of brown trout eggs (compared to glass beads), this may not be solely a consequence of nutrient release from eggs. Other factors, in particular the interaction of egg surface properties and bacterial processes of adhesion must also be of potential importance in governing bacterial loading of living and inert surfaces. Under the same incubation system rainbow trout eggs supported higher numbers of bacteria than brown trout eggs after 14 days of incubation. Whether rainbow trout eggs have slightly different egg surface properties facilitating bacterial adhesion or whether rainbow trout embryo's excrete increased amounts of waste products, thereby supporting higher numbers of bacteria (compared to brown trout eggs) remains doubtful. Increased levels of bacteria on rainbow trout egg surfaces in this trial, may be a consequence of a far simpler process, for although availability of nutrients from normally developing eggs may be considered limiting, the possibility still exists that over protracted periods of incubation more nutrients for potential bacterial growth will become available. In particular, the presence of any dead eggs in trays, possibly due to individual poor egg quality, or poor local environmental conditions, may give rise to more freely available nutrients due to rupturing of the vitelline membrane (Post, Power & Kloppel 1974) leading in turn to considerable nutrient loss. Smith, Armstrong, Springate & Barker (1985) demonstrated that shocked rainbow trout eggs release considerable quantities of metabolites including potassium, phosphate and amino acids all of which favour bacterial growth and reproduction. Thus, increased numbers of dead eggs amongst rainbow trout egg groups (compared to brown trout egg groups) may have led to higher levels of nutrients available locally and in turn supported higher numbers of bacteria. This argument may equally well explain the higher numbers of bacteria found on rainbow trout egg surfaces under the recycled water incubation system (source 2), even though physical conditions (for example, water temperature, flow rates, egg density), were identical to those employed in the

flow-through system. However, such a marked increase in bacterial numbers (3 times that of eggs from source 1) is unlikely to be solely due to increased numbers of dead eggs. Rainbow trout eggs in both incubation systems came from a common genetic pool and therefore, physical characteristics of eggs and their surface properties can be assumed to be identical. However, continual recycling of water may in turn ensure that any nutrients already present in the water, or any leaked from eggs, are not lost to drain but are continually available to bacteria on egg surfaces over the whole incubation period thereby encouraging further bacterial growth.

Generally, bacterial numbers increased with progressive incubation periods, irrespective of the substrate, but species diversity was seen to decline. A similar pattern of bacterial activity was described by Shewan (1961) and Shewan & Hobbs (1967), although specifically in relation to the spoilage of stored fish. The observed loss of diversity, in particular that associated with continued increase in *Pseudomonads*, may be a consequence of either inhibition of other competing microbial organisms by *P. fluorescens* (Cornick, Chudyk & McDermot 1969 ; Dubois-Darnaudpeys 1977 ; Gurusiddaiah, Weller, Sarkar & Cook 1986 ; Hatai & Willoughby 1988) or, a possible variety of environmental factors interacting to enhance the success of a limited group of bacteria. The actual species of bacteria present on rainbow trout and brown trout egg surfaces differed very little, with *P. fluorescens* and *A. hydrophila* dominating surfaces of both egg species. In contrast, *Cytophaga* sp. were isolated far less frequently from surfaces of eggs maintained under a flow-through system, however surfaces of rainbow trout eggs kept under recycling conditions were colonized by considerable numbers of *Cytophaga* sp. as also observed in earlier work (Chapter 2) and by Bell, Hoskins & Hodgkiss (1971) and Trust (1972) albeit under different incubation conditions. As all rainbow trout eggs came from a common genetic pool it is therefore likely, that some aspect of water quality, possibly involving the quality of filtration was responsible for favouring the growth of *Cytophaga* sp. at the expense of *P. fluorescens* and *A. hydrophila*. Alternatively, constraints on numbers of *P. fluorescens* could have resulted in less inhibition of *Cytophaga* sp. and in turn their

subsequent proliferation. Horsley (1973) demonstrated that the bacterial flora of fish tends to reflect that of their environment, and differences in egg surface microflora in this trial would seem to suggest that this might apply equally as well to incubating salmonid eggs.

In this trial, brown trout eggs supported the least number of surface bacteria and experienced the fewest deaths. Rainbow trout eggs (source 1) demonstrated higher numbers of surface bacteria and in turn more deaths but, rainbow trout eggs (source 2) supported the highest number of surface bacteria and also experienced the most deaths. Thus, analysis of data by multiple regression found a marked correlation between egg surface bacteria and egg death ($r = 0.74$). Between all 3 egg groups numbers of surface bacteria remained constant up until 14 days of incubation. During this period (1-14 days of incubation) the majority of total egg deaths, within each egg group was seen to occur ; rainbow trout eggs (source 1) 76% ; brown trout eggs (source 1) 82% and rainbow trout eggs (source 2) 57%. Therefore, although, from 14-28 days differences in bacterial numbers (and species) became more marked between all groups, proportionally fewer egg deaths actually occurred. Thus, if a relationship between bacteria and egg mortality does exist, it is not likely to be simply due to the presence or absence of one particular species of bacteria at a given concentration. Furthermore, it could be argued that higher numbers of egg surface bacteria are a consequence of original poor egg quality (and the availability of increased nutrients) and not a cause of it. However, whatever the causes of egg mortality, they are unlikely to be so simplistic as a considerable variety of other factors (as already outlined), in addition to the presence of large numbers of bacteria may also be of influence. A better understanding of the relationship between "commensal" bacteria and egg mortality might possibly be achieved by manipulation of both numbers and species of the potential egg bacterial flora and close observation of subsequent egg death.

SUMMARY

Analyses of bacterial numbers attached to surfaces of rainbow trout eggs, brown trout eggs and glass beads incubated under identical conditions revealed significant differences between groups. Generally, glass beads supported the least number of bacteria, brown trout eggs supported considerably more bacteria and rainbow trout eggs supported the greatest number of bacteria. However, glass beads were colonized by a more diverse range of bacteria than either rainbow trout or brown trout eggs. In addition, replicate batches of rainbow trout eggs incubated under a different system were colonized by far greater numbers of bacteria and dominated by bacteria from a different genera. Thus, in this study the egg surface bacterial flora is influenced by water quality / treatment, potentially a consequence of the varying filtration systems employed between the two incubation systems. Overall, analysis of egg mortalities between groups revealed a strong correlation between egg surface bacterial numbers and deaths of the incubating eggs.

CHAPTER SIX

EXPOSURE OF NEWLY-FERTILIZED EGGS TO HIGH CONCENTRATIONS OF BACTERIA RELATED TO SUBSEQUENT INCUBATING EGG MORTALITY

INTRODUCTION

In 1987 the United Kingdom certified the importation of 61.5 million eyed rainbow trout (*Salmo gairdneri*) eggs (Bromage & Cumaranatunga 1988) in order to meet the industry's expected demand for healthy adult fish. Importation of such vast numbers of eggs is undesirable on grounds of cost, genetic strategy and furthermore leaves the industry vulnerable to imported diseases. However, it is possible that approximately 48 million home produced rainbow trout eggs are "lost" during the incubation process (Chapter 1). A small reduction in the amount of eggs lost could in turn lead to a significant reduction in eyed egg imports. However, the causes of egg death during incubation are not fully understood and are likely to be many and varied. The period of time from egg ovulation to actual stripping is known to have a profound effect on egg survival rates during subsequent incubation (Sakai, Nomura, Takashima & Oto 1975 ; Hirose, Ishida & Sakai 1977 ; Lam, Nagahema, Chan & Hoar 1978 ; Craik & Harvey 1984 ; Springate, Bromage, Elliott & Hudson 1984) and provision of suitable environmental conditions is fundamental (Leitritz & Lewis 1976). The role of aquatic moulds of the *Saprolegniaceae* is also well documented (Smith, Armstrong, Springate & Barker 1985) and daily treatment with malachite green is fully accepted within the U.K. (Cline & Post 1972). However, the possible role of other micro-organisms such as bacteria on egg development is less clear.

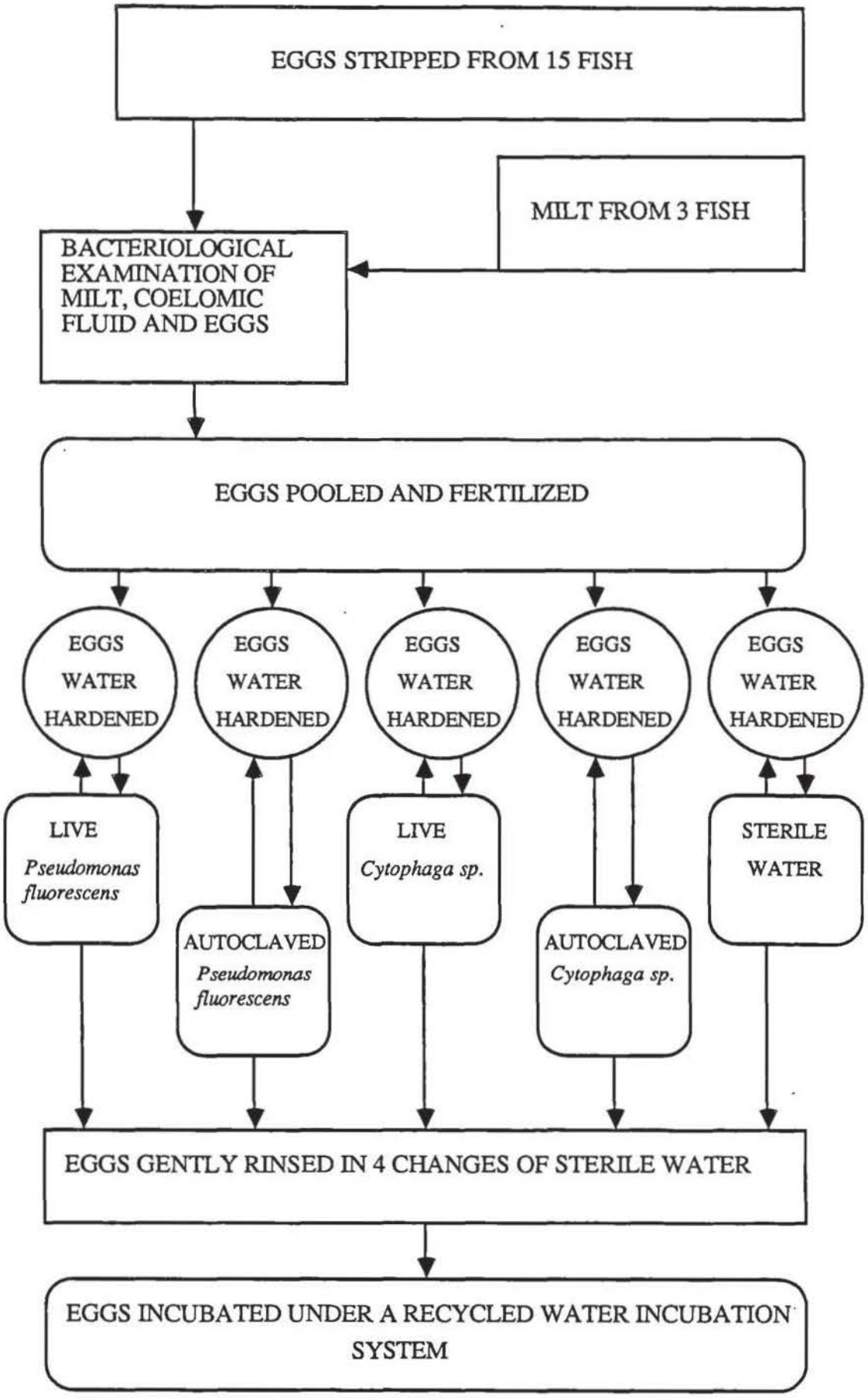
Oppenheimer (1955) first suggested that bacteria might be responsible for mortality of pelagic fish eggs. Further studies on incubating eggs from a wide range of fish have also implicated a relationship between bacteria and incubating egg death (Hunter 1959 ; McNeil, Wells & Brickell 1964 ; Bell, Hoskins & Hodgkiss 1971 ; Trust 1972 ; Yoshimizu, Kimura & Sakai 1980) (as fully described in Chapter 1). Bell (1966), Bell *et al* (1971), Trust (1972) and Yoshimizu *et al* (1980) have all characterized the large and varied bacterial community that occurs in association with surfaces of incubating salmonid eggs under a wide variety of environmental conditions.

However, only Trust (1972) considered it possible for egg surface bacteria to be responsible for salmonid egg death, and then only under certain circumstances, for example, high numbers of egg surface bacteria coupled with poor water circulation, leading in turn to insufficient levels of available oxygen for continued egg survival. Previous studies (Chapters 2 and 5) have also confirmed a specific bacterial community on incubating egg surfaces comprising mainly *Cytophaga* sp., *Pseudomonas fluorescens* and *Aeromonas hydrophila*, largely influenced by the prevailing environmental conditions. Statistical analysis (Chapter 5) suggested a close link between egg surface bacteria and egg death, although a straight forward "cause and effect" relationship was still thought unlikely to exist under optimum incubation conditions. It was therefore considered that a greater understanding of the above relationship might be achieved by careful manipulation of bacterial floras associated with egg surfaces.

In the following trial newly fertilized rainbow trout eggs were exposed to high concentrations of *P. fluorescens* and *Cytophaga* sp. during 45 minutes of water hardening. Both *P. fluorescens* and *Cytophaga* sp. were selected as they are frequently found on live incubating salmonid egg surfaces (Chapters 2 and 5). After exposure to selected bacteria, eggs were laid down under optimum incubation conditions and due to the large number of selected bacterial cells adhering to eggs it was considered likely that colonization by other bacterial species would at least be initially hindered. Thus, early egg mortalities within groups of eggs exposed to bacteria at water hardening might be directly related to the dominant (inoculated) egg surface bacterial flora. Replicate groups of eggs were also exposed to autoclaved suspensions of both *P. fluorescens* and *Cytophaga* sp. as water hardening of eggs in water of inferior quality might disrupt the hardening process in turn reducing egg viability, which could subsequently be interpreted as an effect of bacterial activity on eggs. Egg surface bacterial populations were monitored throughout incubation and all egg mortalities carefully recorded. In addition, exposure of eggs to bacteria at water hardening (when eggs are actively taking up water) might also result in internal egg

contamination via the micropyle. Therefore, throughout incubation internal egg contents were also investigated for bacterial contamination. A schematic outline of procedures and practices followed during this study is summarized in Figure 6 : 1.

FIGURE 6:1 Flow diagram of experimental protocol.



MATERIALS AND METHODS

Experimental design

Incubation system. One, single incubation system comprising of two separate (parallel) troughs was used during this trial. Each trough contained two sectioned aluminium trays to support eggs. Water at 10 °C ± 1 °C after passing over eggs at a flow rate of 3600 l / day, was redirected through a gravel filter bed (as a biological filter), before being recycled. A small volume of fresh "make-up" water (approximately 5% / day) was added to allow for evaporation and spillage (Figure 6 : 2).

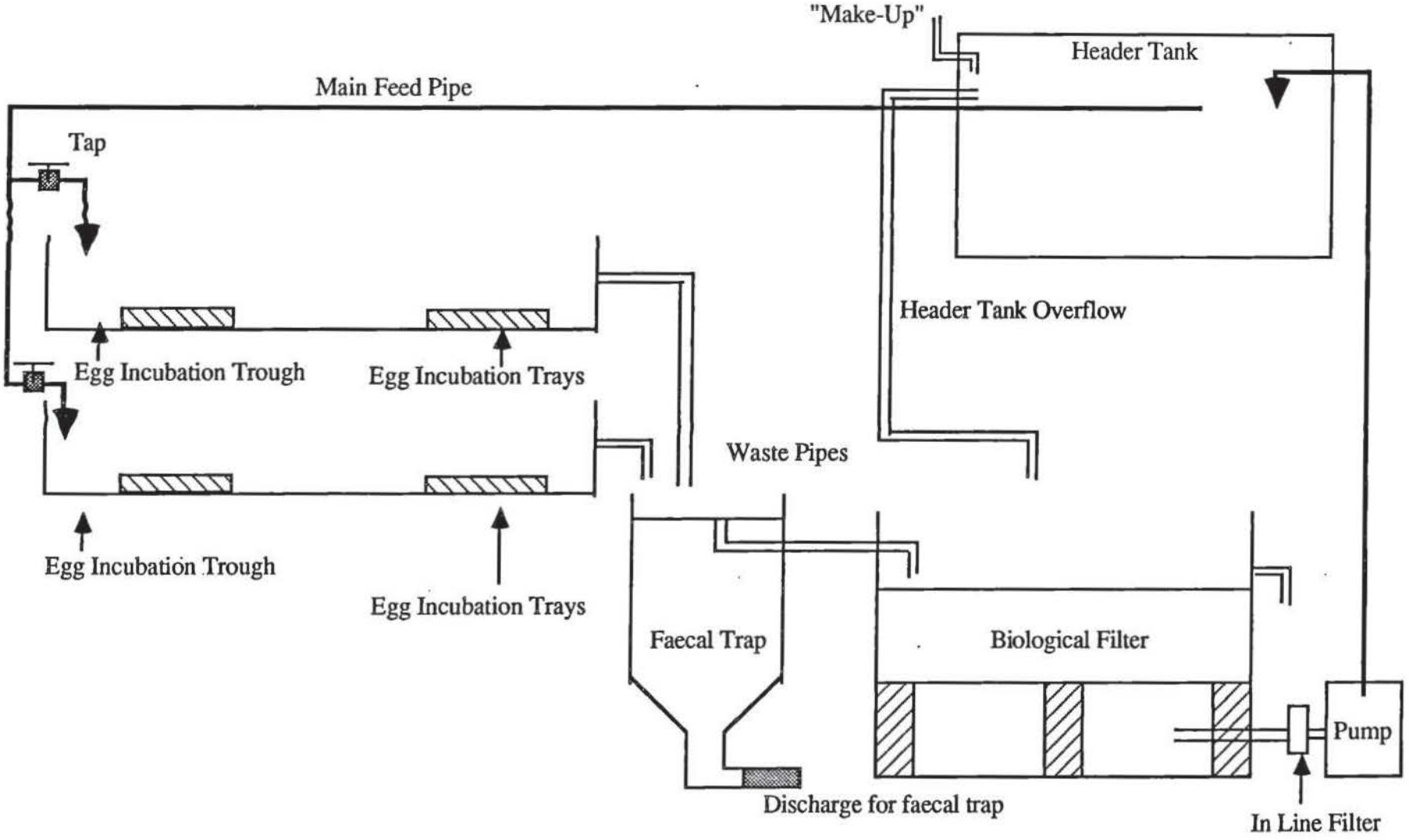
Collection of Eggs and milt

Stripping. Eggs were obtained from 15 ripe rainbow trout from a single commercial fish farm. Eggs of each fish were stripped into individual sterile containers. The first eggs from each fish were discarded to avoid any contamination from the surrounding water or ventrolateral surface of the female. Milt, also collected in sterile containers, was obtained from 3 sex-reversed females. Milt and eggs were immediately put on ice and returned to the laboratory. Eggs, coelomic fluid and milt from each container were removed for bacteriological examination.

Preparation of inocula

Bacteria. P. fluorescens and *Cytophaga* sp. were initially isolated from rainbow trout eggs (Chapter 2), subcultured, checked for purity and after extensive tests identified (Appendix 2 : 10 - 2 : 11). 25 ml flasks of defined sterile medium (Appendix 2 : 1) were inoculated with each of the two bacterial species and placed on a

FIGURE 6 : 2 Pumped recirculatory incubation system



continuous orbital shaker at 100 rpm and incubated at a temperature of 10 °C. Stationary phase cells of *P. fluorescens* were harvested after 48 hours and *Cytophaga* sp. after 72 hours. Cells were spun twice at 10, 000 rpm for 20 minutes and rinsed twice in sterile water derived from the Aston University Fish Culture Unit, before being filtered and finally resuspended in 500 ml volumes of sterile fish unit water. Suspensions were initially checked for optical density (L.K.B., Cambridge) and from a previously obtained standard curve of optical density against colony forming units (cfu's) / ml, a suspension of approximately 10^6 cells / ml was obtained. A more accurate adjustment to the final concentration employed was obtained by direct count using a haemocytometer. Two 500 ml volumes of 10^6 cells / ml were obtained for both species of bacteria. One of each was held at 10 °C whilst the others were sterilized by autoclaving (20 minutes, 115.5 °C, 0.72 Kg / cm^2) and then swiftly returned to 10 °C.

Inoculation of eggs

Water hardening. Eggs were pooled in a sterile container and fertilized. After 5 minutes excess milt was washed off with sterile fish unit water and eggs divided into 5 approximately equal volumes and placed into 5 separate sterile containers. 500 ml of sterile fish unit water was added to one batch of eggs. To the remaining 4 batches, one of the following 500 ml suspensions was added; 10^6 cells / ml *P. fluorescens*, 10^6 cells / ml autoclaved *P. fluorescens*, 10^6 cells / ml *Cytophaga* sp. and 10^6 cells / ml autoclaved *Cytophaga* sp. Eggs were left to water harden for 45 minutes at 10 °C before being rinsed in 4 changes of sterile water to remove excess volumes of bacterial suspensions and any unattached bacteria from egg surfaces.

Egg incubation

Laying down of eggs. Replicate batches of eggs from bacterial treatments were supported on the aluminium trays in incubation troughs. Eggs from each batch were assigned randomly to each individual section.

Egg fertilization rates were determined after 7 days at 10 °C by placing 100 eggs for each species into clearing solution (acetic acid : methanol : water, 1 : 1: 1 $^{v}/_{v}$) as outlined by Springate & Bromage (1984). Fertilized eggs could be clearly distinguished by the presence of a developing neural tube. At eyeing, eggs were "shocked" by siphoning from a height of 1m into a sterile beaker and any unfertilized eggs removed. Eyed eggs were replaced into their relevant sections in the incubators. Eggs were treated with malachite green oxalate (Difco, Surrey) twice a day at a concentration of 2 ppm.

Bacteriological Examination

Coelomic fluid and milt. Aliquots of coelomic fluid from each fish were serially diluted and inoculated in 0.02 ml amounts (drop inoculation method) onto Tryptone soya agar (T.S.A., Oxoid, Basingstoke), peptone beef extract glycogen agar (P.B.G., McCoy & Pilcher 1974) and low nutrient *Cytophaga* agar (Anacker & Ordal 1959). Plates were incubated at 20 °C for up to 10 days and enumeration performed only from drops where total separation of colonies occurred.

Eggs. Eggs were sampled at the green stage (unfertilized), immediately after water hardening and during incubation after 12 hours, 7 days, 14 days, 21 days and 28 days.

External sampling. For, each sampling time / treatment three separate batches of 10 eggs from each of the 5 groups were removed at random from appropriate sections. Subsequently, samples were rinsed in 4 changes of sterile water to remove any detritus or loosely adhered bacteria and added to 5 ml of sterile diluent (peptone - 0.1%, saline - 0.85%) before shaking vigorously for 2 minutes on a vortex stirrer, in a manner adapted from Evelyn, Ketcheson & Prosperi-Porta (1984). Aliquots of diluent were serially diluted and inoculated in 0.02 ml amounts (drop inoculation method) onto *Cytophaga* agar, P.B.G. and T.S.A. The number of viable cells transferred to each of the 3 different media was determined by plate count. Dividing by 10 gave the average count per egg and by taking into account dilution factor, volume of diluent and egg surface area, the number of viable colony forming units (cfu's) per mm^2 was estimated (as described in Chapter 2).

Internal sampling. At each sampling time 15 eggs randomly chosen for each species were removed and surface sterilized by placing in iodophor disinfectant (Buffodine - Evans Vanodine International Ltd, Preston) for 15 minutes at an available iodine concentration of 200 ppm (as described in Chapter 5). Such a process constitutes a longer treatment time and twice the manufacturers recommended concentration of iodine, as egg surface sterility was considered more important in an experimental context than any damage that might have occurred to the embryo. However, an even greater concentration of iodine (and thus guaranteeing surface sterility) could not be employed as there is evidence to suggest that substances of low molecular weight (such as iodine) may pass across the chorion into the perivitelline space (Hayes 1949 ; Potts & Rudy 1969 ; Rudy & Potts 1969) potentially influencing numbers of bacteria isolated. After treatment eggs were removed and rinsed in four changes of sterile water to remove any traces of disinfectant. Eggs were placed individually into small sterile tubes (LP3- Luckhams, Burgess Hill) containing 1ml of tryptone soya broth (T.S.B.) and incubated at 20 °C for 10 days. Surface sterilization was only considered to have been successful when the surrounding media was still clear and no growth occurred after a further 10 days when samples of the media were dropped onto the three agars.

Surface sterilized eggs were lanced in the tube (still containing sterile T.S.B. media) with a 1ml syringe and needle (0.5 x 16 mm) and the egg contents removed. Drops were inoculated directly onto agar media and into further tryptone soya broth.

Identification of bacteria. Bacteria isolated from green eggs and towards the end of incubation were identified by a variety of techniques, procedures and schemes outlined by Krieg and Holt (1984), Cowan (1974), the tables of Allen, Austin & Colwell (1983) and Stanier, Palleroni & Doudoroff (1966). Some Gram negative bacteria, especially *Aeromonas hydrophila*, were more easily identified using API 20E and 20NE identification strips (API laboratory products -Basingstoke). However, for the majority of sampling times *P. fluorescens* and *Cytophaga* sp. were the most frequently isolated organisms greatly facilitating identification processes.

Statistical analysis. Numbers of colony forming units per mm^2 for each species of egg were compared using a two way split-plot analysis of variance (Ridgman 1975; Snedecor & Cochran 1980). Differences between means were compared by calculating the standard errors and 95% confidence limits appropriate to the split-plot design (Snedecor & Cochran 1980). Numbers of hatched eggs against eggs that failed to hatch were compared using a chi square (χ^2) test. Effect of bacterial presence on egg surfaces was analysed by multiple regression chosen to compare numbers of surface bacteria with egg deaths by relating mortality to time (age) and bacterial numbers.

RESULTS

Egg development

Hatching Success. Figures obtained for egg performance are summarized by Table 6 : 1. From this table it can be estimated that overall a 96% fertilization rate of eggs was obtained leading to hatching rates ranging from 74.8% to 87% (Table 6 : 1) with a mean of 80.1%. Utilizing figures outlined in Table 6 : 1 the percentage eyeing of control, autoclaved *P. fluorescens* and autoclaved *Cytophaga* sp. can be calculated (83%, 91.1% and 87.5%) leading in turn to hatching percentages of 77.8%, 87% and 83.1% respectively as noted in Table 6 : 1. In comparison, by following the same exercise slightly lower percentage survivals were obtained for eggs originally exposed to live *P. fluorescens* and live *Cytophaga* sp. ; eyeing, 79.2% and 77.1% leading to 74.8% and 77.1% hatching (Table 6 : 1). It can also be seen from Table 6 : 1 that estimated fertilized egg losses were 21.2% and 18.9% for egg groups treated with live *P. fluorescens* and live *Cytophaga* sp. respectively. Fertilized egg losses for control eggs were 18.2%, and for egg groups treated with autoclaved bacteria losses were 9.0% (autoclaved *P. fluorescens*) and 12.9% (autoclaved *Cytophaga* sp.). Statistical analysis, taking into account total numbers of eggs incubated for each group over the whole incubation period, reveals overall a highly significant difference in successfully hatched eggs compared to eggs that failed to hatch between all groups ($\chi^2 = 70.98$, $P < 0.001$) (Appendix 6 : 1). However, this comparison is made between all 5 groups, including not only control eggs but, also those exposed to autoclaved bacteria, which performed equally as well, if not better, than the control group. If the two groups exposed to autoclaved bacteria are removed from the analysis and control eggs are compared to first, live *P. fluorescens* exposed eggs ($\chi^2 = 2.82$, $P > 0.05$) and secondly, live *Cytophaga* sp. treated eggs ($\chi^2 = 0.14$, $P > 0.05$), then no overall significant difference exists.

TABLE 6 : 1 Eyeing and hatching rates for all egg groups.

EGG SURVIVAL :	Control	*P. fluorescens* (autoclaved)	*Cytophaga* (autoclaved)	*P. fluorescens* (live)	*Cytophaga* (live)
Total number of eggs	1091	1216	870	1212	997
Estimated fertilized eggs	1047	1167	835	1164	957
Actual eyed eggs	906	1108	761	960	807
Actual hatched eggs	849	1058	723	907	769
Actual % hatched eggs	(77.8)	(87.0)	(83.1)	(74.8)	(77.1)
EGG LOSS :					
Deaths to eyeing	132	59	68	212	161
Deaths at shocking	53	49	41	40	29
Deaths eyeing to hatching	57	50	38	53	38
Eggs that failed to hatch	242	158	147	305	228
UNEXPLAINED LOSSES :					
Estimated unfertilized eggs	44	49	35	48	40
Estimated fertilized losses	198	109	112	257	188
Estimated fertilized losses	(18.2)	(9.0)	(12.9)	(21.2)	(18.9)

Figures in brackets = percentages.

Daily egg mortalities are recorded in Figure 6 : 3 from which it can be seen that eggs exposed to live *P. fluorescens* suffer the greatest egg mortality, and in comparison to all other egg groups suffers an exceedingly high mortality rate over the first 5 days of incubation. Indeed statistical analysis of numbers of live eggs and dead eggs for each group, compared not over the whole incubation period (as above) but, at 7 day intervals (Figure 6 : 4) reveals a highly significant difference in "rate" of egg death between control eggs and eggs exposed to live *P. fluorescens*, over the first seven days of incubation (χ^2 = 49.13, P < 0.001). In comparison, a significant difference between control eggs and eggs treated with live *Cytophaga* sp. is not seen at this time (1-7) days but, is found between 14-21 days (χ^2 = 7.08, P < 0.05) (Appendix 6 : 2).

Bacteriological Examination

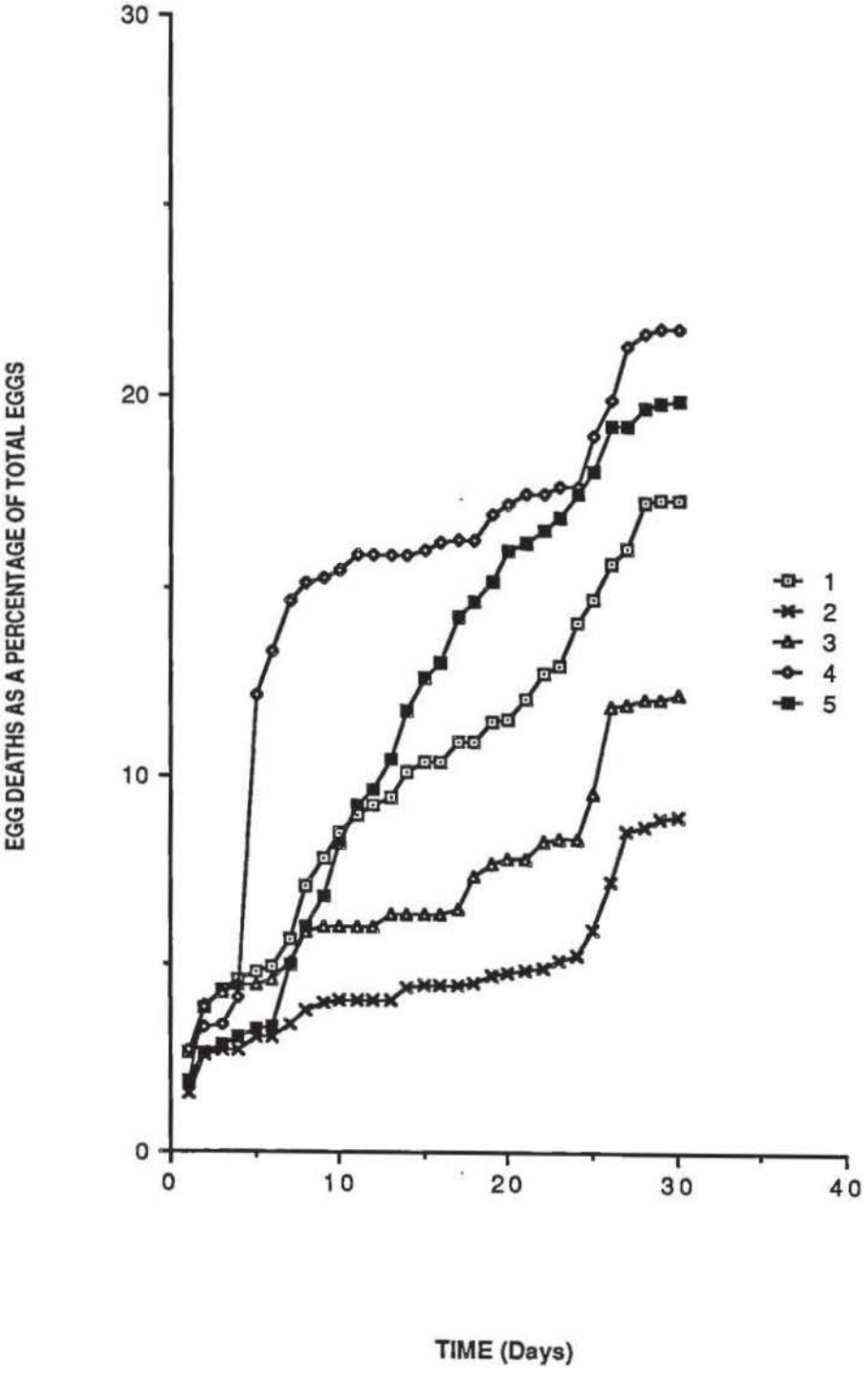
Coelomic fluid. Bacteria were isolated from 10 out of the 15 samples of coelomic fluid. Total numbers of recovered colony forming units (cfu's) ranged from 10 to 100 cells / ml. Bacteria most frequently isolated were ; *Pseudomonas* sp., *Aeromonas hydrophila, Corynebacterium* and *Staphylococcus epidermis.*

Milt. Bacteria were isolated from all samples of milt and were estimated to be in the range of 100 to 1000 cells / ml. However, removal of testis may have allowed some contamination to have occurred (as previously described, Chapter 5). Bacteria isolated were similar in genera and species to those isolated from coelomic fluid.

External Egg sampling. Few bacteria were found attached to surfaces of green (unfertilized) eggs, and for purposes of this study can be considered as virtually "sterile". After water hardening, control eggs and those treated with autoclaved bacteria predictably showed no real increase in numbers of surface adhered bacteria. However, those bacteria exposed to live bacteria had high numbers adhering to their surfaces ; estimated mean figures approaching 10,000 cfu's / mm^2 (*Cytophaga* sp.) and 20,000 cfu's / mm^2 (*P. fluorescens*).After gentle rinsing, eggs were laid down for incubation

FIGURE 6:3 Accumulative total of dead eggs

(recorded daily)



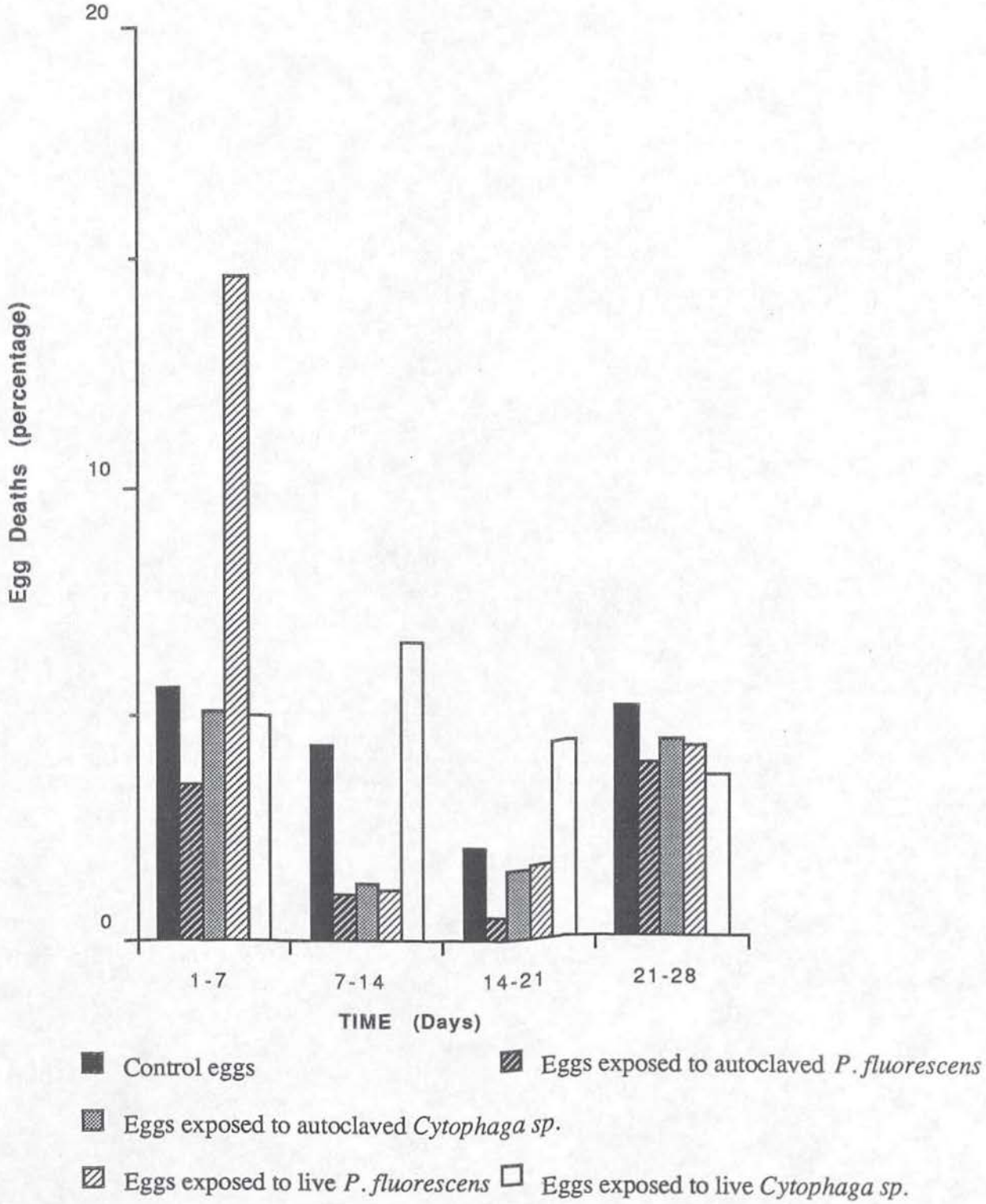
1 = Control eggs, 2 = eggs exposed to autoclaved *P. fluorescens*

3 = Eggs exposed to autoclaved *Cytophaga* sp.

4 = Eggs exposed to live *P. fluorescens*

5 = Eggs exposed to live *Cytophaga* sp.

FIGURE 6 : 4 Egg deaths per 7 day period as a percentage of total number of eggs incubated for each group.



and total numbers of cfu's / mm^2 of egg surface are summarized in Table 6 : 2 for all sampling times, from green eggs to 28 days. From this table it can be seen that during the first 12 hours of incubation many bacteria were "washed off" egg groups treated with live bacteria and in contrast increases in number of surface bacteria were subsequently seen on control eggs and those originally exposed to autoclaved bacteria. Analysis of cfu's recovered for all egg groups (by two way split-plot analysis of variance) revealed overall (from 12 hours to 28 days) a highly significant difference in numbers of surface bacteria between groups ($P < 0.001$). A highly significant effect of time was seen ($P < 0.001$) but, no significant interaction between time and numbers was found ($P > 0.05$) (Appendix 6 : 3).

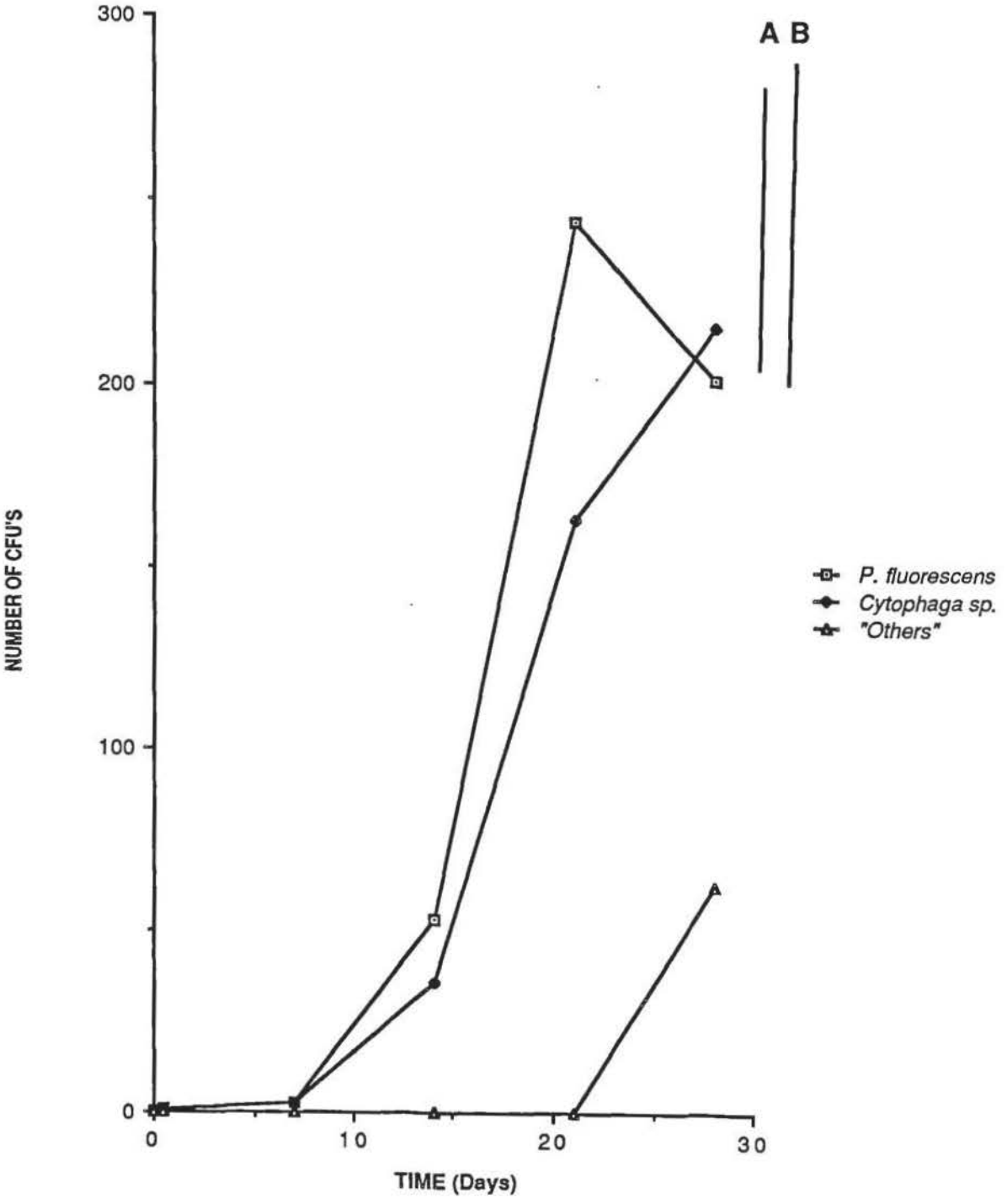
Although, groups of eggs were kept physically apart in incubation trays, it was inevitable that live bacteria from hatchery water or washed off treated eggs would eventually colonize eggs from other groups. Control eggs (Figure 6 : 5) were colonized at virtually the same rate by both *P. fluorescens* and *Cytophaga* sp. All other bacterial species were excluded from these egg surfaces (at least in numbers high enough to recover) until the last sampling at 28 days of incubation. Eggs treated with autoclaved bacteria (Figures 6 : 6 and 6 : 7) were colonized in a similar manner to the control eggs by both *P. fluorescens* and *Cytophaga* sp. although surprisingly *Cytophaga* sp. occurred in far greater concentrations than *P. fluorescens* (about 1.5 times greater) on egg surfaces originally treated with autoclaved *Cytophaga* sp. However, both these two groups of eggs differed from the control group in that "other" bacterial species appeared far earlier on during incubation leading to concentrations of "other" bacteria as much as double that found on control eggs. Eggs originally treated with live bacteria exhibited a different pattern of colonization to the above three groups. *P. fluorescens* treated eggs (Figure 6 : 8) began incubation with large numbers of bacteria on their surfaces and partial domination by this organism (*P. fluorescens*) was seen throughout incubation. However, complete domination of surfaces did not occur since after 14 days *Cytophaga* sp. were recovered and after 21 days "other" bacteria were also isolated. Similarly live *Cytophaga* sp. treated eggs (Figure 6 : 9) started incubation

TABLE 6 : 2 Estimated total colony forming units for all bacteria per sq mm egg surface for all egg groups.

	Control	*P. fluorescens* (autoclaved)	*Cytophaga* sp. (autoclaved)	*P. fluorescens* (Live)	*Cytophaga* sp. (Live)
Green Eggs	0.2	0.2	0.2	0.2	0.2
After Hardening	0.2	0.2	0	19160	9792
12 hours	1.0	4.9	1.0	30.6	48.8
7 Days	4.5	9.0	6.2	28.6	109.9
14 Days	89.2	32.8	24.1	106.5	216.7
21 Days	406.4	706.2	600.3	1315.8	528
28 Days	478.8	597.6	680.7	1075.1	485.1

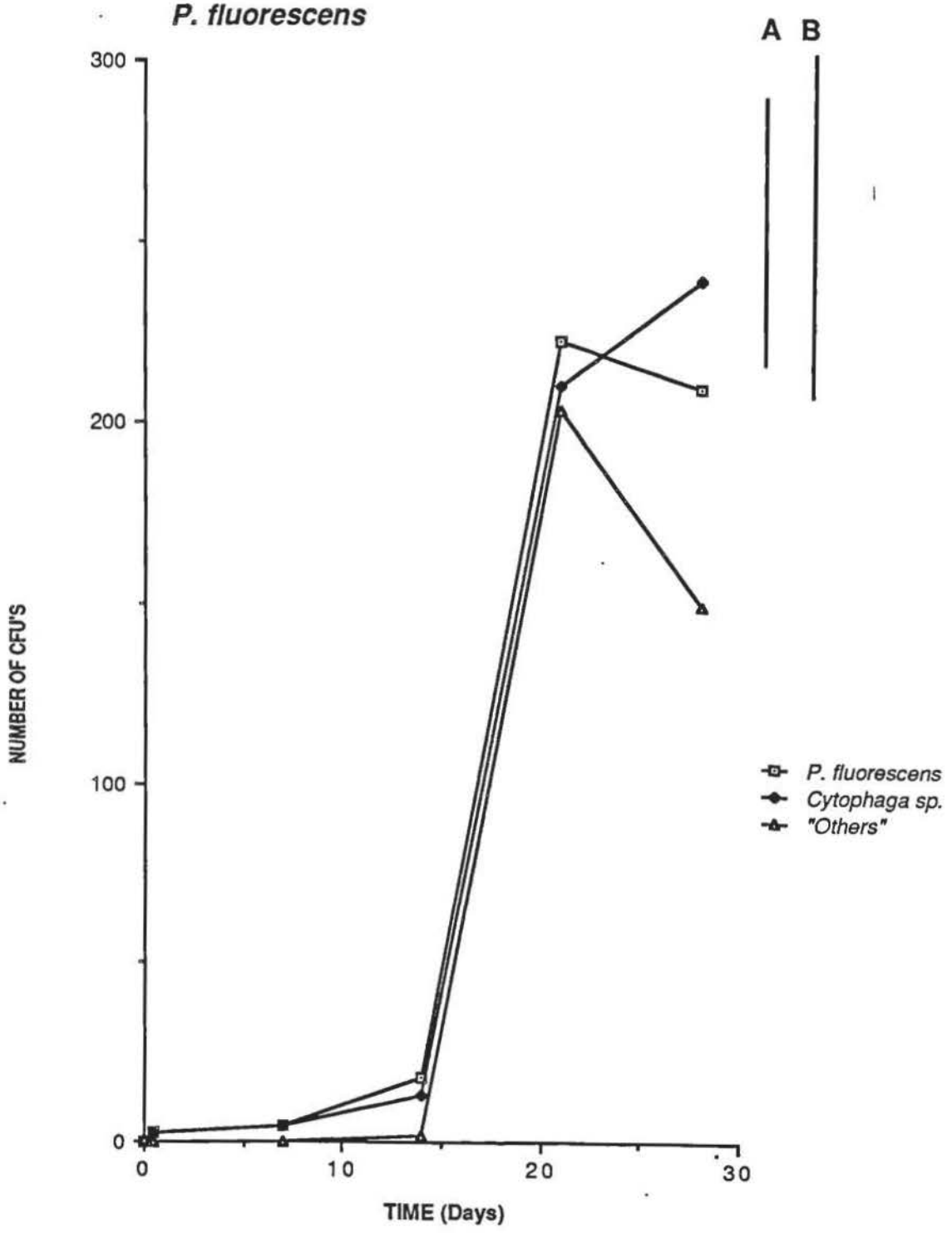
Mean figures obtained from 3 replicates on 3 different media. 95% confidence limits, from 12 hours to 28 days ; differences within a treatment 366.1 and for differences between two treatments at the same or different times 489.4.

FIGURE 6:5 Estimated colony forming units per sq mm egg surface (control eggs)



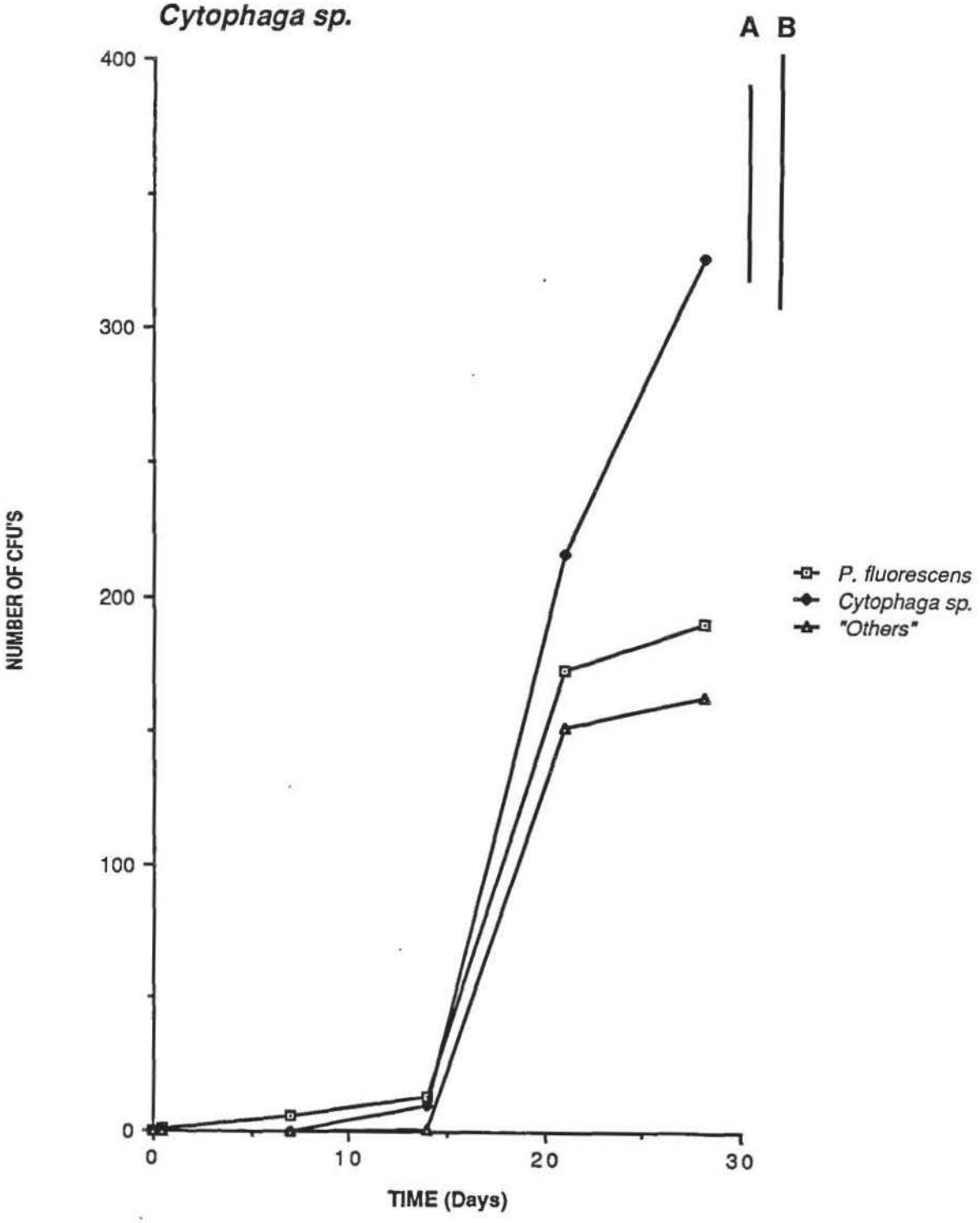
A = 95% confidence limits for differences within a treatment and B = 95% confidence limits between 2 treatments at the same or different times

FIGURE 6:6 Estimated colony forming units per sq mm egg surface for eggs treated with autoclaved *P. fluorescens*



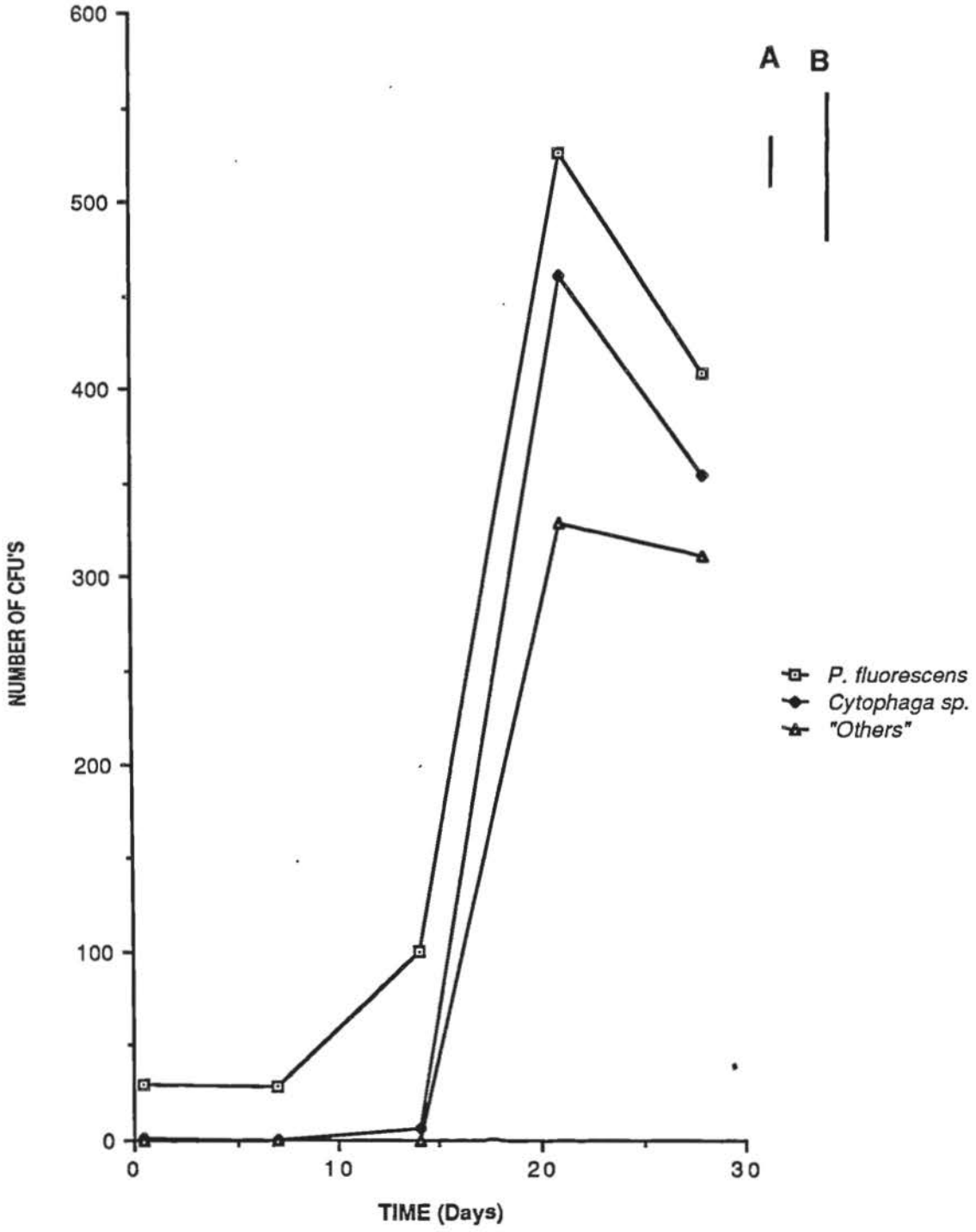
A = 95% confidence limits for differences within a treatment and B = 95% confidence limits between 2 treatments at the same or different times

FIGURE 6:7 Estimated colony forming units per sq mm egg surface for eggs treated with autoclaved *Cytophaga sp.*



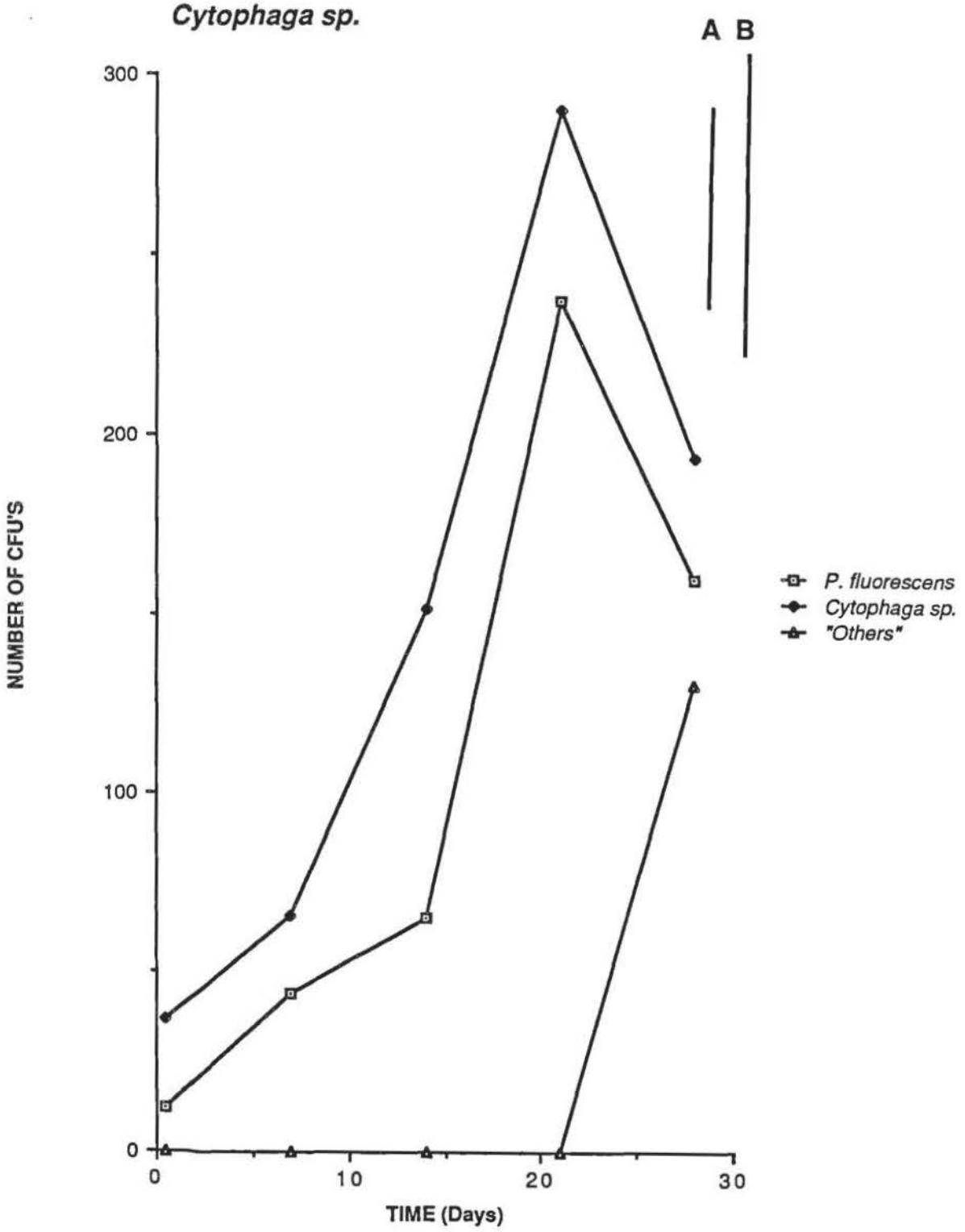
A = 95% confidence limits for differences within a treatment and B = 95% confidence limits between 2 treatments at the same or different times

FIGURE 6:8 Estimated colony forming units per sq mm egg surface for eggs treated with live *P. fluorescens*



A = 95% confidence limits for differences within a treatment and B = 95% confidence limits between 2 treatments at the same or different times

FIGURE 6:9 Estimated colony forming units per sq mm egg surface for eggs treated with live *Cytophaga sp.*



A = 95% confidence limits for differences within a treatment and B = 95% confidence limits between 2 treatments at the same or different times

with considerable numbers of bacteria adhered to their surfaces and throughout incubation were dominated by this particular organism. However, in comparison to the above results (Figure 6 : 8) *P. fluorescens* quickly colonized these egg surfaces and were recorded present after only 12 hours. Once again "other" species of bacteria were not found until 21 days of incubation.

Internal Egg Sampling. During the course of the experiment a total of 75 eggs (15 from each group) were investigated at each of 7 sampling times. Few eggs were found to contain bacteria, although *Pseudomonas* sp. were isolated from 4 eggs and *Aeromonas hydrophila* from 2.

Egg death. As so few eggs were colonized internally by bacteria it was decided to disregard any effect internal contamination might have on incubating egg death. Effect of bacterial presence on egg surfaces was analysed by multiple regression which demonstrated a slight correlation between bacterial numbers and egg deaths ($r = 0.52$) (Appendix 6 : 4).

DISCUSSION

Exposure of newly fertilized eggs to high concentrations of bacteria at water hardening resulted in many bacteria adhering to egg surfaces but not subsequent total domination of egg surfaces by anyone bacterial species. After gentle rinsing 20,000 cfu's / mm^2 of *P. fluorescens* remained attached to egg surfaces and similarly 10,000 cfu's / mm^2 of *Cytophaga* sp. were still adhered to their respective egg surfaces. Within the first 12 hours of incubation approximately 99.8% of *P. fluorescens* cells were "washed off" into recirculating hatchery water, even so many cells still remained attached to egg surfaces. Attachment at such an early stage of incubation of so many cells, in turn permitted continued domination of egg surfaces by *P. fluorescens*, although eventually, *Cytophaga* sp. became established, followed by "other" bacterial species. In a similar vein, eggs originally exposed to live *Cytophaga* sp. during water hardening "lost" 99.6% of their bacteria after 12 hours incubation. On this occasion, despite an already established population of *Cytophaga* sp. on these egg surfaces, colonization by *P. fluorescens* occurred at an early stage of incubation (possibly a reflection of the faster growth rate of *P. fluorescens* compared to *Cytophaga* sp. - Chapter 3). Irrespective of which bacteria predominated both egg groups were eventually colonized by large numbers of "other" bacteria (comprising mainly of *A. hydrophila* and *Pseudomonas* sp.) and although often slow to establish, possibly due to their presence in lower numbers in the hatchery water, their combined numbers on egg surfaces by the end of incubation approached those of *P. fluorescens* and *Cytophaga* sp. In contrast to the two egg groups discussed above, control egg surfaces were considered sterile at time of laying down but subsequently, immediate exposure to relatively high numbers of bacteria probably occurred (washed off from surfaces of egg groups above). Such control egg surfaces were colonized at about the same rate by *P. fluorescens* and *Cytophaga* sp., which may be indicative of their ability to compete successfully in each others presence during initial establishment on "sterile" surfaces.

A major aim of this investigation was to ascertain more fully whether egg mortality could be connected with bacterial loading. Initially a difference in egg surface bacterial flora was achieved between egg groups and analysis by χ^2 revealed that those eggs originally exposed to live *P. fluorescens* experienced a far greater mortality rate. Trout eggs are well known to be at their most fragile during the early part of incubation (Post, Power & Kloppel 1974 ; Leitritz & Lewis 1976) and therefore environmental conditions were designed to minimize losses which can occur through knocking, excess light and low oxygen levels. Irrespective of such care greatest losses experienced during this investigation tended to be amongst young eggs especially within the group exposed to live *P. fluorescens*, possibly indicating that at this early, sensitive stage of incubation the presence of large numbers of bacteria is potentially damaging to hatchery enterprises. The actual mechanisms or processes by which bacteria could effect egg incubation remains unclear. However, as already stated (Chapter 2) Trust (1972) considered it possible that surface bacteria, possibly reaching a theoretical maximum of 10^8 bacterial cells / mm^2 could lead to oxygen deprivation and subsequent death of developing embryo's. However, during this trial great care was taken over such factors as egg density and water flow rate and it is unlikely that poor, local environmental conditions (and hence low levels of available oxygen) were responsible for egg deaths. Bell, Hoskins & Hodgkiss (1971) considered it unlikely that egg surface bacteria could produce sufficient enzymes or toxins to penetrate the extremely tough egg outer membrane (Young & Smith 1956 ; Bell, Hoskins & Bagshaw 1969). Although, there is no evidence to contradict their argument, it is well worth noting that the three most frequently occurring bacteria on egg surfaces, *Cytophaga* sp., *Pseudomonas* sp. and *A. hydrophila* , are all well known opportunistic pathogens and secondary invaders of diseased or injured fish. *Cytophaga* sp. have been implicated in bacterial gill disease of salmonid fish (Borg 1960; Anderson & Conroy 1969). *P. fluorescens* is an organism commonly isolated from many fresh water environments (Allen, Austin & Colwell 1983) and has also been implicated as a secondary invader of damaged fish tissues and even on occasions as a

primary invader (Roberts & Horne 1978). *A. hydrophila* like *A. salmonicida* (the cause of furunculosis) possesses adhesins allowing it to attach closely to selected eukaryotic cells (Trust, Canotice & Atkinson 1980) and is known to produce an extended range of enzymes and exotoxins (Wadström, Lungh & Waetlend 1976). Therefore, although salmonid embryo s enjoy the protection of a tough outer membrane the presence of all three bacterial groups on egg surfaces can not be dismissed as of little consequence, for even if eggs are not damaged during incubation, successfully emerging fry will immediately come into contact with high numbers of potential secondary and primary invaders.

In this trial few bacteria were isolated from bacterial contents, in contrast to Sauter, Williams, Meyer, Celnik, Banks & Leith (1987), who found a large range of bacteria within salmon eggs (*O. tshawytscha*) Walbaum, however this may possibly be a reflection on the large size of salmon eggs compared to rainbow trout eggs (allowing easier access for bacteria) or their particular experimental method of "enrichment" recovery. Results outlined earlier indicate that ovulated eggs are exposed to bacteria from an early stage, even within the coelomic cavity. Whether eggs are exposed to bacteria before ovulation is unclear but, it seems unlikely that most bacterial species could manage to enter eggs before they are shed into the coelomic cavity. One possible exception may be *Renibacterium salmoninarum* (responsible for Bacterial Kidney Disease - B.K.D.), a highly specialised bacterium able to live and reproduce intracellularily (Young & Chapman 1978 ; Bruno & Munro 1986). Other bacteria entering eggs probably do so once eggs have been shed into the coelomic cavity and gain access via the micropyle which for rainbow trout eggs is 3.3-4.3 µm in diameter (Riehl 1980). In comparison *P. fluorescens* is only 0.8 µm wide and *Cytophaga* sp. 0.5 µm wide (Palleroni 1984 ; Strohl & Tait 1978). However,in this study "naturally occurring" bacteria did not appear to be in coelomic fluid in sufficiently high concentrations to promote infection. In contrast, diseased broodstock suffering from B.K.D. infections are often seen to have a coelomic fluid that appears milky, due to considerable numbers of bacteria, often as many as 4.0 to 8.0 x 10^9 cells / ml as

revealed by plate count (Evelyn, Prosperi-Porta & Ketcheson 1981; Evelyn *et al* 1984). Under such conditions a much greater possibility of bacterial entry into eggs (after their shedding into the coelomic cavity) must therefore exist. Samples of milt were also shown to have a bacterial content (albeit low) and it is possible that entry could also occur at fertilization but, perhaps a more likely stage for infection is during water hardening, when eggs themselves actively take up water and possibly any aquatic bacteria that may be present. Trust (1972) indicated that approximately 1.5×10^8 bacterial cells would be needed to cover the entire surface of a rainbow trout egg (and therefore the micropyle). In this study 500 ml of 10^6 cells / ml were used to cover 1000 eggs - a lower concentration than suggested by the above author but one high enough to create an extremely cloudy solution in which to harden eggs and one surely allowing sufficient opportunity for bacterial entry during the 45 minute process of water hardening. Entry via the micropyle would allow access to the perivitelline space but further progress into the yolk might be impaired by the vitelline membrane. However, potentially bacteria might cross the vitelline membrane either taken up by pinocytosis or during a temporary period of disruption to the membrane that occurs at water hardening (Potts & Rudy 1969). Most bacteria though would probably be confined to the perivitelline space and not gain access to the yolk. Once confined in this space bacteria would be denied any nutrients suitable for microbial growth that are often sufficiently abundant in hatchery waters (Willoughby, McGrory & Pickering 1983) and would presumably have to rely on waste products from developing embryos. However, trout embryos are very efficient at using nutrients available to them and only small quantities of carbon dioxide and ammonia are known to be excreted (Hayes 1949 ; Smith 1947; 1957). Therefore, whether bacteria in the perivitelline space could obtain sufficient nutrients for growth or reproduction and inturn disrupt eggs remains unlikely.

In this present study eggs were only taken from a total of 15 fish in order that great care could be taken with stripping, fertilization and with the provision of optimum incubation conditions. High levels of hatching and very low levels of mortality were therefore obtained, regardless of all the factors that have potential to influence egg

survival (in addition to exposure to live bacteria in high concentrations during water hardening). However, under commercial farm conditions it would be impossible to take such care due to pressures of time and sheer numbers of fish. Eggs that are obviously overripe will of course be noticed and discarded but inevitably eggs reflecting a wide range of quality will be pooled together, fertilized and incubated. Presently the only form of prophylactic treatment these eggs are likely to receive is the daily addition of malachite green at a concentration effective for reducing the levels of *Saprolegnia* sp. but totally ineffective at influencing bacterial populations. Furthermore, use of malachite green is often minimized since excessive levels may, in themselves, lead to reduced hatching rates. If bacteria can influence egg survival (as suggested in Chapter 5 and during this trial), even if only under particular conditions such as poor egg or water quality, then some additional form of prophylactic treatment may be considered necessary in order to maximize the number of successfully hatched fry. Additionally great care should be exercised wherever possible to ensure a pure (low bacterial content) source of water for the purposes of both water hardening and subsequent egg incubation.

SUMMARY

Exposure of newly-fertilized salmonid eggs to high concentrations of viable bacteria resulted in considerable numbers of cells adhering to egg surfaces. However, during subsequent incubation many bacteria were removed from eggs, although treated egg surfaces were still colonized by far higher numbers of bacteria (either *Pseudomonas fluorescens* or *Cytophaga* sp.) than might otherwise have occurred during such early stages of incubation. A microbiological examination of egg contents revealed little bacterial contamination within treated eggs. Thus, significantly greater mortalities amongst egg groups previously exposed to *P. fluorescens* , were considered due to the presence in high numbers of such bacterial cells on surfaces of young eggs during early "sensitive" stages of incubation.

CHAPTER SEVEN

TREATMENT OF INCUBATING RAINBOW TROUT EGGS WITH ANTIBIOTICS AND ANTIMICROBIAL COMPOUNDS TO INCREASE EGG HATCHING SUCCESS

INTRODUCTION

Use of antibiotics and antimicrobial compounds has been well established within the agricultural industry for many years (Yeoman 1982). Principally, they have been employed for the treatment of specific diseases against identified pathogens in a manner similar to that of human medicine. However, such compounds have also been added in low levels to animal feeds in order to increase feed efficiency and improve weight gain (Stokstad, Jukes, Pierce, Page & Franklin 1949 ; Stokstad & Jukes 1950). Low level addition of antibiotics in animal feed has undoubtedly resulted in a decline in sensitivity of some bacterial pathogens to particular antibiotics. Concern about such possibilities, which culminated in the Swann report (1969), have since been clearly proven (Levy, Fitzgerald, & Macone 1976a ; Levy, Fitzgerald, & Macone 1976b ; Richmond & Linton 1980).

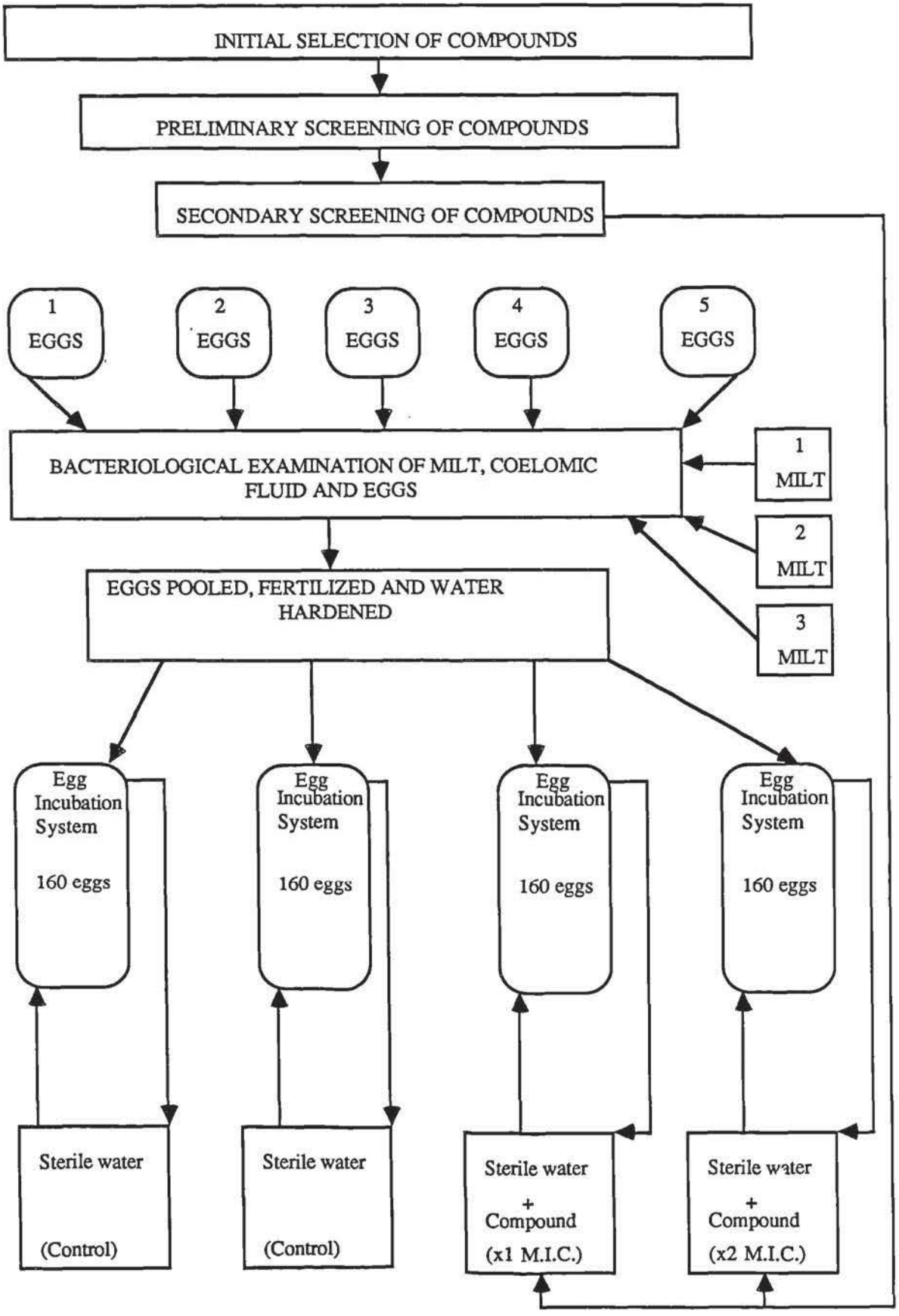
Bacterial resistance to antibiotics may develop within a population through several distinct mechanisms. Aforemost of which includes both selection of bacterial strains carrying plasmids, small extrachromosomal D.N.A. elements coding for resistance and plasmid transfer. Included amongst such plasmids are R factors, which carry genes bestowing antibiotic resistance. Examples of such plasmids are those which carry genes coding for β lactamases, a group of enzymes which split β lactam rings of penicillins and cephalosporins thereby rendering them inactive. In addition practices associated with antimicrobial therapy can often lead to the inadvertent selection of resistant bacterial strains. Frequently, this can occur when a pathogenic organism is originally tested for antibiotic susceptibility, whereby trials are often performed on relatively few colonies "picked" at random from a sub-culture plate. Other colonies on the plate may already posses natural resistance (for example plasmids) and will be overlooked. Thus, subsequent therapy with antimicrobial drugs may well prove to be both ineffective and lead to the selection of resistant strains of the pathogen (Sabath 1982).

In the Aquaculture industry one of the earliest usages of chemicals was the addition of salt to control ectoparasites such as *Costia*. Leger (1909) further improved control of *Costia* with the use of formalin. In the early 1930's other disinfectants such as copper sulphate, potassium permanganate and malachite green were first utilized (Alderman 1988). Development of systemic antimicrobial agents in the late 1930's swiftly led to their use on fish farms: sulphonamides (Litchfield 1939 ; Wolf 1939), nitrofuran (Gutsell 1946), chloramphenicol (Smith 1950), oxytetracycline (Snieszko, Friddle & Griffin 1952) and 4-quinolones (Endo, Ogishima, Hayasaka, Kaneko & Oshima 1973a). However, only a few antimicrobial compounds have been officially licensed for fisheries use in Great Britain (Austin 1985a), although, a wide range of compounds are available (Snieszko 1978 ; Herwig 1979 ; Austin 1984 ; Austin 1985b) and in the past many have, probably, been used on fish farms (Austin & Austin 1987).

In previous studies (Chapters 2,5 and 6) eggs were held in large incubation systems, similar in both design and scale to those found under commercial conditions. These included a flow-through system (Chapter 5), where water passed over eggs once and went to drain, and re-cycling systems (Chapters 2, 5 and 6), where water passed through a gravel filter bed (as a biological filter) and was continually re-cycled over eggs, by pumping. Both these systems involved the use of many litres of water and even the re-cycling system (as well as the flow-through system) allowed excess water to go to drain. However, in this present study, where it was proposed to test antibiotics and antimicrobial compounds for their potential to reduce "commensal" bacteria on incubating salmonid egg surfaces, both at regular intervals and over a protracted period of time, such incubating systems (as described above) were considered to be inappropriate. In the context of previous comments (above) concerning indiscriminate use of antibiotics and antimicrobial compounds, any study which allowed large quantities of such compounds to drain into the environment along with bacteria that might have been exposed to them, would be hard to justify. Naturally, therefore, it was deemed necessary to design and construct a unique, small scale, totally enclosed, egg incubation system (to be described in detail later), whose design was dependant on

several diverse considerations. First, the equipment designed should allow eggs to develop normally throughout incubation leading to successfully hatched fry. Secondly, all water and antimicrobial compounds used in the system had to be kept isolated from the environment and drained after use into a suitable container for sterilization, in theory destroying any surviving bacteria that may have been exposed to test compounds. Thirdly, the equipment had to allow water (and compounds) to be removed and replaced during the course of incubation without excessive disturbance to delicate incubating embryo's. A consideration deemed necessary as it was envisaged that compounds would gradually decline in concentration and their efficacy be reduced with progressive incubation. Thus, it was proposed that all water and dissolved compounds would have to be removed at regular intervals (to be determined experimentally) and replaced with fresh sterile water and renewed concentrations of compound. During this period of water change it was particularly important that the equipment designed allowed some water to remain covering eggs, in order to reduce knocking and jolting of delicate embryos. Irrespective of equipment design it was realized that water flow over eggs would be temporarily disrupted however undesirable, choice of test compounds would therefore be governed in part by their stability and residual activity. These parameters were assessed by a number of independent preliminary trials which determined the potential of compounds to reduce egg surface bacteria and ensured that their efficacy did not over rapidly diminish once placed into the incubation system. Experimental procedures and protocols for both pretrial screening of compounds and for the subsequent main trials are schematically outlined in Figure 7 : 1. Finally, it must be stressed that the objective of the study was not to encourage indiscriminate use of antibiotics in hatchery systems, but to reduce egg surface bacterial numbers, in an attempt to increase eyeing and hatching rates of salmonid eggs under closely controlled laboratory conditions.

FIGURE 7:1 Flow diagram of experimental protocol.



MATERIALS AND METHODS

Selection of antimicrobial compounds

Initial selection. A range of antimicrobial compounds and antibiotics were selected for their potential ability to inhibit the growth of *Pseudomonas fluorescens* and *Cytophaga* sp. (both commonly found on incubating egg surfaces - Chapters 2, 5 and 6). This initial selection of compounds was based on their reported effectiveness against specific fish pathogens in a commercial fish farm environment or in the field of human medicine. The compounds initially tested were ; oxolinic acid, furazolidone, oxytetracycline, chloramphenicol, chlortetracycline, erythromycin, gentamicin, carbenicillin, polymixin in conjunction with streptomycin, malachite green and iodine (iodophor disinfectant). Compounds were dissolved in water or 10% ($^{v}/_{v}$) dimethyl sulphoxide (DMSO) to aid solubility where appropriate (Austin, Morgan & Alderman 1981).

Test protocol for preliminary screening of compounds (minimum inhibitory concentration - M.I.C.). The protocol for the testing of compounds was based on that of Austin *et al* (1981). Compounds, in a range of concentrations were incorporated into sterile, molten (40 °C) tryptone soya agar (T.S.A. - Oxoid, Basingstoke) and poured into 90 mm diameter sterile petri dishes. Plates were stored inverted over night at a temperature of 37 °C to allow removal of excess surface moisture. Subsequently, four evenly spaced 4 mm diameter plugs were removed from the agar using a "cork" borer. A plug of a young actively growing culture was placed into each of these holes and plates were incubated for 20 days at 10 °C. After this time the presence or absence of microbial growth around the plug was noted. Plugs were removed and placed into fresh tryptone soya agar plates (lacking antibiotic). Growth on this occasion would

indicate that earlier inhibitory activity in the presence of the antimicrobial compound was bacteriostatic rather than bacteriocidal. All concentrations used and the results obtained were recorded (Appendix 7:1).

Four compounds, based on their ability to inhibit either *P. fluorescens* and / or *Cytophaga* sp.,were selected for further study ; oxolinic acid, chloramphenicol, chlortetracycline and oxytetracycline. The iodophor disinfectant was equally as effective as the above compounds but was not selected, solely due to it's tendency to "froth" which made it totally unsuitable for the small scale, aerated incubation systems used during this study.

Test protocol for secondary screening of compounds. The decline in efficacy of compounds was tested microbiologically in a similar manner to that outlined by Evelyn, Ketcheson & Prosperi-Porta (1986). These authors were interested in the effect of erythromycin on *Renibacterium salmoninarum* (a Gram +ve organism, responsible for bacterial kidney disease) and, in so doing used a reference strain of *Sarcinia lutea* (also Gram +ve) as a "test" organism. In this study, compounds were selected to reduce levels of *P. fluorescens* and *Cytophaga* sp. (both Gram -ve) and thus, a Gram -ve test organism - a strain of *Escherichia coli* (W 3110), was chosen. This particular organism was selected for two main reasons ; first, it had already been used as a control organism for previous minimum inhibitory concentration trials (above) and secondly, it had exhibited a similar profile of antibiotic susceptibility to test compounds as shown by both *P. fluorescens* and *Cytophaga* sp. Test plates were prepared by first pouring 20 ml of assay medium (Oxoid : Antibiotic Assay Medium No. 1) into standard size petri-dishes. These were then topped with 6 ml of the same medium with 0.1 ml of a phosphate buffered saline suspension of the assay organism (1.0 O.D. at 460 nm ; pH 7.0). Antibiotic assay was determined by regular sampling over 36 hours of circulatory water derived from the egg incubation systems containing antibiotic but no eggs. Dry, sterile blotting paper disks (6 mm diameter) (Oxoid) were briefly soaked in circulatory water, placed onto prepared media and incubated right side up at 25 °C for 5

days, after which time the diameter of growth inhibition was carefully measured. From a previously constructed standard curve for each compound, based on zones of inhibition with known amounts of compound (Appendix 7 : 2), a guide to the decline in concentration of each compound with the passage of time was obtained (Appendix 7 : 3). From these calibration curves it was determined that oxytetracycline would have to be replaced at 12 hour intervals in order to maintain "active" levels just above the determined minimum inhibitory concentration value, leading in turn to greater egg disturbance than would be desirable (as previously discussed).

Experimental design

Construction of equipment. Each incubation system comprised of a reservoir of 3 litres of aerated water which was pumped (Gilson Minipuls 2 - Anachem, Bedfordshire) at a flow rate of 22 ml / min over eggs which were placed into a modified, plastic cell culture tray (Sterilin Ltd, Feltham). Water, after passing over eggs subsequently drained from the rear of each tray (under gravity) back into the original reservoir below (Figure 7 : 2). All water, incubation systems and equipment were kept at 10°C ± 1°C in a chilled air cabinet (Verticold, Evesham). In total, four such identical systems were set up in the cabinet.

During each trial chosen, selected agents (Figure 7 : 3) were assigned at random to one system at minimum inhibitory concentration value and to a second system at twice minimum inhibitory concentration value. The remaining two systems were both used as controls to which sterile water alone was added. All systems had water and compounds (or water alone) replaced at intervals of 36 - 48 hours as determined from the previously described trial.

FIGURE 7 : 2 Diagrammatic representation of egg incubation systems

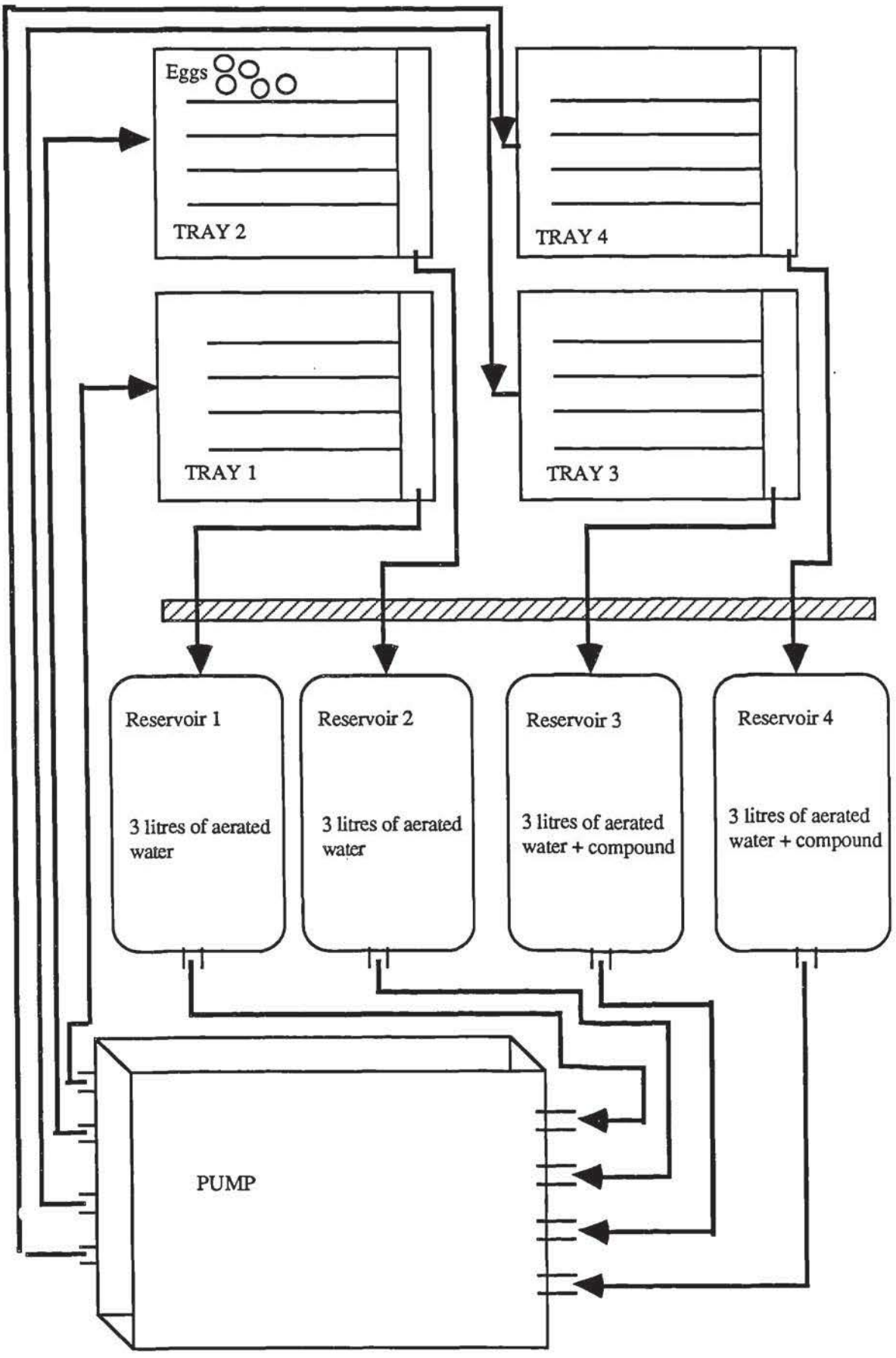
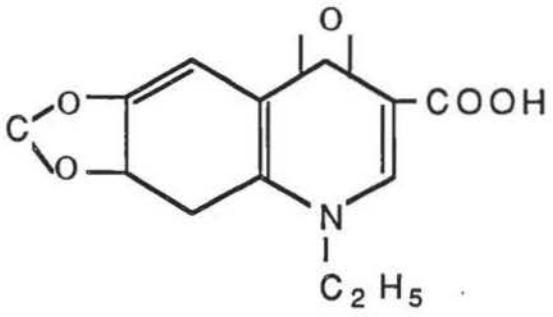
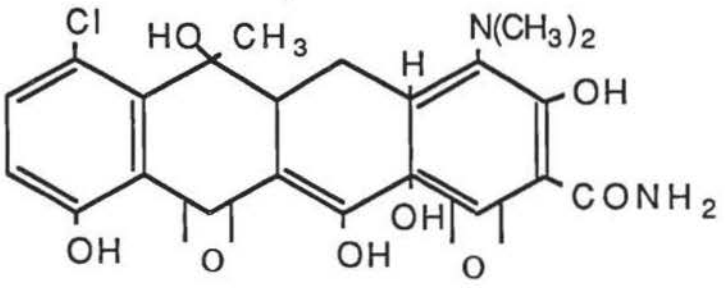


FIGURE 7 : 3 Chemical structure of compounds



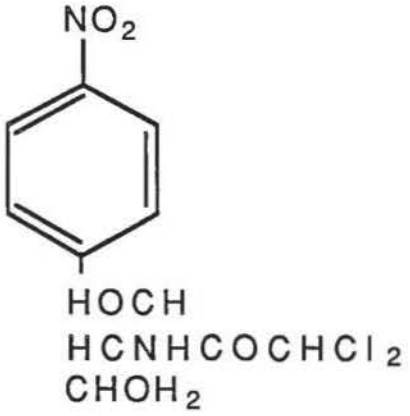
Oxolinic acid

(5-ethyl-5,8 dihydro-8-oxol,3 dioxolo-(4,5) quinolone-carboxylic acid)



Chlortetracycline

7-chloro-4-dimethylamino-1,4,4a,5,5a,6,11,12a octahydro-carboxamide



Chloramphenicol

D(-)-threo-2,2 dichloro-N-(ß hydroxy- α (hydroxy-methyl)-P-nitrophenyethyl) acetamide

Collection of eggs and milt

Stripping and egg incubation. Eggs were obtained for each trial from 5 fish from a single commercial fish farm. All eggs obtained were "out of season eggs" produced by placing broodstock under photoperiod control (Bromage & Cumaranatunga 1988), a process which ensured continued production of eggs over the entire period of this study. Eggs of each fish were stripped into individual sterile containers. The first eggs from each fish were discarded to avoid contamination from the surrounding water or ventrolateral surface of the female. Milt, also collected in sterile containers was obtained from a mixture of sex-reversed females and normal males. (Good quality milt is often hard to find in order to fertilize "out of season eggs"). Eggs, coelomic fluid and milt from each container were removed for bacteriological examination. The remaining eggs, on each occasion, were pooled and fertilized. After 5 minutes excess milt was washed off and eggs allowed to water harden for 45 minutes. Small batches of eggs (160 in each batch) were laid down in each of the 4 trays. This was considered the most suitable number of eggs for each tray to ensure adequate flow rate and egg separation. All egg mortalities were recorded daily. Fertilization rates were determined after 7 days at 10 °C by removing 20 eggs from each tray (80 in total) and placing them into clearing solution (acetic acid : methanol : water, 1:1:1 $^{v}/_{v}$) (Springate & Bromage 1984). Fertilized eggs could be clearly distinguished by the presence of a developing neural tube. At eyeing eggs were "shocked" by siphoning from a height of 1m into a sterile beaker and any unfertilized eggs removed. Eyed eggs were subsequently returned to their relevant sections in incubation trays.

Bacteriological examination

Coelomic fluid. Aliquots of coelomic fluid from each fish were serially diluted and inoculated in 0.02 ml volumes (drop inoculation method) onto tryptone soya agar (T.S.A. - Oxoid, Basingstoke), peptone beef extract glycogen agar (McCoy & Pilcher 1974) and low nutrient *Cytophaga* agar (Anacker & Ordal 1959). Plates were incubated at 20 °C for up to 10 days and enumeration performed only from drops where total separation of colonies occurred.

Milt. Milt for each fish was treated as above.

Eggs. Eggs were sampled at the green stage (unfertilized) and during incubation 7 days, 14 days, 21 days and 28 days after fertilization.

External sampling. At each sampling time 10 eggs were removed at random from each tray and sampled individually. (Batch sampling of eggs as in previous chapters was not possible due to the smaller number of eggs incubated). Each egg was rinsed in 4 changes of sterile water to remove any detritus or loosely adhered bacteria and added to 0.5 ml of sterile diluent (peptone 0.1% , saline 0.85%) and shaken vigorously for 2 minutes on a vortex stirrer, in a manner outlined in previous chapters (chapters 2, 5 and 6). The number of viable cells transferred to each of the 3 different media was determined by plate count. By taking into account the dilution factor, the volume of diluent and the egg surface area, the number of viable colony forming units (cfu's) per mm^2 was estimated.

Identification of bacteria. Bacteria were isolated by a variety of techniques, procedures and schemes outlined by Krieg and Holt (1984), Cowan (1974), the tables of Allen, Austin & Colwell (1983) and Stanier, Palleroni & Doudoroff (1966). Some Gram negative bacteria, especially *Aeromonas hydrophila*, were more easily identified using API 20E and 20NE identification strips (API laboratory products, Basingstoke).

Statistical analysis. Three different compounds were chosen in this study to reduce egg surface bacteria of incubating eggs. They were each tested sequentially on

three different batches of eggs and thus, statistical analysis was used to compare differences between treated eggs and control eggs within a given trial. Numbers of cfu's per mm^2 egg surface for treated and non-treated eggs were compared using a 2 way split-plot analysis of variance (Ridgman 1975 ; Snedecor & Cochran 1980). Differences between means were compared by calculating the standard errors and 95% confidence limits appropriate to the split-plot design (Snedecor & Cochran 1980). Numbers of hatched and non-hatched eggs were compared using a chi square (χ^2) test. The effects of bacterial presence on egg surfaces were analysed by multiple regression, chosen to compare numbers of surface bacteria with egg deaths by relating mortality to time (age) and bacterial numbers.

RESULTS

Oxolinic Acid

Egg development

Hatching success. Figures obtained for eyeing and hatching are summarized in Table 7 : 1. From this table it can be seen that estimated fertilization rates of 85% were obtained in turn leading to hatching rates ranging from 49% to 58%. Estimated fertilized egg losses (calculated by subtracting numbers of "estimated unfertilized eggs" from total number of "eggs that failed to hatch") ranged from 27% to 36%, and represents the actual number of eggs "lost" through premature egg mortality. Treated eggs (x2 M.I.C.) suffered the highest number of eggs lost in this category, mainly due to the high number (10) of eggs lost between eyeing and hatching, perhaps indicative that prolonged exposure to oxolinic acid at relatively high concentrations can be harmful to eyed salmonid eggs. Analysis by χ^2 revealed no overall significant difference in hatching success between the four groups ($\chi^2 = 1.69$) (Appendix 7 : 4). Daily mortalities of eggs are recorded in Figure 7 : 4, from which it can be seen that the two control groups show slightly higher rates of mortality during the mid-point of incubation (7-21 days) than treated egg groups. Indeed, analysis of egg mortality data at 7 day intervals between all 4 groups reveals a significant difference on two occasions between control eggs and treated eggs. Control eggs (group 2) suffered significantly greater mortalities than treated eggs (x2 M.I.C.) between 14-21 days ($\chi^2 = 4.84$, $P < 0.05$) and the same control group (group 2) also showed significantly greater mortalities than treated eggs (x1 M.I.C.) at 7-14 days ($\chi^2 = 4.14$, $P < 0.05$) (Appendix 7 : 5).

TABLE 7 : 1 Eyeing and hatching rates for control eggs and eggs exposed to oxol inic acid

EGG SURVIVAL :	Control (group 1)	Control (group 2)	x1 M.I.C.	x2 M.I.C.
Total number of eggs	100	100	100	100
Estimated fertilized eggs	85	85	85	85
Actual eyed eggs	58	56	61	59
Actual hatched eggs	55	54	58	49
EGG LOSS :				
Deaths to eyeing	29	36	24	21
Deaths at shocking	13	8	15	20
Deaths from eyeing to hatching	3	2	3	10
Eggs that failed to hatch	45	46	42	51
UNEXPLAINED LOSSES :				
Estimated unfertilized eggs	15	15	15	15
Estimated fertilized egg losses	30	31	27	31

FIGURE 7:4 Accumulative total of dead eggs recorded daily, for control eggs and eggs treated with oxolinic acid

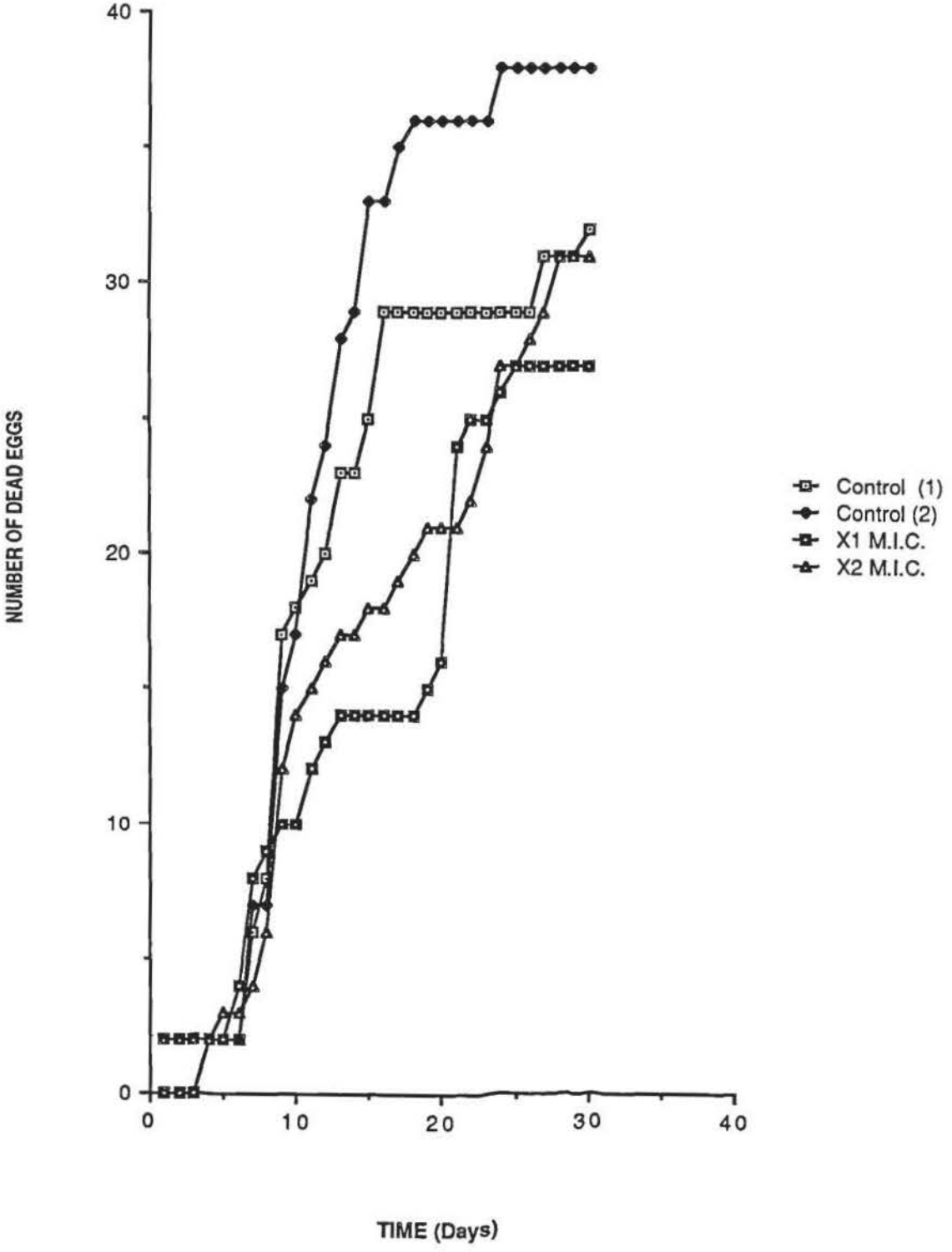
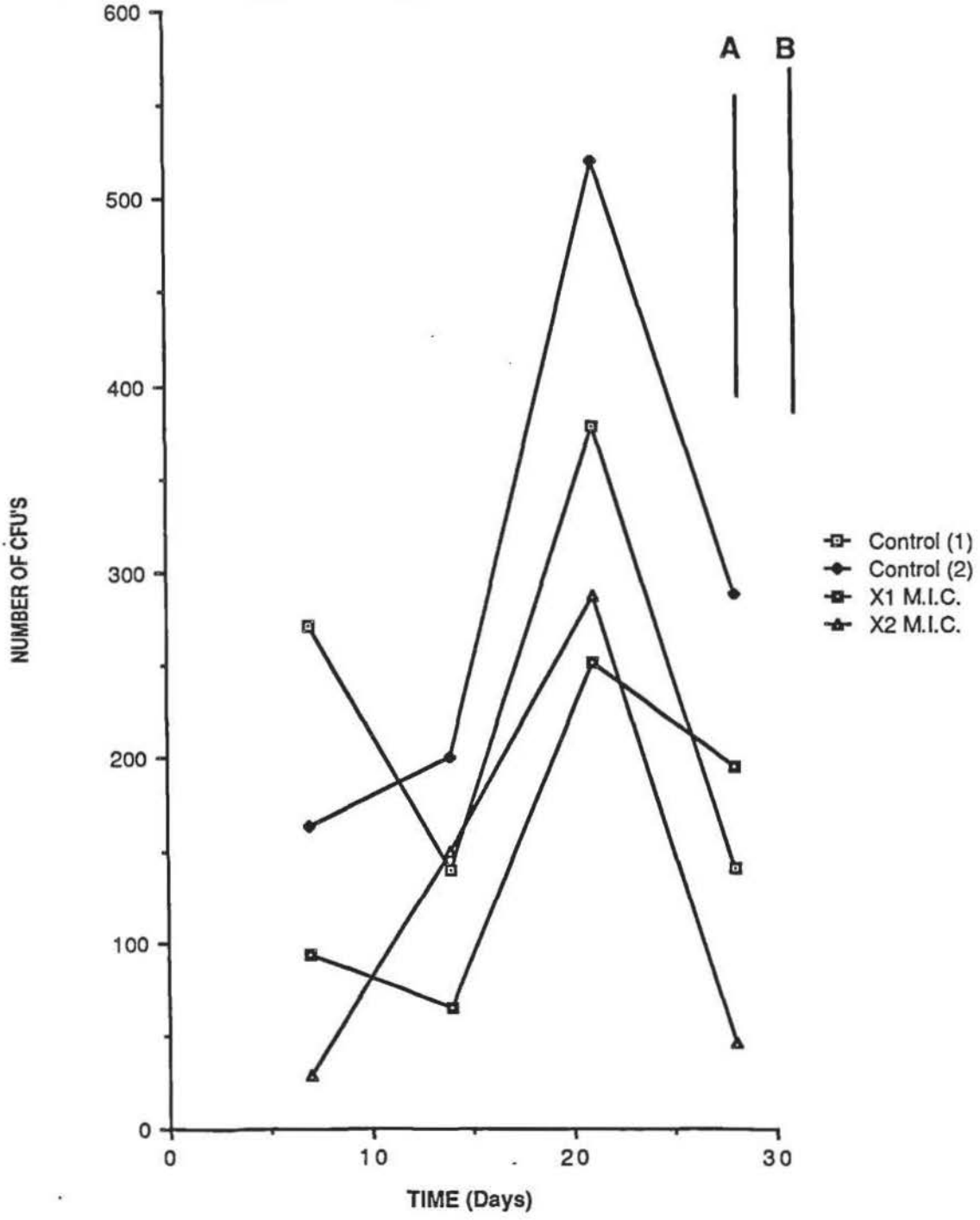


FIGURE 7:5 Estimated total colony forming units for all bacteria per sq mm egg surface for control eggs and eggs treated with oxolinic acid



A = 95% confidence limits for differences within a treatment and B = 95% confidence limits between 2 treatments at the same or different times.

FIGURE 7:6(a) Estimated colony forming units per sq mm egg surface for each bacterial genera (control group 1)

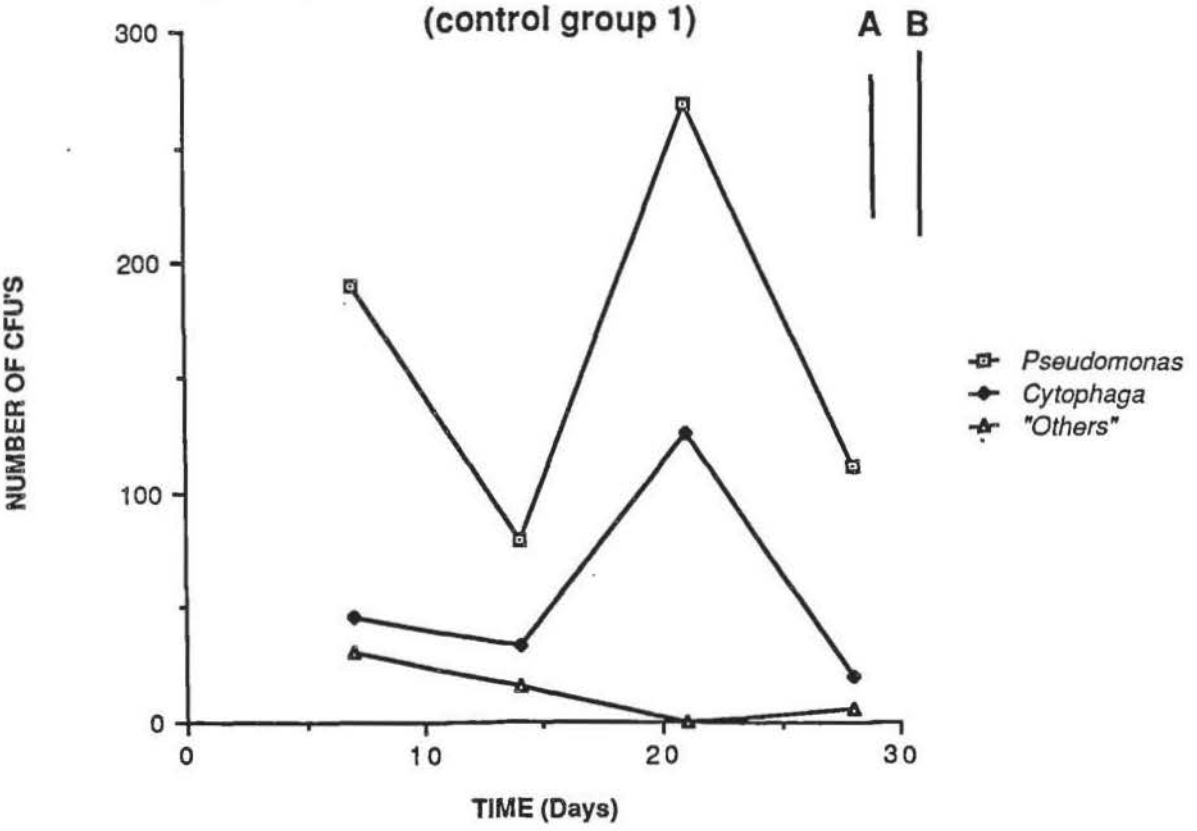


FIGURE 7:6(b) Estimated colony forming units per sq mm egg surface for each bacterial genera (control group 2)

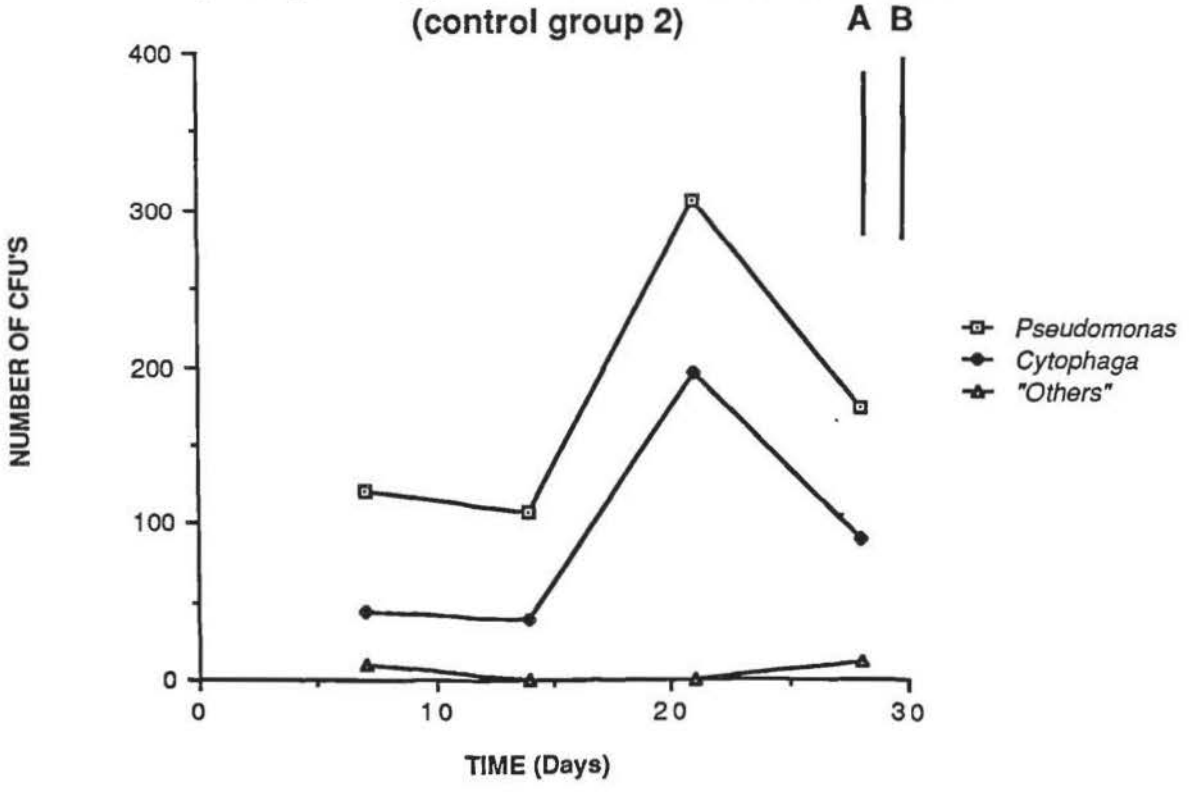


FIGURE 7:6(c) Estimated colony forming units per sq mm egg surface for each bacterial genera during oxolinic acid treatment at x1 minimum inhibitory concentration

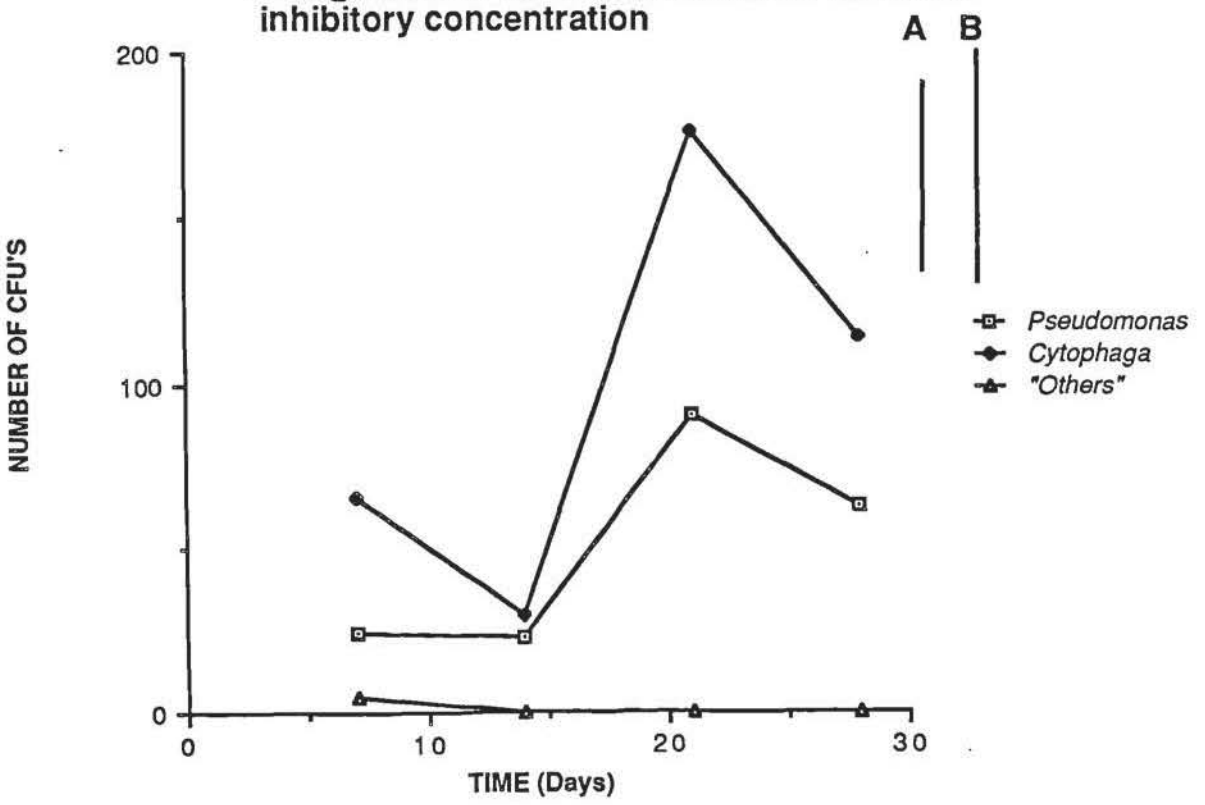
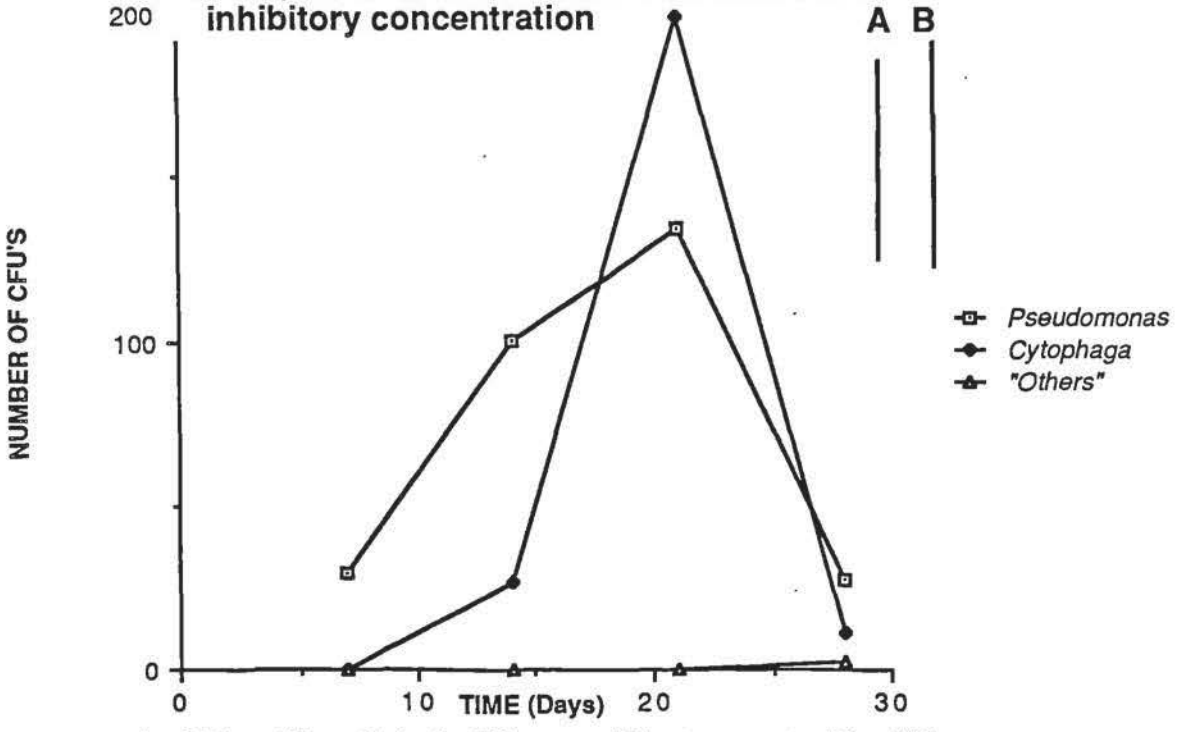


FIGURE 7:6(d) Estimated colony forming units per sq mm egg surface for each bacterial genera during oxolinic acid treatment at x2 minimum inhibitory concentration



A = 95% confidence limits for differences within a treatment and B = 95% confidence limits between 2 treatments at the same or different times

Bacteriological examination

Coelomic fluid Bacteria were isolated from the coelomic fluid of all 5 fish. Genera recovered included *Pseudomonas, Aeromonas, Staphylococcus, Corynebacterium* and yellow pigmented Gram negative rods of the *Flavobacterium - Flexibacter - Cytophaga* group.

Milt Bacteria, of similar genera to the above, were found in all milt samples but present in greater numbers than in coelomic fluid.

External sampling. Few bacteria were found attached to the surface of green (unfertilized) eggs. However, bacteria isolated included *Staphylococcus epidermis*, and *Pseudomonas* sp. During incubation large numbers of bacteria accumulated around egg surfaces and generally, untreated eggs supported higher numbers of bacteria than were isolated during earlier studies (chapters 5 and 6), a possible consequence of the slow flow rate, as discussed (chapter 2). Estimates of total cfu's isolated during the trial are summarized in Figure 7 : 5. It can be seen from this figure that treated eggs (x1 M.I.C.) had the least egg surface bacteria when sampled at both 14 and 21 days and, in general supported far fewer bacteria than untreated groups. Treated eggs (x2 M.I.C.) had the least surface bacteria at 7 days and at 28 days, but during the mid-point of incubation (14-21 days) they supported bacteria in similar numbers to untreated eggs (group 1). Untreated eggs (group 2) mostly supported the highest numbers of bacteria throughout incubation. Analysis of numbers of cfu's recovered for all egg groups (by 2 way split-plot analysis of variance) revealed overall a highly significant difference in numbers of egg surface bacteria between groups ($P < 0.001$). A highly significant effect of time was seen ($P < 0.001$) but, no significant interaction between time and numbers was found (Appendix 7 : 6). It should also be added that although, bacterial numbers were significantly different between groups, they were all subject to great fluctuations over

the experimental period and exhibited none of the stability or gradual increase in numbers found during previous studies (Chapters 2, 5 and 6). Examination of species of bacteria found on egg surfaces revealed that both untreated groups exhibited a very similar pattern of colonization with greater numbers of *Pseudomonas fluorescens* recovered, followed closely by *Cytophaga* sp. (Figures 7 : 6a and 7 : 6b). In comparison, treated egg groups exhibited a greater variation, with *Cytophaga* sp. dominating (x1 M.I.C.) throughout the incubation period and, at 21 days (x2 M.I.C.) (Figures 7 : 6c and 7 : 6d). In all 4 egg groups relatively few "other" bacteria were recovered. Analysis by multiple regression of egg death demonstrates a correlation with bacterial numbers ($r = 0.81$). However, time (6.36) rather than bacterial numbers showed the greatest correlation with egg mortality (Appendix 7 : 7).

Chlortetracycline

Egg development

Hatching Success. Eyeing and hatching rates achieved during this trial are summarized in Table 7 : 2, from which it can be seen that estimated fertilization rates of only 51% were obtained, leading in turn to hatching rates ranging from 19% to 41%. Estimated fertilized egg losses ranged from 10% to 32%, with untreated eggs (group 2) suffering the greatest egg losses in this category. Analysis by χ^2 revealed overall a highly significant difference in hatching success between all four groups ($\chi^2 = 14.97$, $P < 0.001$). Daily mortalities of eggs are recorded in Figure 7 : 7, from which it can be seen that untreated eggs (group 2) suffered far greater egg mortalities than all other groups. Indeed analysis of egg mortality data between untreated groups revealed a very significant difference ($\chi^2 = 9.71$, $P < 0.01$). On the other hand, the remaining untreated group (group 1) and the two treated groups exhibited a relatively similar profile of egg

TABLE 7 : 2 Eyeing and hatching rates for control eggs and eggs exposed to chlortetracycline

EGG SURVIVAL :	Control (group 1)	Control (group 2)	X1 M.I.C.	X2 M.I.C.
Total number of eggs	100	100	100	100
Estimated fertilized eggs	51	51	51	51
Actual eyed eggs	40	41	45	40
Actual hatched eggs	39	19	41	27
EGG LOSS :				
Deaths to eyeing	15	33	8	8
Deaths at shocking	45	26	47	52
Deaths from eyeing to hatching	1	22	4	13
Eggs that failed to hatch	61	81	59	73
UNEXPLAINED LOSSES :				
Estimated unfertilized eggs	49	49	49	49
Estimated fertilized egg losses	12	32	12	24

FIGURE 7:7 Accumulative total of dead eggs recorded daily, for control eggs and eggs treated with chlortetracycline

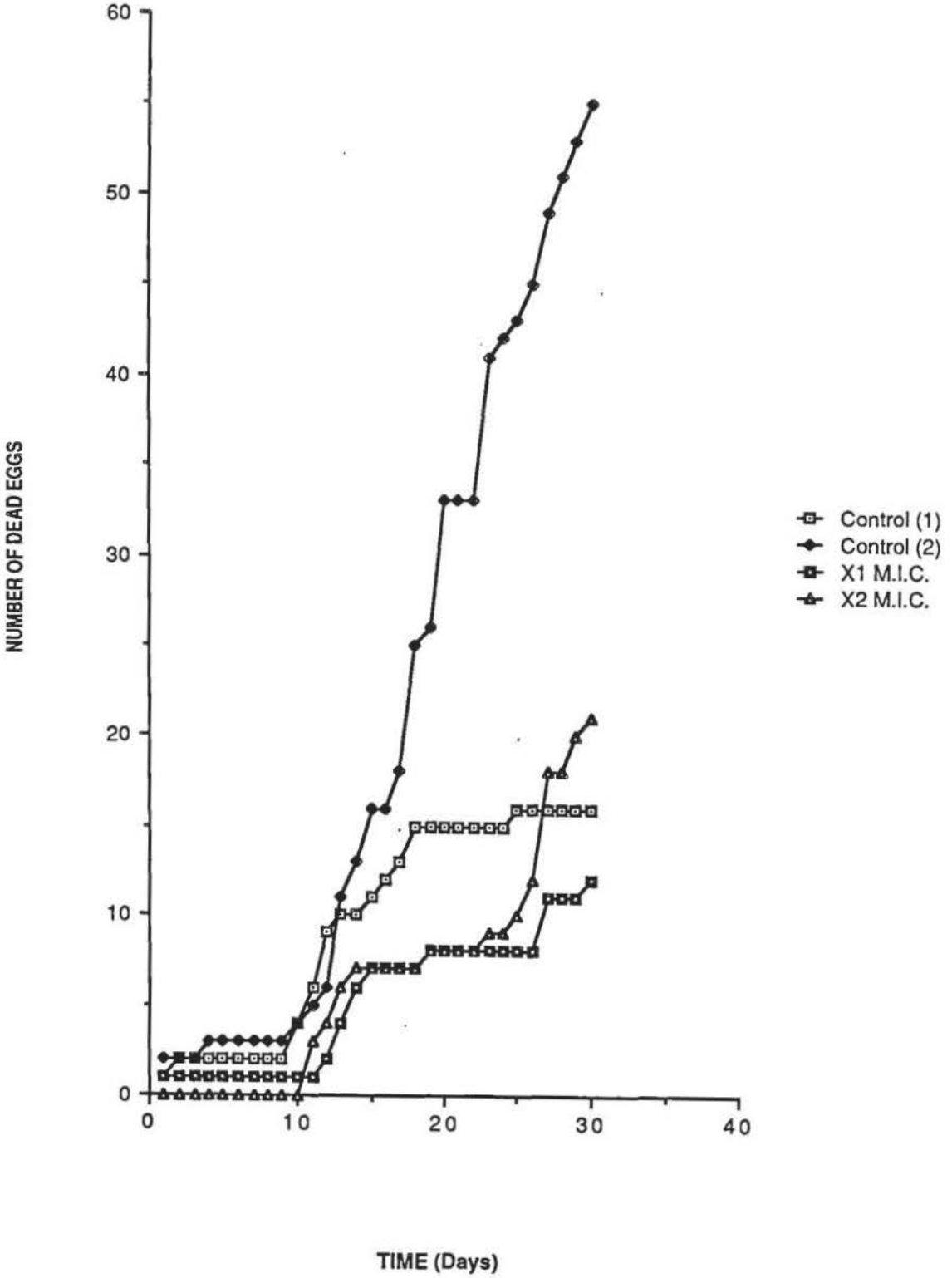
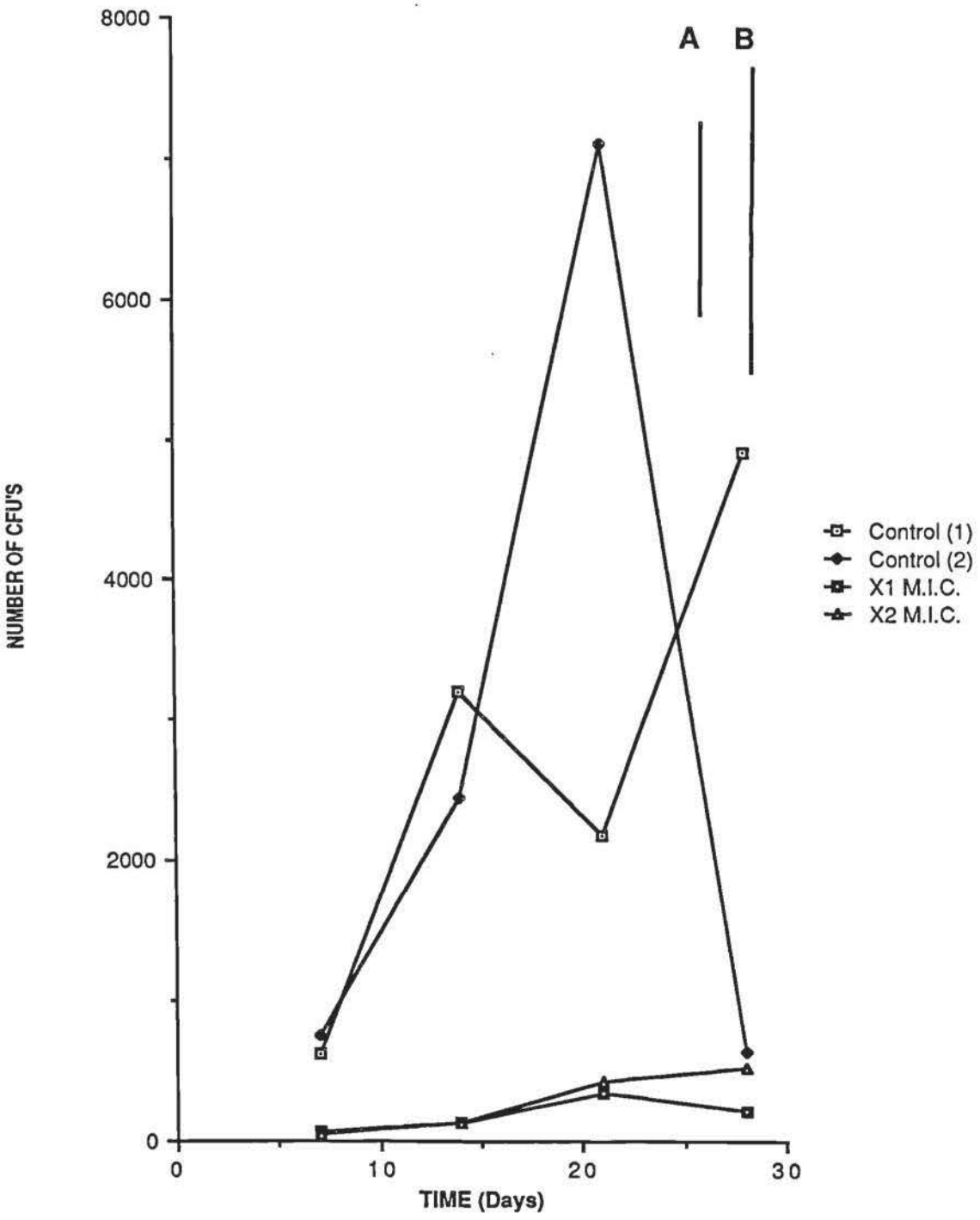


FIGURE 7:8 Estimated total colony forming units for all bacteria per sq mm egg surface for control eggs and eggs treated with chlortetracycline



A = 95% confidence limits for differences within a treatment and B = 95% confidence limits between 2 treatments at the same or different times

FIGURE 7:9(a) Estimated colony forming units per sq mm egg surface for each bacterial genera (control group 1)

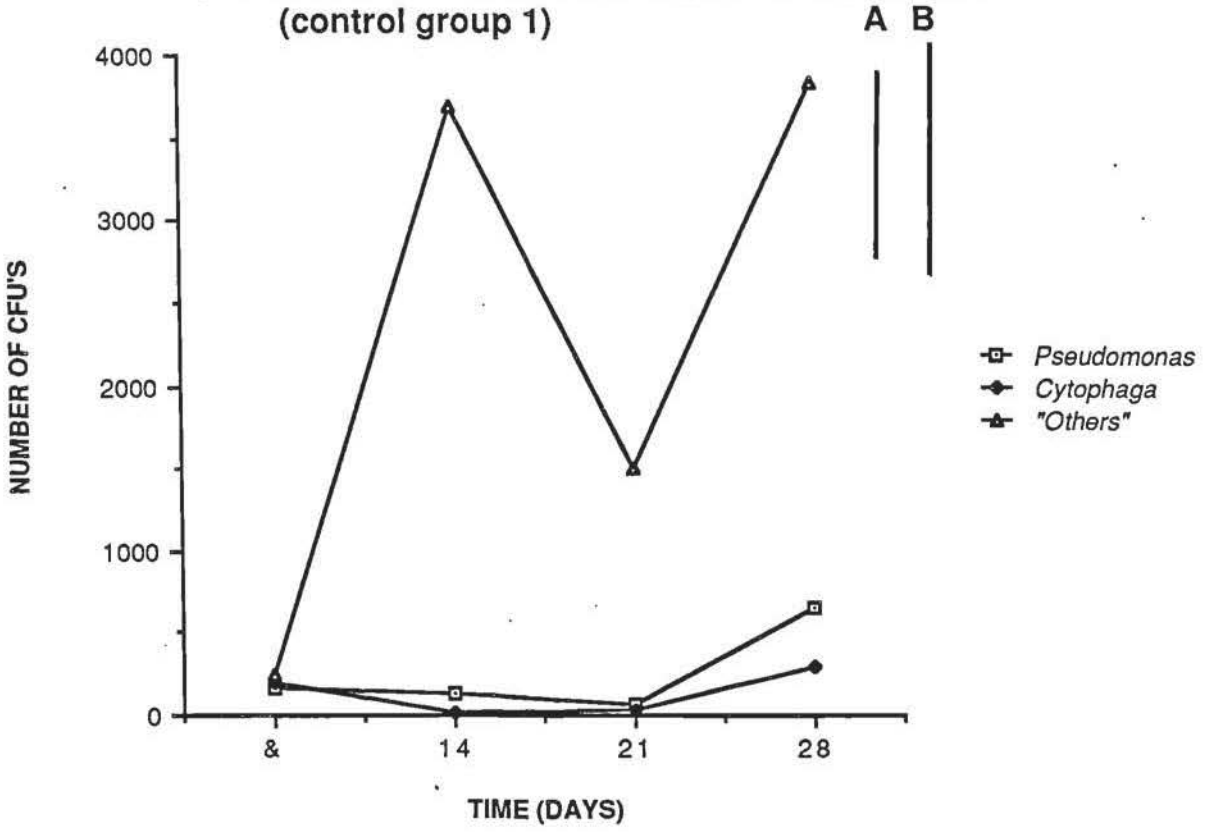


FIGURE 7:9(b) Estimated colony forming units per sq mm egg surface for each bacterial genera (control group 2)

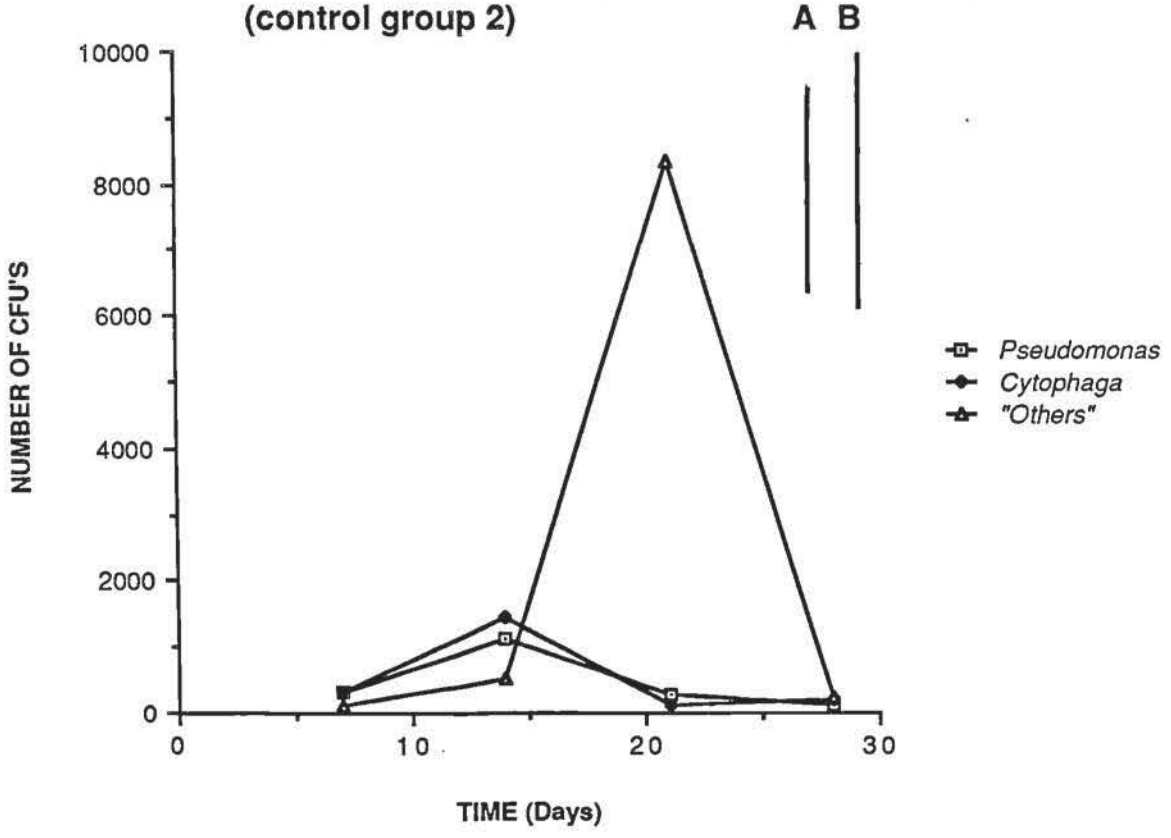


FIGURE 7:9(c) Estimated colony forming units per sq mm egg surface for each bacterial genera during chlortetracycline treatment at x1 minimum inhibitory concentration

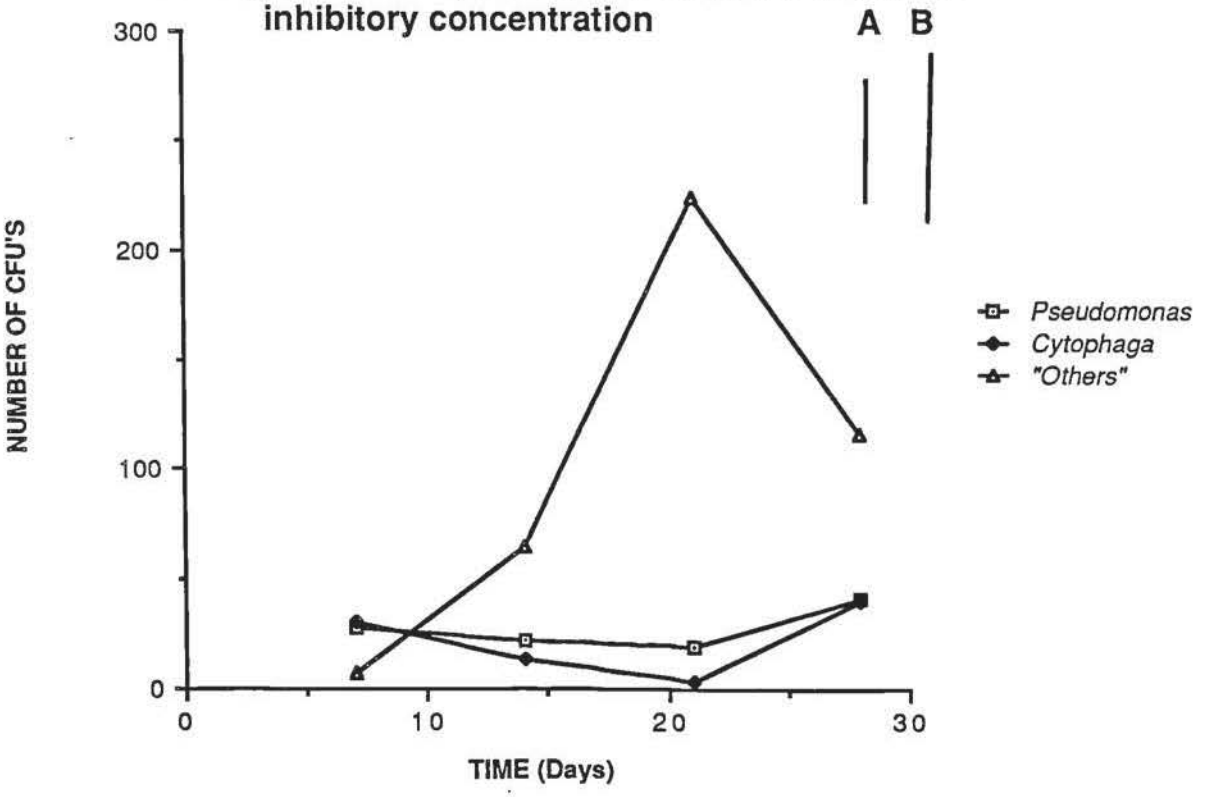
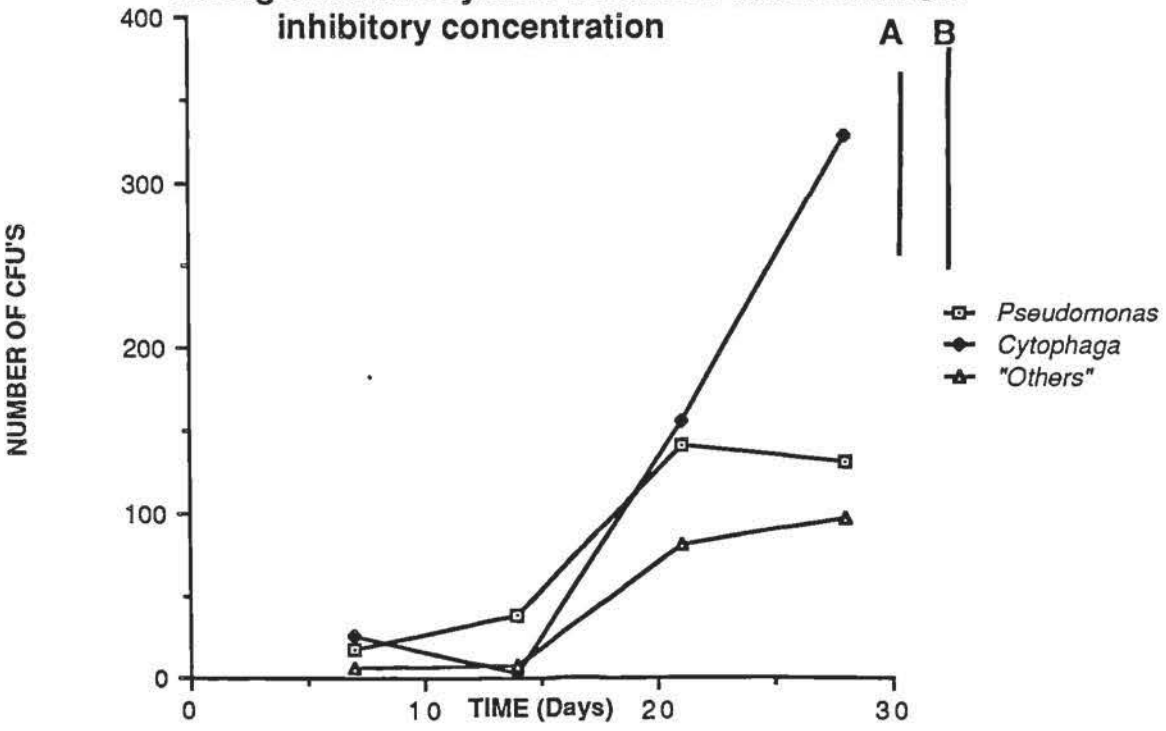


FIGURE 7:9(d) Estimated colony forming units per sq mm egg surface for each bacterial genera during chlortetracycline treatment at x2 minimum inhibitory concentration



A = 95% confidence limits for differences within a treatment and B = 95% confidence limits between 2 treatments at the same or different times.

mortality (Appendix 7 : 8). Analysis of egg mortality data at 7 day intervals revealed that no significant difference occurred between untreated groups for the first 14 days of incubation. However, at 14-21 days there was a significant difference between them ($\chi^2 = 5.83$, $P < 0.05$), followed by a highly significant difference at 21-28 days ($\chi^2 = 19.21$, $P < 0.001$). Furthermore, over equivalent sampling periods highly significant greater mortality occurred amongst untreated eggs (group 2) compared to chlortetracycline treated eggs (x1 M.I.C.) ($\chi^2 = 14.15$, $P < 0.001$ and $\chi^2 = 28.85$, $P < 0.001$) respectively and (x2 M.I.C.) ($\chi^2 = 13.91$, $P < 0.001$ and $\chi^2 = 19.46$, $P < 0.001$) respectively (Appendix 7 : 9).

Bacteriological Examination

Coelomic fluid. Bacteria were again isolated in coelomic fluid from all fish. Genera isolated did not differ from the previous study (oxolinic acid) but, in contrast, higher numbers of bacteria were found. Poor fertility figures (discussed above), coupled with the high number of bacteria in the coelomic fluid might imply that eggs were past their optimum ripeness when stripped, a problem that tends to be compounded by higher water temperatures often associated with "out of season" egg production.

Milt. A similar profile of bacterial genera and number to that of the previous trial (oxolinic acid) were found in the milt.

External sampling. Although, relatively few bacteria were found attached to green (unfertilized) egg surfaces, more bacteria than usual were isolated (compared to previous studies - Chapters 2, 5 and 6) , a possible consequence of egg overripeness as stated above. During incubation, large numbers of bacteria accumulated around egg surfaces, and estimated total numbers of cfu's recovered are summarized in Figure 7 : 8. From this figure it can be seen (with the exception of untreated eggs, group 2, at 28 days) that treated eggs supported far fewer bacteria than untreated eggs, throughout the entire incubation period. Analysis of numbers of cfu's recovered for all egg groups (by

2 way split-plot analysis of variance) revealed overall a highly significant difference in numbers of egg surface bacteria between groups ($P < 0.001$). A highly significant effect of time was seen ($P < 0.001$) and a highly significant interaction between time and numbers was found ($P < 0.001$) (Appendix 7 : 10). Examination of species of bacteria found on eggs revealed that both untreated groups differed greatly in their profile of bacterial species isolated, in contrast to both the previous trial (oxolinic acid) and previous studies (Chapters 2, 5 and 6). Egg surfaces of both untreated groups were dominated by "other" bacteria (mainly *Aeromonas hydrophila*) (Figures 7 : 9a and 7 : 9 b). On treated eggs (x1 M.I.C.) "other" bacteria also predominated throughout most of the incubation period (Figure 7 : 9c). However treated eggs (x2 M.I.C.) revealed a bacterial species profile more frequently observed during past studies (Chapters 2, 5 and 6) with high numbers of *Cytophaga* sp. followed by *P. fluorescens* and "other" sp. (Figure 7 : 9d). Analysis by multiple regression of egg death demonstrated a weak correlation with bacterial numbers ($r = 0.50$). However, as in the previous trial (oxolinic acid), time (2.90) rather than bacterial numbers showed the greatest correlation with egg mortality (Appendix 7 : 11). It should also be added that the greatest factor in egg mortality in this trial was, probably, poor egg quality leading in turn, to low fertilization rates and, subsequently, low hatching rates. However, untreated eggs (group 2) not only suffered the highest estimated "fertilized egg losses", but also, perhaps not coincidentally, supported the highest number of egg surface bacteria.

Chloramphenicol

Egg development

Hatching success. Eyeing and hatching rates achieved are summarized in Table 7 : 3, from which it can be seen that estimated fertilization rates of 58% were obtained, in turn, leading to hatching rates ranging from 39% to 47%.

TABLE 7 : 3 Eyeing and hatching rates for control eggs and eggs exposed to chloramphenicol

EGG SURVIVAL :	Control (group 1)	Control (group 2)	X1 M.I.C.	X2 M.I.C.
Total number of eggs	100	100	100	100
Estimated fertilized eggs	58	58	58	58
Actual eyed eggs	48	42	52	41
Actual hatched eggs	44	39	47	40
EGG LOSS :				
Deaths to eyeing	23	37	28	39
Deaths at shocking	29	21	20	20
Deaths from eyeing to hatching	4	3	5	1
Eggs that failed to hatch	56	61	53	60
UNEXPLAINED LOSSES :				
Estimated unfertilized eggs	42	42	42	42
Estimated fertilized egg losses	14	19	11	18

FIGURE 7:10 Accumulative total of dead eggs recorded daily, for control eggs and eggs treated with chloramphenicol

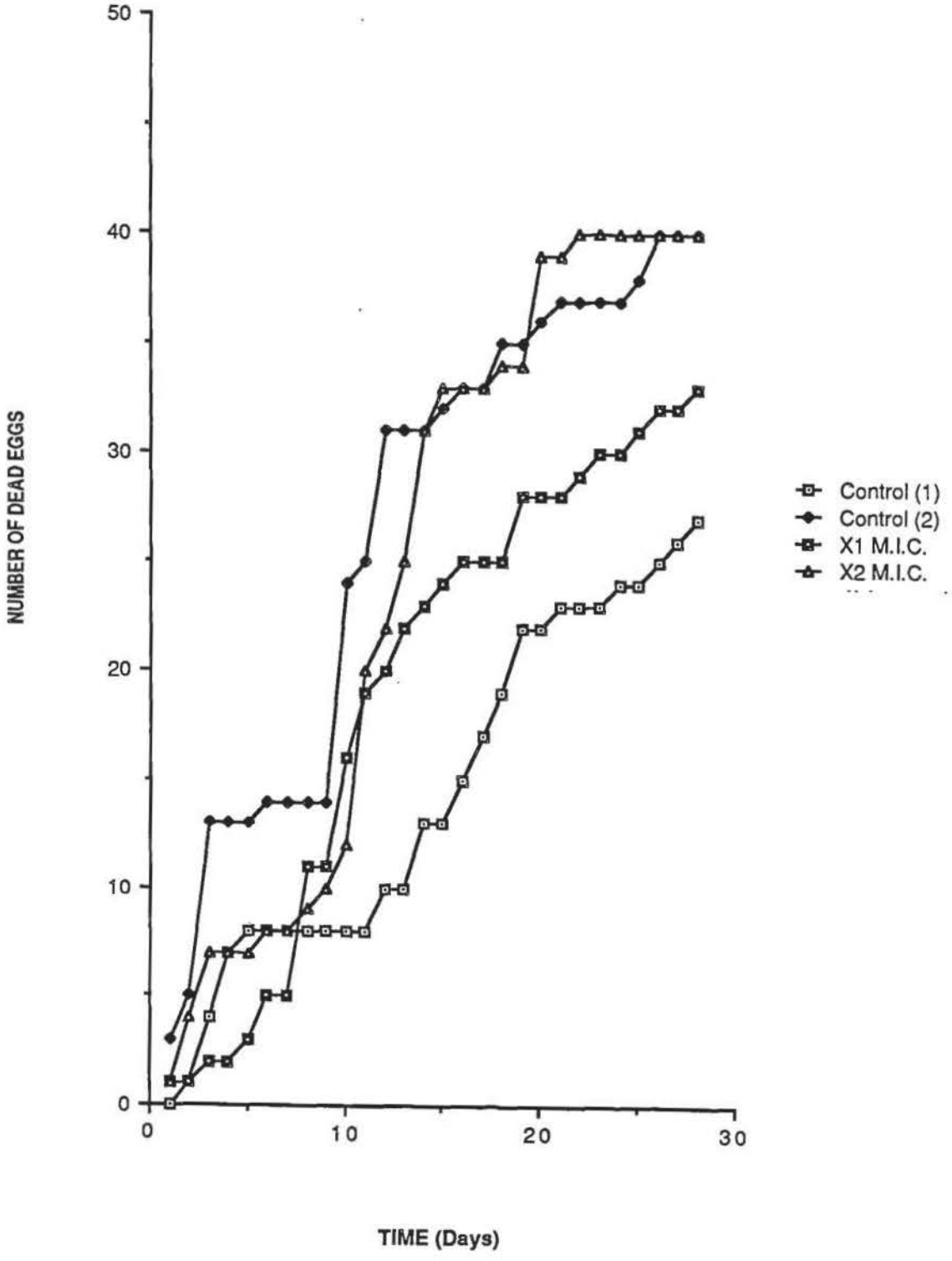
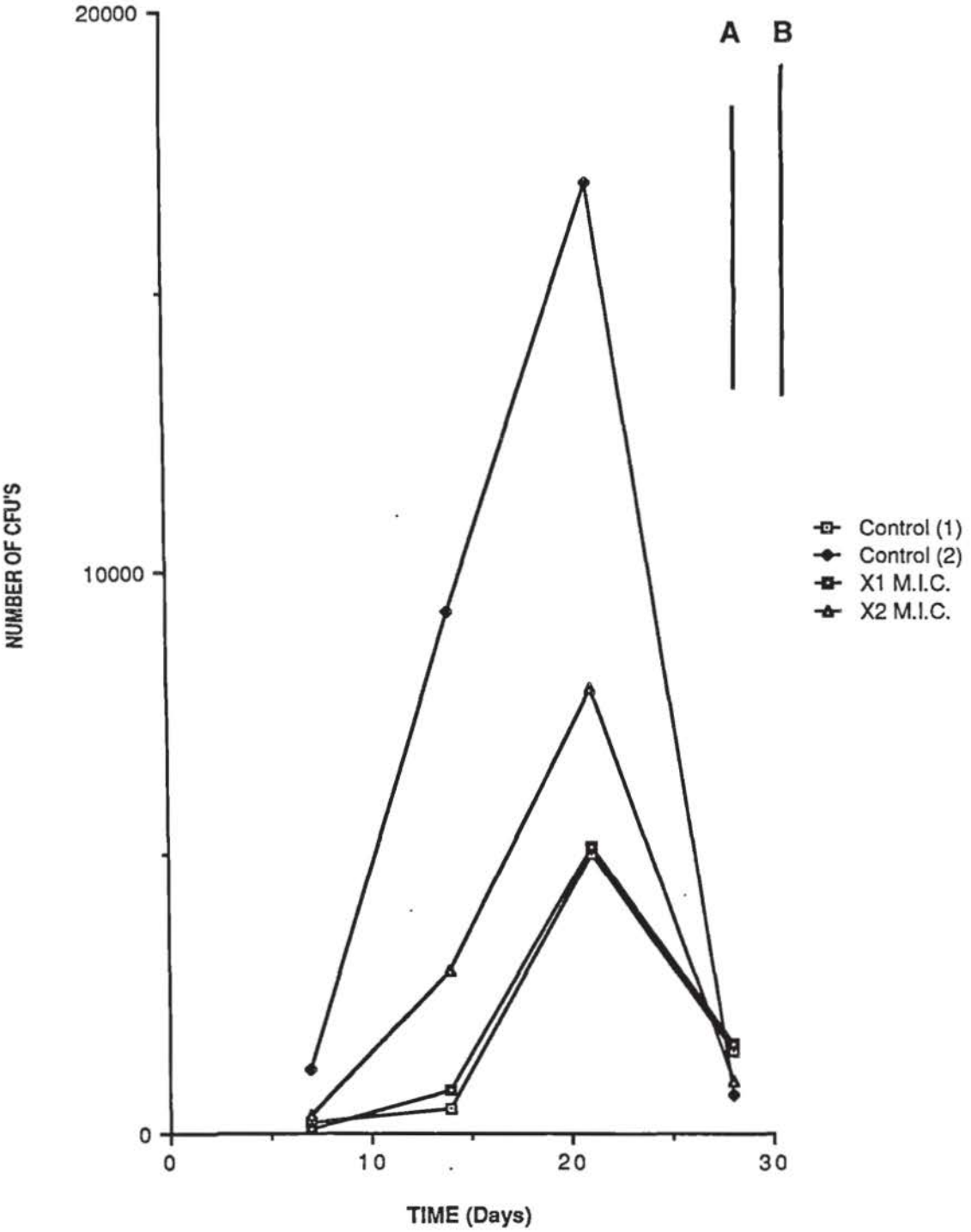


FIGURE 7:11 Estimated total colony forming units for all bacteria per sq mm egg surface for control eggs and eggs treated with chloramphenicol



A = 95% confidence limits for differences within a treatment and B = 95% confidence limits between 2 treatments at the same or different times

FIGURE 7:12(a) Estimated colony forming units per sq mm egg surface for each bacterial genera (control group 1)

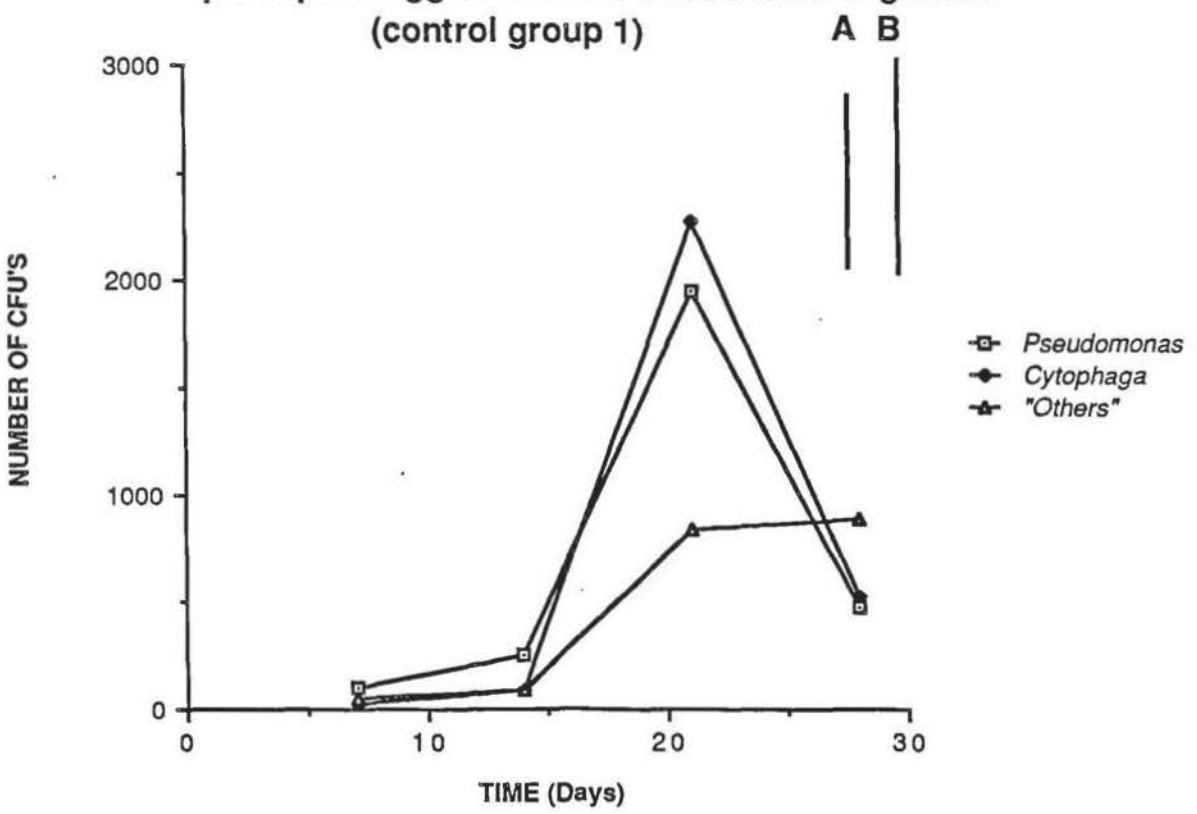


FIGURE 7:12(b) Estimated colony forming units per sq mm egg surface for each bacterial genera (control group 2)

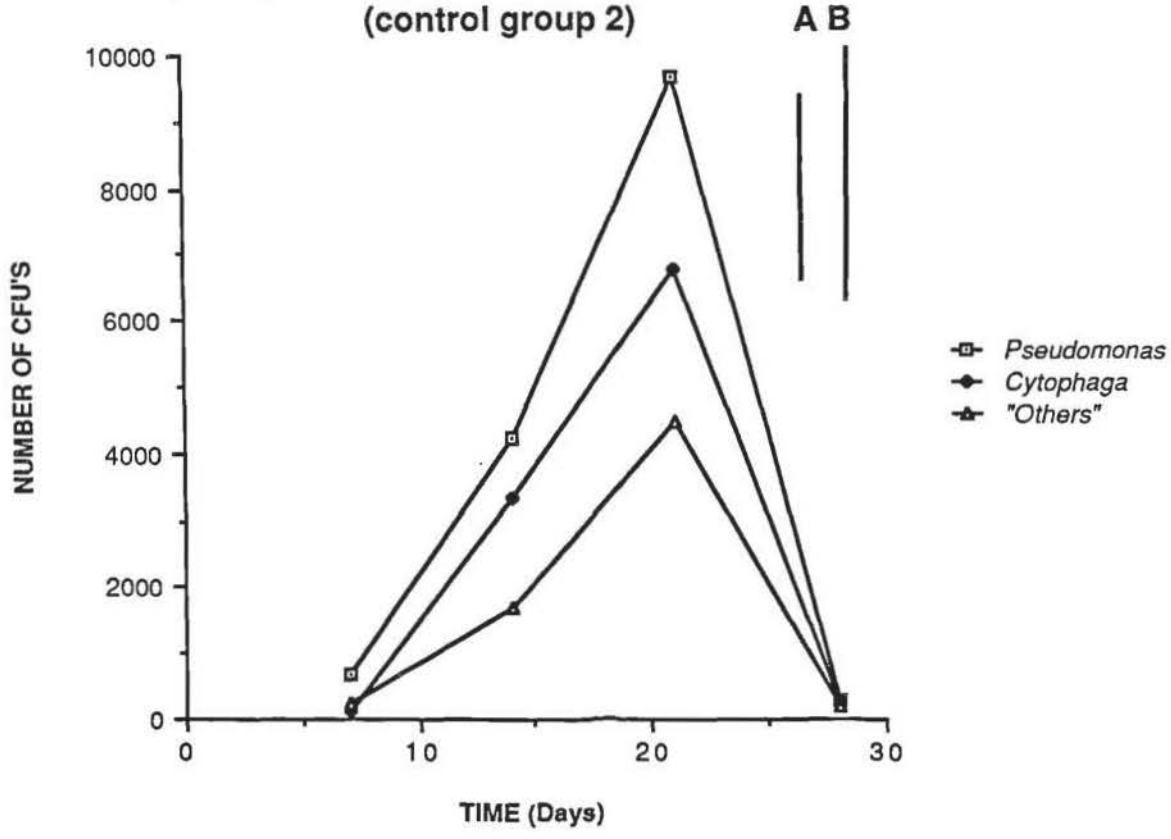


FIGURE 7:12(c) Estimated colony forming units per sq mm egg surface for each bacterial genera during chloramphenicol treatment at x1 minimum inhibitory concentration

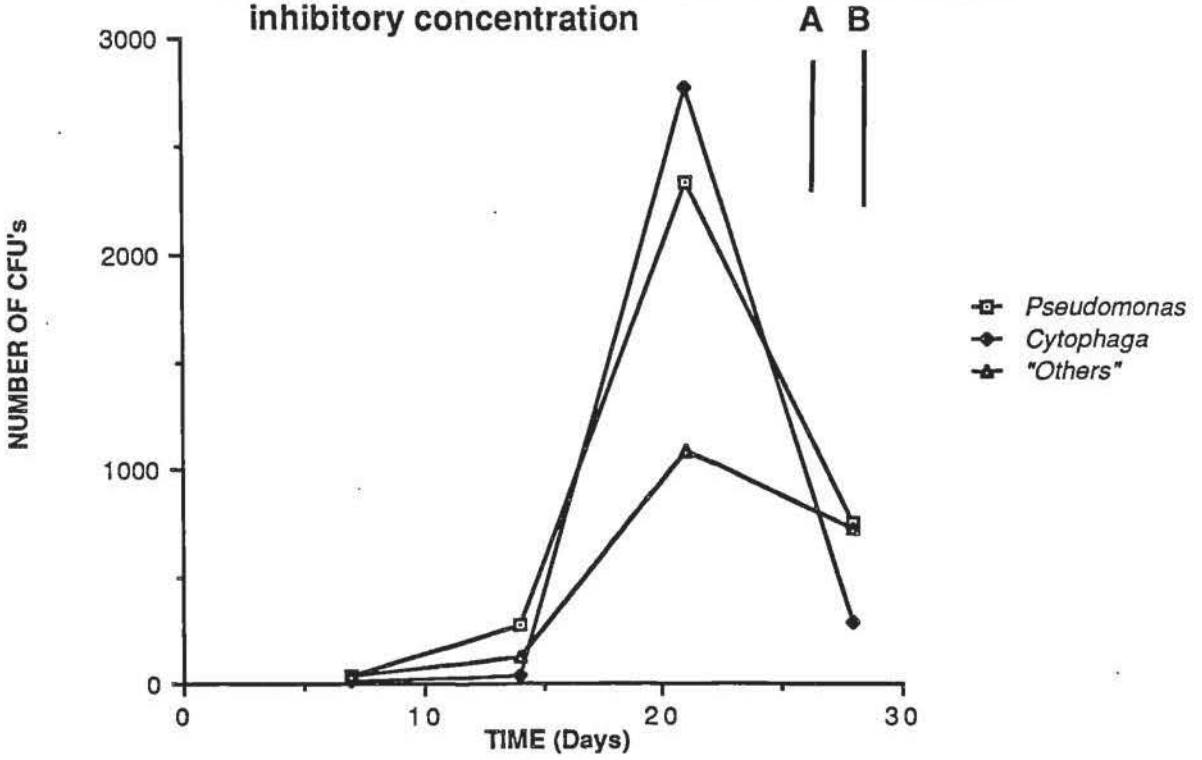
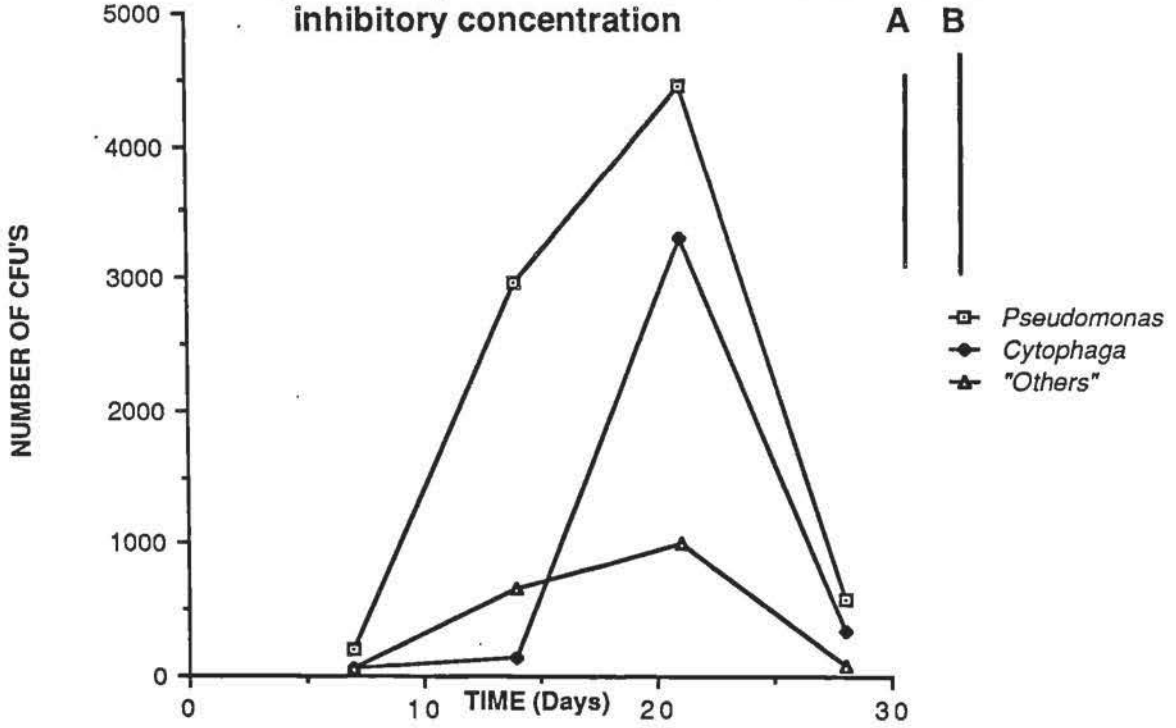


FIGURE7:12(d) Estimated colony forming units per sq mm egg surface for each bacterial genera during chloramphenicol treatment at x2 minimum inhibitory concentration



A = 95% confidence limits for differences within a treatment and B = 95% confidence limits between 2 treatments at the same or different times.

Estimated fertilized egg losses ranged from 11% to 19%. Untreated eggs (group 2) suffered the greatest egg losses in this category, although, in comparison to the previous two trials (oxolinic acid and chlortetracycline) less difference in fertilized egg losses was seen between the four groups, with no overall significant difference in hatching rates ($\chi^2 = 1.68$) (Appendix 7 : 12). Daily egg mortalities are recorded in Figure 7 : 10, revealing a similar pattern of egg mortality between all groups. However, untreated eggs (group 1) suffer the least mortalities, followed closely by treated eggs (x1 M.I.C.). Untreated eggs (group 2) and treated eggs (x2 M.I.C.) show little difference in egg mortality throughout the greater part of the whole incubation period. Analysed at 7 day intervals a very significant difference in mortality was found between untreated eggs (groups 1 and 2) during the early part of incubation at 7-14 days ($\chi^2 = 9.44$, $P < 0.01$) and, 14-21 days a significant difference was found ($\chi^2 = 4.67$, $P < 0.05$). In addition, very significant differences in egg mortality were found between untreated eggs (group 1) and treated eggs (x2 M.I.C.) at 7-14 days ($\chi^2 = 9.44$, $P < 0.01$) and also, a significant difference was found between these two above groups at 14-21 days ($\chi^2 = 5.98$, $P < 0.05$) (Appendix 7 : 13).

Bacteriological examination

Coelomic fluid. In contrast to the two previous trials (oxolinic acid and chlortetracycline) bacteria were only isolated from the coelomic fluid of 4 out of the 5 fish. Bacteria isolated included ; *P. fluorescens*, *Pseudomonas* sp., *A. hydrophila*, *S. epidermis* and *Corynebacterium.*

Milt. Bacteria were isolated from all milt samples in similar numbers and genera to those found in the coelomic fluid (above).

External sampling. Few bacteria were found attached to surfaces of green (unfertilized) eggs, indeed only *S. epidermis* was isolated. During incubation large numbers of bacteria accumulated around egg surfaces, and estimated total numbers of

cfu's recovered are summarized in Figure 7 : 11. Untreated eggs (group 2) were particularly heavily colonized by bacteria, although by 28 days there was little difference between the 4 groups. In a manner similar to the previous two trials (oxolinic acid and chlortetracycline) total egg surface bacterial numbers were seen to rise and fall. Analysis of numbers of cfu's recovered for all egg groups (by two way split-plot analysis of variance) revealed overall, a highly significant difference in numbers of egg surface bacteria between all groups ($P < 0.001$). A highly significant effect of time was seen ($P < 0.001$) and a significant interaction between time and numbers was found ($P < 0.05$) (Appendix 7 : 14). Examination of the species of bacteria found on egg groups revealed little difference between numbers of *Cytophaga* sp. and *P. fluorescens* on surfaces of untreated eggs (group 1) throughout the incubation period (Figure 7 : 12a). In contrast, untreated eggs (group 2) exhibited slightly higher numbers of *P. fluorescens* compared to *Cytophaga* sp. (Figure 7 : 12b). Treated eggs (x1 M.I.C.) were colonized in similar numbers by both *Cytophaga* sp. and *P. fluorescens* (Figure 7 : 12c), while treated eggs (x2 M.I.C.) exhibited greater numbers of *P. fluorescens* compared to *Cytophaga* sp. (Figure 7 : 12d). Analysis by multiple regression of egg death demonstrates a correlation with bacterial numbers ($r = 0.79$). However, time (5.80) rather than bacterial numbers had the greatest correlation to egg mortality (Appendix 7 : 15).

DISCUSSION

Oxolinic acid, along with nalidixic acid and flumequine are 4-quinolone, synthetic, systemic, anti-bacterial agents. Their mode of action is thought to be by interference with the action of bacterial deoxyribose nucleic acid gyrase leading to the prevention of negative supercoiling of the bacterial chromosome. Oxolinic acid along with two other agents - piromodic acid and furnace were developed for fisheries use in Japan (Endo *et al* 1973a), and it s efficacy against a wide range of fish pathogens has been fully investigated ; *Aeromonas salmonicida, A. liquefaciens, Vibrio anguillarum, Chrondococcus columnaris* (Endo *et al* 1973a ; Endo, Sakuma, Tanaka, Ogishima, Hara, Ohshima & Sato 1973b), *A. salmonicida* (Austin, Rayment & Alderman 1983) *V.anguillarum* (Austin, Johnson & Alderman 1982) and *Yersinia ruckeri* (Rodgers & Austin 1983). In addition, oxolinic acid has also proved useful in the field of human medicine (Madsen & Rhodes 1971) and veterinary practice (Ziv 1976).

Oxolinic acid was successful in reducing the bacterial egg surface populations, mainly through lowering numbers of *P. fluorescens*. Oxolinic acid also proved successful in reducing numbers of "other" bacteria, a category mainly comprised of *Aeromonas* and *Pseudomonas* sp. Endo *et al* (1973a) and Jo (1978) have demonstrated the effectiveness of oxolinic acid against *Aeromonas* and *Pseudomonas* sp. respectively. Therefore reductions in numbers of "other" bacteria is probably at the expense of these two species. In contrast *Cytophaga* sp. were largely unaffected by oxolinic acid treatment and numbers remained similar to those found on control eggs. Inhibition of *P. fluorescens* and "other" bacteria could have allowed numbers of *Cytophaga* sp. to proliferate due to reduced competition. However, as numbers of *Cytophaga* sp. did not significantly increase, it is possible that presence of oxolinic acid reduced environmental quality in turn, restricting proliferation of *Cytophaga* sp. Furthermore, oxolinic acid is known to be effective against a wide

range of Gram -ve rods (Alderman 1988) and has been reported to be effective against *Flexibacter* sp., a group closely related to *Cytophaga* sp. (Austin & Austin 1987).

Austin and Al-Zahrani (1988), found treatment with oxolinic acid caused increases in numbers of bacteria throughout the digestive tract of rainbow trout, a consequence of inhibiting sensitive organisms and in turn allowing the proliferation of resistant bacteria. Such an effect was not observed during this study and may be attributed to the limited range of bacterial genera found on egg surfaces (i.e. few Gram -ve bacteria and no Gram +ve bacteria) compared to the adult intestine. It is also likely that the numbers or species of bacteria found on egg surfaces by this study is lower than might be expected under natural or large scale commercial conditions. Cyclical draining and subsequent refilling of egg incubation systems with fresh sterile water may reduce the unattached "free-living" bacterial population, in turn reducing the exposure of eggs to potential colonizers.

Despite lowering bacterial numbers on egg surfaces use of oxolinic acid appears of little value, as hatching success did not markedly increase, implying that the presence of bacteria (*Pseudomonas* sp. and "Other" bacteria) on eggs has little effect on egg mortality. Furthermore, it should also be noted that excessive use of oxolinic acid may even be detrimental. Markedly greater numbers of eggs exposed to x2 M.I.C. failed to hatch after successful eyeing indicating that prolonged exposure to high concentrations of oxolinic acid may be toxic to developing embryos. Although, Alderman (1988) did not report toxicity amongst members of the 4-quinolones to fish at normal recommended therapeutic doses, high daily rates of oxolinic acid administration have been shown to cause liver and blood damage in yellowtail, *Seriola quinqueradiata*, (Miyazaki, Nakauchi & Kubota 1984).

Three tetracyclines have been widely used in fisheries, chlortetracycline, oxytetracycline and doxycycline (Alderman 1988). All are octahydro-napthacenes and within the fish rearing industry are probably the most frequently used broad spectrum antibiotics. Although, of the tetracyclines, oxytetracycline has been preferred by the

aquaculture industry (Alderman 1988) it s use in this study had to be curtailed in favour of chlortetracycline on account of the latter's greater stability under experimental conditions. Chlortetracycline first isolated in 1947 (Albert 1979), in common with other tetracyclines crosses the plasma membrane as a liposoluble magnesium complex, whereupon subsequent binding to the 30S ribosomal unit inhibits protein synthesis. Commercial fisheries initially utilized chlortetracycline in the middle fifties after Snieszko *et al* (1952) demonstrated by *in vitro* techniques the sensitivity of *A. salmonicida* to chlortetracycline. Subsequent widespread and indiscriminate use particularly as a feed additive (Stokstad *et al* 1949 ; Stokstad & Jukes 1950) has encouraged the development of resistant bacterial strains, even so use of chlortetracycline amongst aquaculturists has persisted into the middle seventies for the treatment of trout furunculosis (Glende, Wenzel & Roth 1974).

Irrespective of it's misuse and long history chlortetracycline still retains a degree of effectiveness as eggs exposed to chlortetracycline supported fewer bacteria compared to those left untreated. Such a result may however be relatively unique as untreated eggs were dominated by "other" bacteria and not *Cytophaga* sp. or *P. fluorescens* as in previous trials. Furthermore, predominant within this "other" bacterial group is *A. hydrophila*, which like *A. salmonicida* is probably sensitive to chlortetracycline (Snieszko *et al* 1952), so accounting for the marked difference in bacterial numbers between treatments. In parallel egg mortalities between groups also differed markedly with those exposed to chlortetracycline appearing to show fewer mortalities. However, variations in egg hatching success amongst untreated groups - a possible consequence of their markedly dissimilar bacterial loading at eyeing precludes confident recommendation of chlortetracycline usage in egg husbandry.

Chloramphenicol was originally isolated by Bartz 1948 and has since been successfully employed as a broad-spectrum antibiotic. Chloramphenicol is largely bacteriostatic and is thought to act on the 50S ribosomal unit inhibiting protein synthesis (Hollstein 1979). Smith (1950) demonstrated the efficacy of chloramphenicol

against two fish pathogens *Pseudomonas hydrophila* and *Aeromonas liquefaciens*, leading in turn to it's subsequent use as a treatment of fish diseases, especially within the European carp industry (Evelyn 1968). Chloramphenicol has also been extensively used in human medicine (Austin 1984) and still remains appropriate for the treatment of typhoid fever (Butler, Arnold, Linh & Pollack 1973).

During the trial, numbers of egg surface bacteria varied significantly between groups of untreated eggs. Indeed, no significant difference occurred in overall bacterial numbers between one group of untreated eggs (group 1) and treated egg groups. Additionally, no individual species of bacteria was particularly inhibited by exposure to chloramphenicol and, overall, little difference was seen in estimated fertilized egg losses between groups. Thus, no evidence was provided by the trial for the recommendation of chloramphenicol usage in egg husbandry.

Large fluctuations in egg surface bacterial numbers were demonstrated during the course of all 3 antibiotic trials. Similar fluctuations in numbers of aquatic bacteria have been found to occur seasonally (Jones 1973 ; Allen, Austin & Colwell 1983 ; Austin & Allen-Austin 1985 ; Iriberri, Unanue, Barcina & Egea 1987). In addition, Bell, Hoskins & Hodgkiss (1971) reported fluctuations in microbial numbers on surfaces of stream incubated salmon eggs, and Trust (1972) demonstrated a similar pattern of bacterial colonization on eggs maintained in a vertical upwelling incubator. In contrast, previous studies (Chapters 2, 5 and 6) have revealed that eggs placed under a carefully controlled horizontal flow of water tend to be colonized by a microbial population that increases in number with progressive incubation. Therefore, fluctuations in bacterial numbers during antibiotic trials (compared to previous trials) may be due to the unique experimental design and the regular refilling of systems, perhaps leading to the partial removal of unattached "free living" bacteria in turn reducing the regular exposure of eggs to potential colonizers.

Overall hatching success rate between antibiotic trials ranged from 19% to 58%. In comparison to previous trials (Chapters 2, 5 and 6) such egg hatching rates

would appear to be poor. Eggs for all 3 antibiotic trials were obtained by photoperiodic manipulation of broodstock (Bromage & Cumaranatunga 1988). Although there has been much debate on the quality of such "out of season" eggs, it is now generally accepted that under commercial farming conditions their performance is perfectly satisfactory. However, due to higher water temperatures often associated with "out of season", more frequent stripping of broodstock is required (Escaffre & Billard 1979). Low egg hatching rates obtained during these trials may therefore be due to egg overripeness and subsequent poor egg quality.

In previous trials (Chapters 2, 5 and 6) great care was also taken to ensure that eggs were placed under optimum incubation conditions. However, during the antibiotic trials far more emphasis was placed on preventing antibiotics (and any resistant bacteria) from entering the natural environment. Thus, although adequate conditions for egg development were provided, slow water flow rate, low levels of oxygen and increased levels of egg disturbance, a consequence of the unique experimental design, may not have provided "optimum" conditions for salmonid egg survival. Even so, if eggs are incubated under less than ideal environmental conditions, then any subsequent form of prophylactic treatment might be especially beneficial for egg survival. However, results obtained during these trials do not support the premise that antimicrobial therapy will reduce fertilized egg losses, despite the potential to limit numbers of egg surface bacteria.

Preparatory M.I.C. trials revealed that both *P. fluorescens* and *Cytophaga* sp. could tolerate high concentrations of a wide range of antibiotics and antimicrobial compounds. Thus, during subsequent trials (and with the introduction of organic matter - eggs) it was decided to use twice *in vitro* M.I.C. values. Even so, despite continual high levels of circulating compounds, many bacteria were still able to colonize egg surfaces. In natural environments most bacterial cells are surrounded by a "polysaccharide component" outside the cell wall, termed the glycocalyx (Costerton, Irvin & Cheng 1981), and in aquatic ecosystems it has been demonstrated that the majority of bacterial populations exist in glycocalyx enclosed microcolonies (Geesey,

Richardson, Yeomans, Irvin & Costerton 1977) (Chapters 4). The glycocalyx may act as a barrier, influencing access of molecules, ions and protons (Cheng, Ingram & Costerton 1970) to the bacterial cell wall and cytoplasmic membrane (Costerton *et al* 1981), and therefore may also give some measure of protection against antimicrobial compounds (Govan & Fyfe 1978). Thus, addition of antimicrobial compounds to hatchery waters to reduce established populations of egg surface bacteria may only ever have a limited effect. However, results of all studies (Chapters 2, 5, 6 and 7) have shown that at the time of stripping eggs are colonized by few bacteria (mainly *S. epidermis*), subsequently rarely isolated from incubating egg surfaces. Indeed, at time of laying down egg surfaces are virtually sterile and are only colonized by large numbers of bacteria (*P. fluorescens* and *Cytophaga* sp.) during incubation. Once colonized, egg surfaces support a large and prolific bacterial population and thus commercially it may prove more prudent to prevent initial bacterial colonization of surfaces rather than attempt to reduce numbers of already established populations. Water treatment methods, particularly those that avoid the use of long term pollutants, for example ozone disinfection (Conrad, Holt & Kreps 1975) or ultra violet (U.V.) light (Spanier 1978 ; Brown & Russo 1979 ; Kimura, Yoshimizu & Atoda 1980) might prove suitable for reducing numbers of circulating, "unattached" bacteria and thus inhibit initial colonization of egg surfaces. However, neither methods is ideal for all situations, as amount of suspended solids in the water, water flow rate and, of course, financial expense will all be important limiting factors.

At present, little can be achieved on a commercial basis to actively reduce potentially detrimental populations of bacteria developing on incubating egg surfaces. However, manipulation of the hatchery environment could contribute to ensuring that the formation of such populations is not encouraged (Chapter 2). Water should be taken from a pure source such as a spring or borehole, with a reduced bacterial flora (Conrad *et al* 1975) and furthermore, should pass over eggs once, go to drain and not be continually re-cycled (as this process may encourage bacterial proliferation - Chapter 5). Ideally water flow rate should be as fast as possible (but without physically

disturbing eggs) and the creation of aerosols (such as when water enters hatchery troughs) should be avoided, as they may be a potential source of bacterial entry into hatchery waters (Trust 1972).

SUMMARY

Rainbow trout eggs placed in small, totally enclosed incubation systems were constantly exposed to antibiotics and antimicrobial compounds throughout incubation. Three compounds, oxolinic acid, chlortetracycline and chloramphenicol were tested separately at two different concentrations on batches of eggs. Regular treatment of eggs with both oxolinic acid and chlortetracycline resulted in a marked decrease in numbers of egg surface bacterial populations. However, no significant correlation could subsequently be drawn between decreased egg surface bacterial numbers and increased egg hatching success. Thus, although oxolinic acid and chlortetracycline reduced egg surface bacterial numbers, subsequent failure to increase egg hatching rates would in turn suggest, that in these trials, no correlation existed between bacteria on egg surfaces and the deaths of incubating eggs.

CHAPTER EIGHT

GENERAL DISCUSSION AND APPENDICES

GENERAL DISCUSSION

Present investigations have clearly shown that there is a close association between salmonid eggs and bacteria, beginning before eggs are stripped and continuing throughout incubation (to be discussed further below). Early bacterial associations with eggs held in the coelomic cavity of healthy, disease free broodstock would not appear to be detrimental to overall egg quality. Furthermore, subsequent bacterial contamination of good quality eggs incubated under carefully controlled conditions does not seem to be a significant cause of egg loss. From these present investigations it appears that bacteria are almost a constant, ubiquitous component of the salmonid egg environment. In turn, healthy salmonid eggs successfully meet this perpetual microbial challenge. Only when other unfavourable environmental conditions combine to cause "stress" amongst incubating eggs does the presence of bacteria appear to detrimentally influence development and hatching success of salmonid eggs.

Using the scientific literature it is possible to speculate that bacterial associations with salmonid eggs might commence at a very early stage of egg development. For example, when Sauter, Williams, Meyer, Celnik, Banks & Leith (1987) examined the blood of 30 healthy female chinook salmon, *Onchorynchus tshawytscha,* they noted that bacteria were present in half the samples. Predominantly, Gram -ve bacteria in particular, *Aeromonas hydrophila,* were recovered from most of the samples, although occasionally Gram +ve bacteria such as *Listeria* sp. were also found. Whether such bacteria could contaminate significant numbers of developing eggs either internally or externally is unlikely but, the distinct possibility of limited contact must however exist. In contrast, in diseased fish infected with *Renibacterium salmoninarum* (responsible for bacterial kidney disease - B.K.D.) there is evidence to suggest that during early egg development significant contamination of eggs can take place. During the development of salmonid eggs, vitellogenin, a large protein chain (molecular weight 250 000 - 600 000) is secreted by the liver and selectively taken up

by the oocytes (Yusko, Roth & Smith 1981), probably, by a process of micropinocytosis (Mommsen & Walsh 1988). *R. salmoninarum* is a highly specialized bacterium able to live and reproduce intracellularily (Young & Chapman 1978 ; Bruno & Munro 1986) and is of such a size that it may also be taken up by developing eggs during the process of vitellogenin incorporation by micropinocytosis (as described) (Evelyn, Ketcheson & Prosperi-Porta 1984).

As the eggs develop, the ripe ovaries enlarge to a point where they may constitute 20% of the total weight of the fish (Frost & Brown 1967). Each egg may reach a size of about 0.5 cm and the surrounding follicular layer becomes stretched, thin and finally disappears. The ovaries are surrounded by a thin, transparent membrane which also becomes stretched, eventually breaking, allowing the eggs to lie loose in the body or coelomic cavity (Frost & Brown 1967). Under commercial conditions eggs will remain in the coelomic cavity until pressure is applied to the outside of the fish and eggs are manually "stripped" from the cavity. However, while eggs remain in the coelomic cavity they are bathed by fluid, termed coelomic or ascitic fluid. Observations suggest that the volume and constituency of this fluid varies greatly between fish. Generally the fluid can be described as "watery" and possibly comprises of surrounding water that has entered the cavity via the urino-genital pore. In addition, it seems likely that the coelomic fluid will also contain some constituents of the circulating fish plasma including erythrocytes and macrophages (Cone 1982).

The intensive culture techniques employed to farm salmonids commercially often leads to high levels of excrement and undigested feed present in the surrounding water of tanks, ponds or raceways (Allen, Austin & Colwell 1983 ; Austin & Allen-Austin 1985). Under such conditions, a suitable nutrient rich "medium" is unintentionally provided for bacterial growth and reproduction in turn potentially leading to relatively high numbers of bacteria present in the surrounding water (Allen *et al* 1983 ; Austin & Allen-Austin 1985). Thus, it is not surprising that aquatic bacteria will enter the coelomic cavity via the urino-genital pore, along with water from the tank or pond. Cone (1982) recovered *Lactobacillus* sp. from the coelomic fluid of rainbow

trout and, in addition, Sauter *et al* (1987) isolated both *A. hydrophila* and *Corynebacterium* sp. from the coelomic fluid of 5 out of 30 chinook salmon. Furthermore, present studies revealed the presence of *A. hydrophila, Pseudomonas fluorescens, Pseudomonas* sp., *Corynebacterium* sp. and *Staphylococcus epidermis,* in samples of coelomic fluid taken from healthy rainbow trout. However, in many cases the coelomic fluid samples were considered bacteriologically sterile. Thus, it seems that under normal (disease free) conditions rainbow trout are able to limit bacterial infection of the coelomic cavity, possibly by the presence of macrophages in the coelomic fluid as reported by Cone (1982) and mentioned above.

The presence of low numbers of bacteria in the coelomic fluid is probably not detrimental to egg quality. In order to enter eggs that are held in the coelomic cavity, bacteria would need to pass through the egg micropyle. The micropyle of rainbow trout eggs is approximately 3.3 - 4.3 μm in diameter (Riehl 1980) and is thus sufficiently large to allow bacteria to enter the egg (for example, *P. fluorescens* is only 0.8 μm wide - Palleroni 1984). However, in order to guarantee internal egg contamination bacteria would need to be present in the coelomic fluid in high concentrations. Trust (1972) calculated that approximately 1.5×10^8 bacterial cells would be needed to cover the entire surface (and thus the micropyle) of a single rainbow trout egg. It therefore seems unlikely that under conditions of good health, bacteria will be able to reach sufficient concentration in the coelomic fluid to internally contaminate a significant number of eggs. In contrast, diseased broodstock suffering from bacterial kidney disease infections are often observed to have a coelomic fluid that appears milky due to the presence of considerable numbers of bacteria, often as many as $4.0 - 8.0 \times 10^9$ cells / ml as revealed by plate count (Evelyn, Prosperi-Porta & Ketcheson 1981 ; Evelyn *et al* 1984). Under such conditions a much greater possibility of bacterial entry into eggs whilst held in the coelomic cavity must therefore exist.

Generally, under commercial conditions, ripe eggs are manually stripped from broodstock into separate bowls or containers to allow a visual check of egg quality (Springate & Bromage 1985). During stripping eggs are vulnerable to surface bacterial

contamination from the urogenital pore, faecal contact and from the air. However, present studies have shown that the majority of newly-stripped eggs contain few bacteria on their surfaces, although *S. epidermis,* possibly from the hands of farm staff (Woodroffe & Shaw 1974) is sometimes isolated.

During the present studies eggs were dry fertilized with milt taken from sex-reversed female fish (i.e. fish with a female genotype but masculinized with male steroids). There are few reports in the scientific literature of the bacterial content of milt, although Sauter *et al* (1987) reported bacterial contamination in the milt of one out of 6 normal male fish, species included ; *Vibrio fluvialis, Pseudomonas putrefaciens, Pasturella multicida* and *A. hydrophila.* In contrast, the present studies showed greater bacterial contamination of milt samples. However, in order to obtain milt from sex-reversed females the selected fish must be sacrificed, cut open, the testis removed and then subsequently punctured to release the milt. This rather protracted process is prone to bacterial contamination from the hands of staff (Woodroffe & Shaw 1974) and from many other sources including, the air, dissecting instruments, containers and bench surfaces. Thus, it may not be surprising that during these present trials a greater number of bacteria, from a greater range of bacterial genera were found in the milt compared to coelomic fluid. Therefore, at fertilization when milt from sex-reversed fish is added to the eggs, further exposure to bacteria is likely to occur.

The recommended sperm to egg ratio is 1 ml of sperm to 10 000 eggs (Springate & Bromage 1985). Of course milt and eggs need to come into contact and are thus immediately stirred (frequently with a less than sterile finger), left for one minute and then excess sperm is carefully rinsed off with slowly running water (Leitritz & Lewis 1976). Subsequently, eggs are left with a covering of water (about 2-3 times their own volume) for a period of about 40 minutes to fully water harden (Springate & Bromage 1985). The source from which water is taken for water hardening is extremely important. As stated above, it is likely that the bacterial content of the water in broodstock ponds will be very high. Consequently, utilization of such water for egg hardening purposes will inturn introduce eggs to large numbers of bacteria for a period

of about 40 minutes. During water hardening eggs actively take-up water and it is feasible that if sufficient numbers of bacteria are present in the surrounding water they could be "sucked-up" into eggs during this process. Entry via the micropyle would allow access to the perivitelline space but, further progress into the yolk might be impaired by the vitelline membrane. However, it is possible for bacteria to cross the vitelline membrane, either by pinocytosis or during a temporary period of disruption to the membrane that is thought to occur at water hardening (Potts & Rudy 1969). Present studies have revealed, however, that even when eggs are hardened in the presence of considerable numbers of bacteria (at a concentration of 10^6 cells / ml), few eggs were subsequently internally contaminated by the bacteria utilized. However, under such conditions, although few eggs become internally contaminated, in contrast, many eggs will have considerable numbers of bacteria attached to their external surfaces. Prudence suggests therefore, that eggs should be hardened in water taken from the purest (lowest bacterial count) source available.

Salmonid eggs are often incubated in water derived from a spring or borehole. Such waters guarantee constant temperatures (8 °C - 10 °C) and an absence of fish pathogens (Stevenson 1980). Both spring and borehole water (ground waters) collect in permeable rocks which lie below the natural water table, a consequence of water percolating through overlying soil (termed vadose water). Many dissolved nutrients in such water are filtered out during the process of percolation and thus ground water can only support a limited microbial population (Fletcher 1979). Even so, sufficient nutrients may exist to support continuous bacterial growth at extremely low rates or alternatively, bacteria may exist in resting or dormant stages (Stevenson 1978). Thus, hatchery waters will probably contain a limited bacterial population prior to the addition of salmonid eggs.

After water hardening, when eggs are laid down for incubation, additional bacteria perhaps from the milt, coelomic fluid or attached to egg surfaces will also be added to hatchery water. These bacteria will join those already present in turn

increasing the chances of subsequent egg contamination throughout the forthcoming period of incubation.

Most bacteria in natural aquatic environments do not occur "freely-suspended" but attach themselves to available surfaces (Marshall, Stout & Mitchell 1971). The incubation of glass beads during one of the present trials highlights the fact that considerable numbers of bacteria will accumulate on inert surfaces. Nutrients which support limited microbial growth have been shown to exist in hatchery waters (Willoughby, McGrory & Pickering 1983). Any surfaces in the hatchery environment, for example trays, glass beads or eggs will in turn provide an interface to which nutrients present in the water (both organic and inorganic) will be attracted by non-specific electrical forces (Marshall *et al* 1971). Indeed Zobell & Anderson (1936) first demonstrated that microbial growth on surfaces was probably the result of increased nutrient concentration. Thus, it is not surprising that during incubation bacteria tend to accumulate around the external surfaces of incubating salmonid eggs (Bell 1966 ; Bell, Hoskins & Hodgkiss 1971 ; Trust 1972 ; Yoshimizu, Kimura & Sakai 1980).

Initial bacterial colonization of egg surfaces could occur through chemotaxis. In particular dead salmonid eggs have been shown to loose amino acids, phosphate and potassium (Smith, Armstrong, Springate & Barker 1985), all agents which have the potential to attract microbes. However, in practice under relatively fast flow rates the effectiveness of such a response may be diminished, and therefore it is likely that initial egg colonization is probably fortuitous - the result of bacteria being swept onto egg surfaces by the flow of water. As bacteria approach a surface they will experience weak Van de Waals attraction induced by the fluctuation of dipoles within the molecules of the two approaching surfaces. Attraction between the surfaces increases as the distance between them decreases. However, as both surfaces are negatively charged there will also be a repulsion, the magnitude of which is dependant on surface potentials, ionic strength, pH and the dielectric constant of the surrounding medium (Marshall 1980 ; Rutter & Vincent 1980). Attractive Van de Waals forces tend to operate over a greater range than repulsive electrostatic forces thus allowing surfaces to remain in contact.

Bacteria in contact with a surface proceed through this phase of adherence termed "reversible" adherence to a further phase of "irreversible" adherence, involving the production of a range of cell surface components including the production of fimbriae (or surface fibrils) (Stenström & Kjelleberg 1985), glycocalyx fibres, pili or spinae, all providing firmer anchorage to the chosen surface (Marshall 1976 ; Costerton, Irvin & Cheng 1981). Once established on the surface aquatic bacteria frequently produce large amounts of exopolysaccharide (termed the glycocalyx) (Costerton *et al* 1981), within which bacterial replication can occur to form an adherent microcolony (Costerton, Geesey & Cheng 1978). Subsequent cell division within the microcolonies and additional bacteria from the surrounding water leads to the production of a "biofilm" overlaying the surface, increasing both nutrient availability and promoting firmer adherance (Costerton, Cheng, Geesey, Ladd, Curtis-Nickel, Dasgupta & Marrie 1987).

One important factor in determining both phases of adhesion (described above) is the hydrophobic nature of the cell surface (Pethica 1980 ; Klotz, Drutz & Zajic 1985), directly influencing the "stickiness" of the cell. Bacteria in low nutrient environments (such as hatchery water) exhibit a range of different characteristics including increased cell surface hydrophobicity (Kjelleberg & Hermansson 1984). A variety of techniques exist to measure bacterial cell surface hydrophobicity and a series of tests were employed in the present study to investigate the cell surface properties of bacteria colonizing egg surfaces. All the selected adherence tests indicated that bacteria colonizing egg surfaces possess a highly hydrophobic cell surface. Such a property will be important for both initial egg colonization and the permanent establishment of colonies on egg surfaces, and in addition, of special value to bacteria in fast flowing environments (such as hatcheries) where bacteria might otherwise be "washed-off" selected surfaces. Furthermore, tests also showed that increasing cell surface hydrophobicity occurred with increasing cell age. Such a phenomenon might be significant in the process of bacterial dispersal, for old (and therefore more hydrophobic) cells within colonies might produce daughter cells of a relatively lower hydrophobic nature, which in turn might not effectively adhere to the colony but pass

"freely-suspended" into the water. In this state bacterial cells could be dispersed from established colonies on one egg, to new uncolonized eggs, where with increasing cell age and therefore hydrophobicity, the establishment of new colonies on fresh surfaces would be promoted.

During incubation, as previously described, considerable populations of bacteria tend to accumulate around the surfaces of salmonid eggs. Such a phenomenon possibly indicates that a supply of nutrients is available to support both microbial growth and reproduction. Studies revealed that increased numbers of bacteria (per mm^2) might be found on live egg surfaces compared to glass beads, in turn suggesting that further supplementary nutrients (in addition to those obtained from the hatchery water) might be available for bacterial colonies on live salmonid egg surfaces. A biochemical analysis of salmonid eggs during the present investigation showed considerable amounts of the following structural amino acids ; alanine, arginine, glutamine and serine, in agreement with earlier studies by Suyama & Suzuki (1978). In addition, analysis of the structural fatty acids of eggs demonstrated relatively high levels of oleic acid, palmitic acid and a polyunsaturated fatty acid, confirming earlier studies of Atchinson (1975) and Nakagawa & Tsuchiya (1976). Therefore, potentially salmonid eggs might provide an abundance of nutrients suitable to support microbial growth. However, the salmonid egg is protected by a tough ichthulokeratin outer membrane (Young & Smith 1956 ; Bell, Hoskins & Bagshaw 1969), which is seemingly impervious to microbial extracellular products such as enzymes (to be discussed further below) and thus these nutrients normally remain unavailable for microbial growth. However, a series of independent studies revealed that small amounts of amino acids, phosphate and potassium, nutrients with the potential to support microbial growth, are "lost" by incubating live eggs. In addition, bacteria colonizing live egg surfaces might also obtain some nutritional benefit from waste products produced by embryos, such as ammonia and carbon dioxide (Hayes 1949 ; Smith 1947, 1957).

In comparison, dead egg surfaces are colonized by significantly higher numbers of bacteria than live egg surfaces. As above, this would again suggest that possibly bacteria on dead egg surfaces have access to enhanced nutrient sources compared to those colonizing live egg surfaces. Observations of dead eggs suggest that a general softening of the egg shell slowly occurs. This may be a result of various chemical / biological processes but, possibly may also be due to colonization by moulds such as *Saprolegnia* sp. Successful colonization by such moulds, weakening the egg shell, may in turn allow greater bacterial access to the above amino acids and fatty acids, supporting increased growth and proliferation. In addition, present studies have also demonstrated that shocked rainbow trout eggs loose significantly higher quantities of amino acids, phosphate and potassium compared to live eggs, thereby supporting further bacterial growth and reproduction.

The state of the salmonid egg (i.e. alive or dead) has been clearly shown to influence the number of bacteria surrounding the egg surface. The present studies have also shown a variety of other factors that are likely to significantly influence the numbers of bacteria colonizing egg surfaces. For example, water flow rates were found to markedly influence numbers of bacteria on egg surfaces. Water flow rate may be an important factor in influencing initial "reversible" bacterial adherence to egg surfaces, for the physical movement of water flowing over eggs may be (dependant on rates) sufficient to dislodge bacteria held in place solely by a weak electrical attraction (Marshall *et al* 1971). Indeed under slow flow rates bacteria were recovered from egg surfaces in markedly higher numbers than from eggs maintained under fast flow rates, possibly indicative of more suitable surface conditions for bacterial colonization, growth and proliferation.

Bacteria were also recovered in greater numbers from surfaces of eggs maintained at high density levels compared to low density levels. Poor water circulation (and thus reduced water flow rates) amongst "egg piles" may facilitate "initial reversible" bacterial adherence (for reasons described above). In addition, under conditions of high egg density, bacteria washed from one egg surface are likely to be

carried by the flow of water onto another nearby egg surface. Thus "chance" bacterial spread from one egg to the next is likely to be encouraged when egg density is high, compared to when egg density is low and adjacent egg surfaces are further apart.

Trout eggs incubated at 15 °C (compared to 10 °C) also supported markedly greater numbers of surface bacteria. Egg incubation at such high temperatures may lead to increased nutrient availability for colonizing bacteria. As trout embryos are poikilothermic, incubation of live eggs at 15 °C compared to 10 °C would increase respiration rates, leading to increased levels of waste by-products over a given period and potentially higher bacterial numbers. Additionally, trout eggs are possibly less well suited to higher incubation temperatures (Stevenson 1980), and under such conditions stressed eggs may loose more nutrient material leading to still further bacterial proliferation. Furthermore, although temperature may influence rates of nutrient loss from dead eggs, the greater numbers of bacteria associated with the higher temperature is probably also a consequence of increased bacterial growth rates, in turn a function of increased cell metabolism (Jawetz, Melnick & Adelberg 1984).

Overall, most of the present investigations have shown that of the bacterial genera colonizing live eggs, *Cytophaga* sp. are the most abundant followed by *A. hydrophila* and *Pseudomonas fluorescens,* closely concurring with earlier work by Bell *et al* (1971), Trust (1972) and Yoshimizu *et al* (1980). Generally, as incubation progresses bacterial numbers increase with progressive incubation periods, but species diversity is seen to decline. A similar pattern of bacterial activity was described by Shewan (1961) and Shewan & Hobbs (1967), although specifically in relation to the spoilage of stored fish. In contrast, dead egg surfaces are almost exclusively dominated by *P. fluorescens* and *Pseudomonas* sp. Other bacterial genera such as *Cytophaga* sp. *Flavobacterium* sp and *A. hydrophila* were often recovered from dead egg surfaces but, proportionally in extremely low numbers.

General external environmental factors such as egg density, water flow rate and water temperature were found to have little influence on the bacterial genera colonizing egg surfaces. However, replicate batches of rainbow trout eggs placed under

different incubation systems were indeed dominated by different bacterial genera. Rainbow trout eggs incubated under a system of recycling water were colonized mainly by *Cytophaga* sp. In contrast, rainbow trout eggs incubated under a flow-through system were dominated by by *P. fluorescens* and *A. hydrophila* and colonized by only a few *Cytophaga sp.* It is likely that in this particular trial some aspect of water quality, possibly involving the source of water was responsible for inhibiting or suppressing the growth of *Cytophaga* sp. compared to both *P. fluorescens* and *A. hydrophila.* Chemical analysis of water taken from both systems revealed much higher levels of chlorine present in the flow-through system compared to the recycling system. Even so, chlorine levels were sufficiently low enough not to affect detrimentally the development of salmonid embryos and in addition, subsequent *in vitro* studies with chlorine failed to reveal *Cytophaga* sp. as especially chlorine sensitive. Whatever, the ultimate cause for the suppression of *Cytophaga* sp., the differing results obtained serve to underline the great variation often experienced in studies of a microbial and / or ecological nature.

The colonization of live and dead egg surfaces by *Cytophaga* sp and *Pseudomonas* sp. (respectively) in turn suggests that these bacterial genera possess special properties or fitness traits that contribute to their success. Such properties are likely to include an ability to adhere to egg surfaces (as previously discussed) and an ability to efficiently utilize any nutrients that might be available in the immediate environment of the incubating egg. The relative abundance of *P. fluorescens* on dead egg surfaces (compared to *Cytophaga* sp.) could be due to a variety of factors including perhaps tolerance of a wide range of fatty acids, which can be potentially toxic to some bacteria (Atlas & Bartha 1981). Furthermore, *P. fluorescens* is known to produce a wide range of extracellular lipases (Sugiura & Isobe 1975 ; Macrae 1983) and, in addition, can utilize alanine and serine (abundant in salmonid eggs) as sole carbon sources. Moreover, *P. fluorescens* has a significantly faster growth rate than *Cytophaga* sp. and a well documented ability to inhibit other competing microbial organisms (Cornick, Chudyk & McDermot 1969 ; Dubois-Darnaudpeys 1977 ;

Gurusiddaiah, Weller, Sarkar & Cook 1986 ; Hatai & Willoughby 1988). In contrast, on live egg surfaces the limited availability of nutrients may restrict the growth and dominance of *P. fluorescens* allowing other bacterial genera (such as *Cytophaga* sp.), that might be better suited to thriving in low nutrient environments, to proliferate unhindered.

The percentage of young fish that survive from eggs to maturity amongst wild populations under natural conditions is extremely small. Nikolsky (1969) estimated percentage survivals of only 0.1% for sturgeon, 0.006% - 0.022% for bream, 0.125% for Atlantic salmon and 0.13% - 0.58% for chum salmon. In contrast, in modern hatcheries, where close control of environmental conditions is exercised and predators are mainly absent, percentage survivals are considerably higher. However, for a variety of reasons 100% survivals are never achieved. Some losses may be the result of the intensive nature of commercial farming, lowering water quality and causing "stress" to developing eggs. Other losses may also potentially be attributed to higher pathogen levels and as such an area closely investigated in these present studies.

Overall, the three most frequently isolated bacteria from live egg surfaces were *Cytophaga* sp., *Pseudomonas* sp. and *A. hydrophila,* all well known opportunistic pathogens and secondary invaders of diseased or injured fish. *Cytophaga* sp. have been implicated in bacterial gill disease of salmonid fish (Borg 1960 ; Anderson & Conroy 1969). *P. fluorescens* is an organism commonly isolated from many fresh water environments (Allen, Austin & Colwell 1983) and has also been implicated as a secondary invader of damaged fish tissues and even on occasions as a primary invader (Roberts & Horne 1978 ; Richards & Roberts 1978). *A. hydrophila* like *A. salmonicida* (the cause of furunculosis) possesses adhesins allowing it to attach closely to selected eukaryotic cells (Trust, Canotice & Atkinson 1980), is associated with spawning mortality in salmonids (Richards & Roberts 1978) and is known to produce an extended range of enzymes and exotoxins (Wadström, Lungh & Waetlend 1976). Thus, colonization of live eggs by considerable numbers of the above bacterial genera would seem potentially detrimental to egg survival. However, (as stated above)

the salmonid embryo is protected by a tough ichthulokeratin radiate membrane (Young & Smith 1956) which is resistant to the action of most proteolytic enzymes (Kanoch & Yamamoto 1957 ; Bell *et al* 1969). Indeed, only "hatching enzyme" released by the mature embryo would seem able to digest this membrane (Bell *et al* 1969). Thus, in agreement with Bell *et al* (1971) it seems unlikely that under normal incubation conditions the typical (commensal) surface bacterial flora of salmonid eggs is responsible for incubating egg death by the secretion of extracellular products. Indeed, if the bacteria frequently isolated from incubating egg surfaces were capable of secreting products that were able to penetrate the egg shell, it is likely that during incubation sufficient opportunity would occur for bacteria to spread throughout hatchery trays causing widespread egg mortality. During many trials, with healthy salmonid eggs, such a phenomenon was not observed.

Occasionally, under experimental and commercial conditions batches of eggs with zero survivals are recorded. These so called "blanks" could be due to a variety of reasons, possibly including adverse microbial involvement. However, without a regular and "predictable" supply of such eggs a definite microbial association will remain unestablished.

Under poor incubation conditions, for example raised water temperatures, slow water flow rates and high egg densities bacterial numbers on egg surfaces can significantly increase. In conjunction, present trials have shown that dissolved oxygen levels, needed to support developing embryos, could fall to critically low levels (Chapter 2). Trust (1972) calculated that if all the bacteria on an egg surface were *Pseudomonas* sp. (dry weight of 10^{-11} g / cell, Brock 1966) then the oxygen demand by bacteria alone on an egg surface could be as much as 15 µl / hour. In comparison, incubating salmon eggs require an estimated 0.2 µl per egg per hour after fertilization rising to 3.4 µl per egg per hour at hatching (Blaxter 1969). Thus, in agreement with Trust (1972), when levels of oxygen are low (higher water temperatures, slow flow rates and high egg density) it is possible that bacterial consumption of oxygen could cause local areas of oxygen deprivation in turn causing potential mortality amongst

incubating salmonid eggs. Indeed, insufficient levels of dissolved oxygen (for a variety of unrelated reasons) are thought to be the cause of many mortalities of natural stream-spawned salmonid eggs (McNeil, Wells & Brickell 1964 ; Servizi, Martens & Gordon 1970).

From a microbiological point of view significant reductions in numbers of eggs lost during incubation might simply be achieved by a combination of different approaches and a more "scientific" policy towards egg incubation. Good quality eggs, milt and great care at fertilization are prerequisites for successful egg incubation. After fertilization eggs should be hardened in water taken from the purest (low bacterial count) source of water available and carefully laid down in incubation trays to avoid accumulation of eggs in "piles". Appropriate egg density will depend on many factors including water flow rate, physical space available and total number of eggs. However, small eggs will have a larger surface area to volume ratio (Bromage & Cumaranatunga 1988) than larger eggs and may in turn suffer from a proportionally higher bacterial loading. Therefore, commercially it may be prudent to allocate more space to smaller eggs than to larger ones. Increased egg density leads to both poor water circulation and high surface bacterial populations and thus should be avoided. Ideally water taken from a spring or borehole should pass over eggs at an appropriate temperature (approximately 10 °C) and at the fastest possible flow rate that does not cause excessive disturbance of eggs. Significantly higher water temperatures further increase bacterial growth and proliferation, and slow water flow rates are not only inefficient at removing potentially toxic waste products produced by salmonid embryos but, will also encourage bacterial colonization of egg surfaces.

A variety of prophylactic treatments are available to reduce microbial contamination of eggs, including the regular application of malachite green to discourage the establishment of *Saprolegnia* sp. on incubating eggs (Cline & Post 1972 ; Oláh & Farkas 1978). Although, suppression of *Saprolegnia* activity may reduce the possibility of interaction between major microbial groups, *in vitro* studies have shown that malachite green alone is ineffectual at reducing numbers of bacteria

colonizing egg surfaces. Presence of such a compound is therefore unlikely to halt the proliferation of bacteria throughout incubation systems, particularly from such potential loci as moribund eggs. Additional removal of pockets of dead eggs from incubation trays, even though tedious and to be avoided if excessive disturbance is likely to occur to nearby healthy eggs, may therefore be necessary to prevent bacteria from increasing. These simple measures alone could lead to a marked decrease in numbers of salmonid eggs lost through microbial activity without recourse to further potentially more expensive and sophisticated prophylactic and husbandry measures.

Present studies have, however, also closely considered the possible contribution that additional prophylactic treatment of incubating eggs might make to increased hatching levels, by reducing numbers of egg surface bacteria. In all other previous studies large, commercial scale incubation systems were employed to incubate large batches of eggs. All these systems involved the use of many litres of water and made provision for excess water to go to drain. However, in this particular study where it was proposed to test antibiotics and antimicrobial compounds for their potential to reduce "commensal" bacteria on incubating egg surfaces, both at regular intervals and over a protracted period of time, such incubation systems would have been inappropriate, allowing compounds and any bacteria exposed to them to drain unchecked into the environment. Naturally, therefore, it was deemed necessary to design and construct small scale egg incubation systems where all water and compounds could be kept isolated from the environment and carefully disposed of after sterilization. After preliminary trials evaluating the efficacy and suitability of compounds for use in the designed systems, three compounds were finally selected. The first, oxolinic acid, was specifically designed for fisheries use in Japan (Endo Ogishima, Hagasaka, Kaneko & Oshina 1973a), and it s efficacy against a wide range of fish pathogens has been fully documented ; *Aeromonas salmonicida, A. liquefaciens, Vibrio anguillarum, Chrondococcus columnaris* (Endo *et al* 1973a ; Endo, Sakuma, Tanaka, Ogishima, Hara, Ohshima & Sato 1973b), *A. salmonicida* (Austin, Rayment & Alderman 1983) *V.anguillarum* (Austin, Johnson & Alderman

1982) and *Y. ruckeri* (Rodgers & Austin 1983). The second compound, chlortetracycline, belongs to the group of antibiotics (tetracyclines) that are probably the most frequently used broad spectrum antibiotics in the fisheries industry (Alderman 1988). Snieszko, Friddle & Griffin (1952) first demonstrated the sensitivity of *A. salmonicida* to chlortetracycline by *in vitro* techniques. Smith (1950) first demonstrated the efficacy of the final selected compound, chloramphenicol, against two fish pathogens, *Pseudomonas hydrophila* and *Aeromonas liquefaciens*, leading in turn to its subsequent use as a treatment of fish disease, especially within the European carp industry (Evelyn 1968).

In previous trials, overall, studies revealed that eggs incubated under a carefully controlled horizontal flow of water were colonized by a microbial population that generally increased in number as incubation progressed. However, during the course of all three antibiotic trials, large fluctuations in bacterial numbers were recorded. Similar fluctuations in numbers of aquatic bacteria have been found to occur seasonally (Jones 1973 ; Allen, Austin & Colwell 1983 ; Austin & Allen-Austin 1985 ; Iriberri, Unanue, Barcina & Egea 1987). In addition, Bell *et al* (1971) reported fluctuations in microbial numbers on surfaces of stream incubated salmon eggs, and Trust (1972) demonstrated a similar pattern of bacterial colonization on eggs maintained in a vertical upwelling incubator. Fluctuations in bacterial numbers during antibiotic trials (compared to previous trials) may thus be due to the unique experimental equipment design and the regular refilling of systems, perhaps leading to the partial removal of unattached "free living" bacteria in turn reducing the regular exposure of eggs to potential colonizers. Certainly the results of the study underline once again the great inherent variation involved in microbial and / or ecological studies.

During antibiotic trials the overall hatching success rates ranged between 19% to 58%. In comparison to all previous trials such egg hatching rates would appear to be poor and possibly due to a variety of causes. Eggs for all 3 antibiotic trials were obtained by photoperiodic manipulation of broodstock (Bromage & Cumaranatunga 1988). Although there has been much debate on the quality of such "out of season"

eggs, it is now generally accepted that under commercial farming conditions their performance is perfectly satisfactory. However, due to higher water temperatures often associated with "out of season", more frequent stripping of broodstock is required (Escaffre & Billard 1979). If this is not carried out then overripening may occur, in turn, low egg hatching rates amongst the "photoperiod" eggs used in the above antibiotic trials may possibly be due to overripeness and subsequent poor egg quality.

In addition, in previous trials great care was also taken to ensure that eggs were placed under optimum incubation conditions. However, during the antibiotic trials far more emphasis was placed on preventing antibiotics (and any resistant bacteria) from entering the natural environment. Thus, although adequate conditions for egg development were provided, slow water flow rate, low levels of oxygen and increased levels of egg disturbance, a consequence of the unique experimental design, may not have provided "optimum" conditions for salmonid egg survival. Even so, if eggs are incubated under less than ideal environmental conditions, then any subsequent form of prophylactic treatment might be especially beneficial for egg survival. However, although numbers of bacteria on egg surfaces were markedly lowered after treatment with two of the three selected compounds (oxolinic acid and chlortetracycline), a corresponding increase in egg hatching success rate was not shown. Thus, no evidence was provided by these trials to recommend routine usage of antibiotics / antimicrobial compounds in commercial egg husbandry. However, a recommendation is made for further studies for there is some evidence to suggest that larger batches of eggs, perhaps incubated under different systems, might indeed benefit from a reduced surface microflora.

The use of antibiotics / antimicrobial compounds though, may not be the most efficient method to reduce the numbers of bacteria on egg surfaces. For, in natural environments, most bacterial cells are surrounded by a "polysaccharide component" outside the cell wall, termed the glycocalyx (Costerton *et al* 1981), and in aquatic ecosystems it has been demonstrated that the majority of bacterial populations exist in glycocalyx enclosed microcolonies (Geesey, Richardson, Yeomans, Irvin & Costerton

1977). The glycocalyx may in turn act as a barrier, influencing access of molecules, ions and protons (Cheng, Ingram & Costerton 1970) to the bacterial cell wall and cytoplasmic membrane (Costerton *et al* 1981), and therefore may also give some measure of protection against antimicrobial compounds (Govan & Fyfe 1978).

Present studies have shown that at time of egg incubation, although eggs will have been exposed to bacteria, few will have been significantly contaminated. Thus, commercially it may prove more prudent to prevent initial bacterial colonization of surfaces rather than attempt to reduce numbers of already established populations. Water treatment methods, particularly those that avoid the use of long term pollutants, for example ozone disinfection (Conrad, Holt & Kreps 1975) or ultra violet (U.V.) sterilization (Spanier 1978 ; Brown & Russo 1979 ; Kimura, Yoshimizu & Atoda 1980) might prove suitable for reducing numbers of circulating, "unattached" bacteria, thus inhibiting initial egg colonization.

Further studies might also concentrate on whether a relationship can be established between bacterial presence and initial egg quality. Potentially, levels of bacteria in the coelomic fluid or on surfaces of young incubating eggs might, overall, be a reflection of general egg quality. For example, high numbers of bacteria within the coelomic fluid might indicate that eggs are overripe. If such a relationship could be determined, it could in turn become a worthwhile "predictive / diagnostic" tool for establishing egg quality and subsequently the prospective egg hatching success rates under the type of incubation conditions that could be provided.

Continual improvements in initial quality of both eggs and milt, coupled with close and careful supervision of hatchery practices should in turn lead to significant reductions in incubating egg losses. Such reductions will reduce the need for ever increasing numbers of egg importations into U.K. hatcheries and subsequently will reduce the risk of introducing non endemic diseases. In addition, it should ultimately lead to self sufficiency in the U. K. industry and the potential, through exportation, to take advantage of other European markets.

APPENDICES

APPENDIX 2 : 1 Bacterial cell culture media.

Cytophaga agar (Anacker & Ordal 1959)

Formula (per litre)

Tryptone	0.5 g
Yeast extract	0.5 g
Sodium acetate	0.2 g
Beef extract	0.2 g
Agar	9.0 g

(pH 7.3)

Method

Suspend in 1 litre of distilled water. Sterilize by autoclaving at 115 °C, 20 mins (0.72 Kg / cm^2).

APPENDIX 2 : 1(continued) Bacterial cell culture media.

Peptone beef extract glycogen agar

(McCoy & Pilcher 1974)

Formula (per litre)

Bacterio peptone	10.0 g
Bacto beef extract	10.0 g
Glycogen	4.0 g
Sodium chloride	5.0 g
Sodium lauryl sulphate	0.1 g
Brom thymol blue	0.1 g
Agar	15.0 g

(pH 6.9 - 7.1)

Method

Suspend in 1 litre of distilled water. Sterilize by autoclaving at 115 °C, 20 mins (0.72 Kg / cm^2).

After agar poured, gelled and dried, overlay with 2% non-nutrient agar in distilled water.

APPENDIX 2 : 1(continued) Bacterial cell culture media.

Tryptone soya agar

(Oxoid)

Formula (per litre)

Tryptone	15.0 g
Soya peptone	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g

(pH 7.3)

Method

Suspend in 1 litre of distilled water. Sterilize by autoclaving at 121 °C, 15 mins (0.72 Kg / cm^2).

APPENDIX 2 : 1(continued) Bacterial cell culture media.

Synthetic basal medium / chemically defined medium

(Davis & Mingioli ; Cruickshank *et al* 1975)

Formula (per litre)

Basal medium

Glucose sterile 10% solution	20 ml
Dipotassium hydrogen phosphate K_2HPO_4	7.0 g
Potassium dihydrogen phosphate KH_2PO_4	3.0 g
Sodium citrate $Na_3\ C_6H_5O_7.2H_2O$	0.5 g
Magnesium sulphate $MgSO_4.7H_2O$	0.1 g
Ammonium sulphate $(NH_4)_2\ SO_4$	1.0 g
Agar (if required)	20.0 g

Formula (per litre)

Trace element solution

Ferrous sulphate $FeSO_4.7H_2O$	0.5 g
Zinc sulphate $ZnSO_4.7H_2O$	0.5 g
Magnesium sulphate $MnSO_4.3H_2O$	0.5 g
Sulphuric acid H_2SO_4, 0.1 N	10.0 ml

Method

Add 5 ml of trace element solution to 1 litre of basal medium. Sterilize by autoclaving at 115 °C, 20 mins (0.72 Kg / cm^2) without the glucose solution. Add sterile glucose solution while media still molten and mix before pouring.

APPENDIX 2 : 2

STATISTICAL NOTATION

The following notation is used throughout ;

Probability (P)		Symbol and interpretation
$P > 0.05$		N.S. i.e. Not Significant
$P < 0.05$	*	Significant
$P < 0.01$	**	Very Significant
$P < 0.001$	***	Highly Significant

APPENDIX 2 : 2 (continued) Analysis of variance of mean cfu's / sq mm egg surface after 7 days incubation (Live eggs).

Temperature :	10 °C				15 °C			
Flow rate :	Fast flow		Slow flow		Fast flow		Slow flow	
Egg density :	Low	High	Low	High	Low	High	Low	High
1)	2.5	122.7	282.3	810.0	22.9	222.1	2120	2847.3
2)	1.6	61.4	130.1	490.9	200.6	121.2	1809	2874.3
3)	2.0	34.4	338.7	515.5	95.6	215.4	2141	4981

APPENDIX 2 : 2 (continued) Analysis of variance of mean cfu's / sq mm egg surface after 7 days incubation (Live eggs).

Statistical Analysis

Table for analysis of variance using a 3 way analysis of variance in a randomized design.

Variable (VAR)	Sums of Squares (SS)	Degrees of Freedom (DF)	Mean Square (MS)	
A	9199454.55	1	9199454.55	
B	13859816.1	1	13859816.1	
C	1575783.75	1	1575783.75	
AB	7652927.34	1	7652927.34	
AC	538291.367	1	538291.367	
BC	1146557.04	1	1146557.04	
Error	3179466.72	16	198716.67	
F RATIO (A) =			46.2943272	**
F RATIO (B) =			69.7466202	**
F RATIO (C) =			7.92980151	*
F RATIO (AB) =			38.5117531	**
F RATIO (AC) =			2.70883851	N.S.
F RATIO (BC) =			5.76980804	N.S.

A = Water temperature. B = Water flow rate. C = Egg density

The above notation is used throughout.

APPENDIX 2 : 3 Analysis of variance of mean cfu's / sq mm egg surface after 14 days incubation (Live eggs).

Temperature :	10 °C				15 °C			
Flow rate :	Fast flow		Slow flow		Fast flow		Slow flow	
Egg density :	Low	High	Low	High	Low	High	Low	High
1)	23.9	110.5	220.9	417.3	242.3	175	3150	5115
2)	7.4	159.5	368.2	1847.1	228.9	141.4	6301	1717
3)	56	138.1	156.5	902.1	80.8	215.4	807.8	605.9

Statistical analysis summary table

F RATIO (A) =	5.03765356	N.S.
F RATIO (B) =	9.78313393	*
F RATIO (C) =	0.000238034	N.S.
F RATIO (AB) =	4.24650094	N.S.
F RATIO (AC) =	0.759878636	N.S.
F RATIO (BC) =	0.0011948947	N.S.

APPENDIX 2 : 4 Analysis of variance of mean cfu's / sq mm egg surface after 21 days incubation (Live eggs).

Temperature :	10 °C				15 °C			
Flow rate :	Fast flow		Slow flow		Fast flow		Slow flow	
Egg density :	Low	High	Low	High	Low	High	Low	High
1)	72.4	98.2	270	8026.5	121.1	262.5	7876	5250
2)	51.5	55.2	303.8	28719	218.5	323.1	5385	3635
3)	40.5	159.6	349.8	13770	121.2	343.3	5654	4645

Statistical analysis summary table

F RATIO (A) =	0.934332706	N.S.
F RATIO (B) =	19.2226945	*
F RATIO (C) =	5.74145664	N.S.
F RATIO (AB) =	1.1324077	N.S.
F RATIO (AC) =	8.53702515	*
F RATIO (BC) =	5.42983143	N.S.

APPENDIX 2 : 5 Analysis of variance of mean cfu's / sq mm egg surface after 28 days incubation (Live eggs).

Temperature :	10 °C				15 °C			
Flow rate :	Fast flow		Slow flow		Fast flow		Slow flow	
Egg density :	Low	High	Low	High	Low	High	Low	High
1)	52.3	209.9	2927.1	10554.8	-	-	-	-
2)	54.5	211.7	927.9	7486.5	-	-	-	-
3)	50.3	276.2	1523.8	7952.9	-	-	-	-

Statistical analysis summary table

F RATIO (A) =	81.9435457	**
F RATIO (B) =	39.3795719	**
F RATIO (AB) =	35.4566659	**

APPENDIX 2 : 6 Analysis of variance of mean cfu's / sq mm egg surface after 7 days incubation (Dead eggs).

Temperature :	10 °C				15 °C			
Flow rate :	Fast flow		Slow flow		Fast flow		Slow flow	
Egg density :	Low	High	Low	High	Low	High	Low	High
1)	3774	8714	4510	75785	48465	492730	60582	277329
2)	2761	14973	3375	14666	79429	91546	40388	235595
3)	6075	15955	4234	28596	40388	68659	57889	324114

Statistical analysis summary table

F RATIO (A) =	14.720682	*
F RATIO (B) =	0.354703517	N.S.
F RATIO (C) =	9.27526712	*
F RATIO (AB) =	0.0050582004	N.S.
F RATIO (AC) =	5.8386157	N.S.
F RATIO (BC) =	0.412344371	N.S.

APPENDIX 2 : 7 Analysis of variance of mean cfu's / sq mm egg surface after 14 days incubation (Dead eggs).

Temperature :	10 °C				15 °C			
Flow rate :	Fast flow		Slow flow		Fast flow		Slow flow	
Egg density :	Low	High	Low	High	Low	High	Low	High
1)	13807	3682	9205	51546	6866	269252	15145	282714
2)	7364	5523	20619	34058	103662	1063543	44426	60582
3)	4523	15096	25773	22091	23156	57351	42407	475229

Statistical analysis summary table

F RATIO (A) =	5.06263714	N.S.
F RATIO (B) =	0.244231423	N.S.
F RATIO (C) =	4.16540764	N.S.
F RATIO (AB) =	0.52232464	N.S.
F RATIO (AC) =	3.75840301	N.S.
F RATIO (BC) =	0.240652546	N.S.

APPENDIX 2 : 8 Analysis of variance of mean cfu's / sq mm egg surface after 21 days incubation (Dead eggs).

Temperature :	10 °C				15 °C			
Flow rate :	Fast flow		Slow flow		Fast flow		Slow flow	
Egg density :	Low	High	Low	High	Low	High	Low	High
1)	4909	21171	24301	45655	52504	619278	108374	258481
2)	7364	28929	9205	3866	31772	222133	88180	262520
3)	3682	9205	47865	44183	40388	258481	271406	371567

Statistical analysis summary table

F RATIO (A) =	28.5851187	*
F RATIO (B) =	0.291538544	N.S.
F RATIO (C) =	11.1095641	*
F RATIO (AB) =	0.008558716	N.S.
F RATIO (AC) =	9.47452723	*
F RATIO (BC) =	1.41580641	N.S.

APPENDIX 2 : 9 Analysis of variance of mean cfu's / sq mm egg surface after 28 days incubation (Dead eggs).

Temperature :	10 °C				15 °C			
Flow rate :	Fast flow		Slow flow		Fast flow		Slow flow	
Egg density :	Low	High	Low	High	Low	High	Low	High
1)	12067	65047	81922	50074	73708	82391	102316	575525
2)	8560	31910	69956	84489	101777	70275	83468	210689
3)	1227	83456	39580	63819	42811	74717	58966	235595

Statistical analysis summary table

F RATIO (A) =	9.4162843	*
F RATIO (B) =	7.63226731	*
F RATIO (C) =	6.79636471	*
F RATIO (AB) =	3.01003791	N.S.
F RATIO (AC) =	2.89105991	N.S.
F RATIO (BC) =	2.8508882	N.S.

APPENDIX 2 : 10 Biochemical tests selected for identification of *Cytophaga* sp.

Characteristic	
Gliding motility	+
Fruiting bodies	-
Spreading colony	+
Colony pigments :	
2% tryptone / nutrient agar	orange
Anaerobic growth	+
Nitrate reduced to nitrite	+
Cellulose degredation	-
Chitin degredation	+ / -
Starch hydrolysis	+
Casein hydrolysis	+
Gelatin hydrolysis	+ / -
Peptone utilization	+
Catalase production	+
H_2S production	-
Indole production	-
Citrate utilization	-
Methyl red test	-
Glucose oxidation	+
Galactose oxidation	-
Sucrose oxidation	-
Lactose oxidation	-
Maltose oxidation	+
Mannitol oxidation	-

+ = positive result, - = negative result, + / - = weak positive result

APPENDIX 2 : 11 Biochemical tests selected for identification of *Pseudomonas fluorescens*

First stage table

Shape	Gram negative rod
Motility	+
Growth aerobically	+
Growth anaerobically	-
Catalase	+
Oxidase	+
Glucose acid	+
Carbohydrates :	
fermentative	-
oxidative	+

+ = positive reaction, - = negative reaction

APPENDIX 2 : 11 (continued) Biochemical tests selected for identification of *Pseudomonas fluorescens*

Second stage table

Fluorescence in	
U.V light	+
Growth at 5 °C	+
Growth at 42 °C	-
Growth on MacConkey agar	+
Growth in KCN	+ / -
Citrate utilization	+
Glucose "	+
Lactose "	-
Maltose "	-
Mannitol "	+
Sucrose "	+
Xylose "	+
Starch hydrolysis	-
Nitrate to Nitrite	-
Gelatin hydrolysis	+
Casein hydrolysis	+
Urease	-
Arginine dihydrolase	+
Lysine decarboxylase	-
Ornithine decarboxylase	-

+ = positive result, - = negative result, + / - = weak positive result

APPENDIX 2 : 12 Identification of *Aeromonas hydrophila* and *Pseudomonas fluorescens* using API 20 NE identification strips (24 - 48 hrs at 30 °C).

API Test	*Pseudomonas fluorescens*	*Aeromonas hydrophila*
Nitrate to nitrite	-	+
Indole production	-	+
Glucose (acidification)	-	+
Arginine dihydrolase	+	+
Urease production	-	-
Hydrolysis β glucosidase	-	+
Gelatin hydrolysis	(+)	+
β galactosidase	-	+
Glucose assimilation	+	+
Arabinose " "	+	+
Mannose " "	+	+
Mannitol " "	+	+
N-acetyl-glucosamine	+	+
Maltose assimilation	-	+
Gluconate " "	+	+
Caprate " "	+	+
Adipate " "	-	-
Malate " "	+	+
Citrate " "	+	-
Phenyl-acetate "	-	-
Cytochrome oxidase	+	+

+ = positive reaction, - = negative reaction, (+) = most strains tested positive.

APPENDIX 2 : 13 Biochemical tests selected for identification of *Pseudomonas* sp.

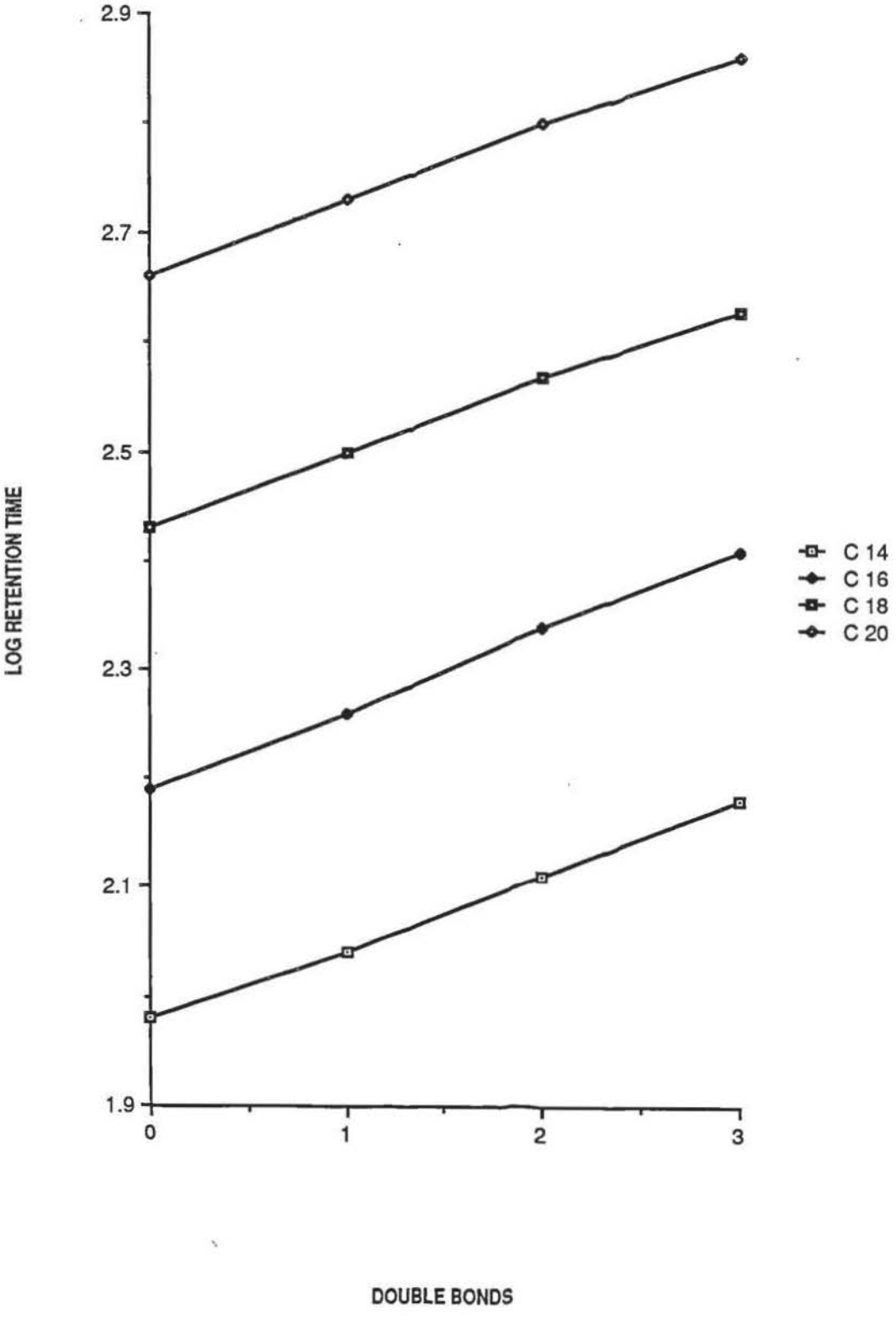
Shape	Gram negative rod
Motility	+
Growth aerobically	+
Growth anaerobically	-
Catalase	+
Oxidase	+
Glucose acid	+
Carbohydrates :	
fermentative	-
oxidative	+

+ = positive reaction, - = negative reaction

APPENDIX 3 : 1 Amino acid standards. Area of trace from 25 nmol of each amino acid.

Amino acid	Area of Trace
L-Aspartate	0.098
L-Threonine	0.095
L-Serine	0.103
L-Glutamine	0.093
L-Proline	0.024
Glycine	0.084
L-Alanine	0.094
L-Valine	0.088
L-Methionine	0.087
L-Isoleucine	0.103
L-Leucine	0.105
L-Tyrosine	0.093
L-Phenylalanine	0.096
L-Histidine	0.091
L-Lysine	0.100
L-Arginine	0.080

APPENDIX 3:2 Fatty acids standard curve



APPENDIX 3 : 3 Increase in cell concentration of *P. fluorescens* in C.D.M. (chemically defined medium - Appendix 2 : 1) at 10 °C by measurement of Optical Density.

	TIME (HOURS)								
replicates	0	6	12	18	24	36	48	60	72
1)	0.038	0.063	0.09	0.176	0.49	1.17	3.01	4.4	5.0
2)	0.036	0.06	0.077	0.185	0.35	1.26	3.26	5.2	5.6
3)	0.04	0.065	0.081	0.187	0.38	1.25	3.2	4.5	5.2
4)	0.048	0.067	0.09	0.19	0.36	1.39	3.26	5.8	5.4
5)	0.06	0.065	0.081	0.156	0.31	1.32	3.66	5.0	5.2
mean	0.044	0.064	0.084	0.179	0.38	1.28	3.28	4.98	5.28
± s.e.	0.004	0.002	0.003	0.006	0.003	0.004	0.106	0.254	0.102

s.e. = Standard error

APPENDIX 3 : 3 (continued) Increase in cell concentration of *P. fluorescens* in C.D.M. at 25 °C by measurement of Optical Density.

	TIME (HOURS)								
replicates	0	6	12	18	24	30	48	60	72
1)	0.011	0.064	0.45	1.6	5.3	5.7	-	-	-
2)	0.013	0.059	0.35	1.5	4.8	5.4	-	-	-
3)	0.013	0.052	0.35	1.4	5.2	5.9	-	-	-
4)	0.012	0.066	0.4	1.7	5.5	5.0	-	-	-
5)	0.011	0.054	0.28	1.6	5.8	5.3	-	-	-
mean	0.012	0.059	0.367	1.56	5.32	5.46			
± s.e.	0.0004	0.003	0.028	0.051	0.166	0.157			

APPENDIX 3 : 4 Increase in cell concentration of *Serratia* sp. in C.D.M. at 10 °C by measurement of Optical Density.

	TIME (HOURS)									
replicates	0	6	12	18	24	36	48	60	72	84
1)	0.034	0.049	0.062	0.102	0.215	0.696	1.15	2.50	4.71	5.08
2)	0.04	0.053	0.068	0.11	0.235	0.714	1.44	2.75	4.93	5.15
3)	0.038	0.044	0.06	0.09	0.198	0.7	1.38	2.64	4.54	4.96
4)	0.036	0.047	0.059	0.095	0.206	0.699	1.3	2.58	4.5	4.9
5)	0.036	0.05	0.063	0.106	0.214	0.721	1.51	2.71	4.8	5.2
mean	0.037	0.049	0.062	0.101	0.214	0.706	1.36	2.64	4.7	5.06
± s.e.	0.001	0.002	0.002	0.004	0.006	0.005	0.004	0.045	0.08	0.056

APPENDIX 3 : 4 (continued) Increase in cell concentration of *Serratia* sp. in C.D.M. at 25 °C by measurement of Optical Density.

	TIME (HOURS)									
replicates	0	6	12	18	24	36	48	60	72	84
1)	0.025	0.043	0.25	0.53	1.3	4.8	5.1	-	-	-
2)	0.031	0.046	0.34	0.53	1.36	5.1	5.2	-	-	-
3)	0.028	0.04	0.29	0.48	1.25	4.7	4.9	-	-	-
4)	0.029	0.039	0.31	0.59	1.2	4.4	5.0	-	-	-
5)	0.03	0.044	0.33	0.65	1.4	5.5	5.6	-	-	-
mean	0.029	0.042	0.304	0.556	1.30	4.9	5.2	-	-	-
± s.e.	0.001	0.001	0.016	0.029	0.036	0.188	0.121			

APPENDIX 3 : 5 Increase in cell concentration of *Cytophaga* sp. in C.D.M. at 10 °C by measurement of Optical Density.

	TIME (HOURS)					
replicates	0	12	24	36	48	60
1)	0.031	0.052	0.104	0.39	1.04	2.01
2)	0.034	0.055	0.092	0.42	0.84	1.93
3)	0.029	0.05	0.094	0.3	0.87	1.77
4)	0.029	0.05	0.099	0.36	1.13	1.88
5)	0.039	0.066	0.156	0.41	1.57	2.88
mean	0.032	0.055	0.109	0.375	1.09	2.09
± s.e.	0.002	0.003	0.012	0.38	0.13	0.2

	TIME (HOURS)				
replicates	72	84	96	120	132
1)	2.89	3.9	4.8	5.6	5.8
2)	2.75	4.7	4.9	6.3	6.5
3)	2.62	2.9	3.3	5.4	5.4
4)	2.85	3.3	3.8	5.7	5.9
5)	3.32	4.3	4.3	6.0	6.0
mean	2.89	3.82	4.22	5.8	5.92
± s.e.	0.112	0.326	0.302	0.158	0.178

APPENDIX 3 : 5 Increase in cell concentration of *Cytophaga* sp. in C.D.M. at 25 °C by measurement of Optical Density.

	TIME (HOURS)					
replicates	0	12	24	36	48	60
1)	0.016	0.034	0.273	1.13	5.9	6.1
2)	0.014	0.029	0.226	1.02	5.4	5.8
3)	0.009	0.028	0.298	1.35	5.9	6.3
4)	0.014	0.034	0.273	1.29	5.5	5.5
5)	0.019	0.035	0.235	1.11	5.4	5.4
mean	0.014	0.032	0.261	1.18	5.62	5.82
± s.e.	0.002	0.002	0.013	0.061	0.116	0.171

APPENDIX 3 : 6 Increase in cell concentration of *P. fluorescens* in Live egg medium at 10 °C by measurement of Optical Density.

	TIME (HOURS)								
replicates	0	6	12	18	24	36	48	60	72
1)	0.030	-	0.05	-	0.082	0.084	0.082	-	0.075
2)	0.027	-	0.057	-	0.08	0.077	0.077	-	0.061
3)	0.025	-	0.050	-	0.075	0.077	0.077	-	0.056
4)	0.015	-	0.051	-	0.077	0.071	0.069	-	0.050
5)	0.013	-	0.051	-	0.079	0.079	0.065	-	0.048
mean	0.022		0.052		0.079	0.078	0.074		0.058
± s.e.	0.003		0.001		0.001	0.002	0.003		0.005

APPENDIX 3 : 6 (continued) Increase in cell concentration of *P. fluorescens* in Dead egg medium at 10 °C by measurement of Optical Density

	TIME (HOURS)								
replicates	0	6	12	18	24	36	48	60	72
1)	0.046	0.09	-	0.118	0.110	-	0.091	-	0.10
2)	0.046	0.089	-	0.127	0.115	-	0.104	-	0.12
3)	0.045	0.087	-	0.118	0.107	-	0.093	-	0.09
4)	0.045	0.087	-	0.119	0.105	-	0.088	-	0.08
5)	0.046	0.096	-	0.129	0.114	-	0.104	-	0.11
mean	0.046	0.09		0.122	0.110		0.096		0.103
± s.e.	0.0002	0.002		0.003	0.002		0.004		0.007

APPENDIX 3 : 6 (continued) Increase in cell concentration of *P. fluorescens* in Sterile fish unit water at 10 °C by measurement of Optical Density.

	TIME (HOURS)								
replicates	0	6	12	18	24	36	48	60	72
1)	0.053	-	0.048	-	0.038	-	0.040	-	0.038
2)	0.052	-	0.044	-	0.034	-	0.039	-	0.041
3)	0.053	-	0.052	-	0.031	-	0.033	-	0.036
4)	0.050	-	0.044	-	0.03	-	0.037	-	0.032
5)	0.046	-	0.053	-	0.031	-	0.031	-	0.029
mean	0.051		0.048		0.033		0.036		0.035
± s.e.	0.001		0.002		0.002		0.002		0.002

APPENDIX 3 : 7 Increase in cell concentration of *Serratia* sp. in Live egg medium at 10 °C by measurement of Optical Density.

	TIME (HOURS)						
replicates	0	6	24	60	72	120	168
1)	0.020	0.021	0.024	0.030	0.040	0.083	0.061
2)	0.026	0.026	0.028	0.036	0.038	0.070	0.040
3)	0.028	0.030	0.030	0.040	0.046	0.090	0.042
4)	0.029	0.029	0.029	0.041	0.040	0.065	0.024
5)	0.024	0.024	0.028	0.039	0.044	0.085	0.030
mean	0.025	0.026	0.028	0.037	0.042	0.079	0.039
± s.e.	0.002	0.002	0.001	0.002	0.002	0.005	0.006

APPENDIX 3 : 7 (continued) Increase in cell concentration of *Serratia* sp. in Dead egg medium at 10 °C by measurement of Optical Density.

	TIME (HOURS)						
replicates	0	6	24	60	72	120	168
1)	0.037	0.039	0.045	0.079	0.14	0.165	0.16
2)	0.04	0.041	0.053	0.1	0.130	0.150	0.151
3)	0.038	0.037	0.05	0.091	0.094	0.145	0.13
4)	0.038	0.037	0.051	0.084	0.11	0.135	0.132
5)	0.040	0.040	0.051	0.095	0.155	0.175	0.150
mean	0.039	0.039	0.05	0.09	0.126	0.154	0.145
± s.e.	0.0006	0.0008	0.001	0.004	0.011	0.007	0.006

APPENDIX 3 : 7 (continued) Increase in cell concentration of *Serratia* sp. in Sterile fish unit water at 10 °C by measurement of Optical Density.

	TIME (HOURS)						
replicates	0	6	24	60	72	120	168
1)	0.034	0.032	0.03	0.024	-	0.018	0.017
2)	0.028	0.028	0.029	0.027	-	0.020	0.018
3)	0.029	0.026	0.027	0.024	-	0.012	0.013
4)	0.031	0.031	0.03	0.026	-	0.017	0.020
5)	0.029	0.028	0.029	0.026	-	0.020	0.017
mean	0.030	0.029	0.029	0.025		0.017	0.017
± s.e.	0.001	0.001	0.0006	0.0006		0.002	0.001

APPENDIX 3 : 8 Increase in cell concentration of *Cytophaga* sp. in Live egg medium at 10 °C by measurement of Optical Density.

	TIME (HOURS)								
replicates	0	12	24	36	48	60	72	84	96
1)	0.033	0.026	-	0.025	0.033	-	-	-	0.018
2)	0.032	0.029	-	0.021	0.021	-	-	-	0.016
3)	0.029	0.03	-	0.021	0.024	-	-	-	0.022
4)	0.032	0.024	-	0.024	0.029	-	-	-	0.010
5)	0.032	0.036	-	0.023	0.027	-	-	-	0.008
mean	0.032	0.029		0.023	0.027				0.015
± s.e.	0.0006	0.002		0.0008	0.002				0.003

APPENDIX 3 : 8 (continued) Increase in cell concentration of *Cytophaga* sp. in Dead egg medium at 10 °C by measurement of Optical Density.

	TIME (HOURS)								
replicates	0	12	24	36	48	60	72	84	96
1)	0.015	0.026	0.041	-	-	0.077	0.096	-	0.075
2)	0.019	0.020	0.035	-	-	0.071	0.099	-	0.086
3)	0.024	0.029	0.041	-	-	0.072	0.095	-	0.088
4)	0.020	0.029	0.043	-	-	0.078	0.099	-	0.087
5)	0.016	0.021	0.035	-	-	0.068	0.096	-	0.080
mean	0.019	0.025	0.039			0.073	0.097		0.083
± s.e.	0.002	0.002	0.002			0.002	0.0008		0.003

APPENDIX 3 : 8 (continued) Increase in cell concentration of *Cytophaga* sp. in Sterile fish unit water at 10 °C by measurement of Optical Density.

	TIME (HOURS)								
replicates	0	12	24	36	48	60	72	84	96
1)	0.030	0.029	-	-	0.018	-	0.019	-	0.017
2)	0.027	0.030	-	-	0.020	-	0.020	-	0.009
3)	0.034	0.028	-	-	0.016	-	0.017	-	0.025
4)	0.038	0.025	-	-	0.019	-	0.012	-	0.020
5)	0.034	0.035	-	-	0.024	-	0.020	-	0.018
mean	0.033	0.029			0.019		0.018		0.018
± s.e.	0.002	0.002			0.002		0.002		0.003

APPENDIX 3 : 9 Viable count of *P. fluorescens* in sterile fish unit water over a 29 day period.

	DAYS								
	inoculum	1	3	5	7	9	11	13	15	17
1)	34000	48750	92500	63333	85000	14000	120000	56667	75000	92500
2)	31500	25000	65000	75000	110000	190000	120000	57500	125000	66667
3)	26000	50000	50000	67500	65000	110000	70000	66667	85000	50000
mean	30500	41250	69167	68611	86666	146666	103333	60278	95000	69722
s.e.	2363	8133	12444	3413	13017	23333	16667	3204	15275	12363

	DAYS					
	19	21	23	25	27	29
1)	75000	41250	57500	29750	20000	18333
2)	50000	26250	29750	25000	20750	32500
3)	30000	31429	40000	50000	16000	18500
mean	51667	32976	42417	34916	18917	23111
s.e.	13017	4399	8101	7665	1474	4695

APPENDIX 3 : 9 (continued) Viable count of *Serratia* sp. in sterile fish unit water over a 29 day period.

	DAYS									
	inoculum	1	3	5	7	9	11	13	15	17
1)	36667	41667	20000	28000	21500	16000	31667	20000	25000	17000
2)	65000	32500	30000	13333	22000	7000	31667	27750	17500	18500
3)	46667	27500	22500	14417	19583	12583	35000	24400	21250	33500
mean	49445	33889	24167	18583	21028	11861	32778	24050	21250	23000
s.e.	8296	4148	3004	4719	736	2623	1111	2244	2165	5268

	DAYS					
	19	21	23	25	27	29
1)	22500	15000	16000	17500	10750	13250
2)	14444	22500	27500	8250	8250	10000
3)	33500	20750	10500	45000	10000	25000
mean	15815	19417	29500	11917	14667	11333
s.e.	3531	2265	8431	2837	5216	983

APPENDIX 3 : 9 (continued) Viable count of *Cytophaga* sp. in sterile fish unit water over a 29 day period.

		DAYS								
	inoculum	1	3	5	7	9	11	13	15	17
1)	52500	75000	91667	27500	40000	20000	42500	49667	55000	18000
2)	40000	55000	155000	50000	42500	15000	51667	27000	37500	16250
3)	100000	116667	150000	73333	133333	17500	66667	32500	11144	26333
mean	64167	82222	132222	50278	71944	17500	53611	36389	34548	20194
s.e.	18276	18164	20329	13232	30702	1443	7044	6826	12746	3111

	DAYS					
	19	21	23	25	27	29
1)	22500	47500	30000	37500	30000	35000
2)	15000	15000	40000	25000	20500	17500
3)	37000	28333	35417	29292	26833	25278
mean	24833	30278	35417	29292	26833	25278
s.e.	6457	9432	2917	4106	3167	5145

APPENDIX 3 : 10 Levels of amino acids "leaked" from live and dead eggs (mg / L / g dry weight).

Live Eggs

Reps	TIME (Hours)						
	0	0.5	1	6	12	24	48
1)	0	27	40.2	43.8	43.8	45.3	62.1
2)	3	32.1	50.4	44.8	41.7	51.9	70.8
3)	6	42.2	38.7	63.6	40.2	42.2	52.9
4)	0	40.8	43.8	42.2	45.8	117.6	76.4
5)	0	50.4	40.2	61.1	47.3	65.7	61.6

Dead Eggs

Reps	TIME (Hours)						
	0	0.5	1	6	12	24	48
1)	17	215	171.3	210.4	334.6	330.5	243.3
2)	33	184.8	290.4	111.6	326.4	118.7	315.1
3)	5	97.9	153.1	98.1	284.4	219.6	383.8
4)	30	156	126.3	176.2	272.4	303.1	211.9
5)	23	143	118.9	183.7	203.8	296.4	264.7

APPENDIX 3 : 10 (continued) Analysis of levels of amino acids leaked between live and dead eggs by 2 way split-plot analysis of variance in a randomized design.

Variable (VAR)	Sums of Squares (SS)	Degrees of Freedom (DF)	Mean Square (MS)
Ma. F	374169.669	1	374169.669
MP. Error	9419.01709	8	1177.37714
MI.F	189532.656	6	31588.776
A X B	86639.5286	6	14439.9214
SP Error	91339.7544	48	1902.91155

F Ratio (Ma. F) =	317.799334	***
F Ratio (Mi. F) =	16.6002335	***
F Ratio (A X B) =	7.58833033	***

Ma = major factor = State of egg (e.g. live eggs / dead eggs)

Mi = minor factor = Time

A X B = Interaction of the two factors.

APPENDIX 3 : 11 Levels of phosphate "leaked" from live and dead eggs (mg / L / g dry weight).

Live Eggs

Reps	TIME (Hours)						
	0	0.5	1	6	12	24	48
1)	0	3.6	5.6	0	0.9	1.8	0
2)	0	1.8	10.7	5.6	3.6	0	3.6
3)	0	0.9	0	1.8	1.8	0	5.6
4)	0.4	0	3.6	0	5.6	0	3.6
5)	0.7	0	3.6	0.9	0	2.7	2

Dead Eggs

Reps	TIME (Hours)						
	0	0.5	1	6	12	24	48
1)	16	92.6	78.4	116	92.6	69.7	98.2
2)	21.4	69.7	92.6	142.5	71.3	81.9	128.3
3)	8.6	62.6	96.2	92.6	105.4	87.5	116
4)	20.7	87.5	82	146.1	71.3	73.3	89
5)	29.6	84	103.3	116.1	76.9	87.5	92.6

APPENDIX 3 : 11 (continued) Analysis of levels of phosphate leaked between live and dead eggs by 2 way split-plot analysis of variance in a randomized design.

Variable (VAR)	Sums of Squares (SS)	Degrees of Freedom (DF)	Mean Square (MS)
Ma. F	114380.517	1	114380.517
MP. Error	218.498291	8	27.3122864
MI.F	16248.5518	6	2708.09196
A X B	14774.0895	6	2462.34825
SP Error	5436.96179	48	113.270037

F Ratio (Ma. F) =	4187.87775	***
F Ratio (Mi. F) =	23.9082817	***
F Ratio (A X B) =	21.7387431	***

Ma = major factor = State of egg (e.g. live eggs / dead eggs)

Mi = minor factor = Time

A X B = Interaction of the two factors.

APPENDIX 3 : 12 Levels of potassium "leaked" from live and dead eggs (ppm / g dry weight).

Live Eggs

Reps	TIME (Hours)						
	0	0.5	1	6	12	24	48
1)	0	0	0	0	0	2.4	0.8
2)	0.5	0	0	0	0	0	2.4
3)	0.3	0	0.4	0.8	0	4.8	1.3
4)	0	0.8	0	1.4	0	0	0
5)	0	1.6	0	0	0	11.1	0.8

Dead Eggs

Reps	TIME (Hours)						
	0	0.5	1	6	12	24	48
1)	0	50.8	55.6	65	47.6	34.9	46.1
2)	0	49.2	63.5	81	36.5	36.5	58.8
3)	1	50.8	74.1	47.6	31.8	36	55.6
4)	1.6	47.6	66.2	81	31.8	36.5	42.9
5)	0.8	42.9	65.1	55.6	31.8	34.9	41.3

APPENDIX 3 : 12 (continued) Analysis of levels of potassium leaked between live and dead eggs by 2 way split-plot analysis of variance in a randomized design.

Variable (VAR)	Sums of Squares (SS)	Degrees of Freedom (DF)	Mean Square (MS)
Ma. F	30996.1286	1	30996.1286
MP. Error	224.122223	8	28.0152779
MI.F	7382.41541	6	1230.40257
A X B	7503.63545	6	1250.60591
SP Error	1420.21774	48	29.5878696

F Ratio (Ma. F) =	1106.4009	***
F Ratio (Mi. F) =	41.5846961	***
F Ratio (A X B) =	42.2675212	***

Ma = major factor = State of egg (e.g. live eggs / dead eggs)

Mi = minor factor = Time

A X B = Interaction of the two factors.

APPENDIX 3 : 13 Free egg amino acids.

Amino acid	Amount (n mols / 10 eggs)			
	1	2	3	mean
L-Aspartate	47.5	33.6	37.7	39.6
L-Threonine	12.0	15.6	18.3	15.3
L-Serine	34.6	30.1	25.2	30.0
L-Glutamine	23.3	27.7	25.6	25.5
L-Proline	8.6	8.9	11.5	9.6
Glycine	7.6	10.1	9.0	8.9
L-Alanine	12.1	7.7	12.1	10.6
L-Valine	4.0	3.9	7.8	5.2
L-Methionine	4.8	4.6	6.0	5.1
L-Isoleucine	7.2	5.5	5.2	6.0
L-Leucine	6.8	6.9	4.7	6.1
L-Tyrosine	4.2	4.9	4.9	4.7
L-Phenylalanine	5.3	4.8	4.2	4.8
L-Histidine	6.4	5.5	7.0	6.3
L-Lysine	4.6	4.7	7.0	5.4
L-Arginine	53.4	47.8	42.0	48.1

APPENDIX 3 : 13 (continued) Structural egg amino acids.

Amino acid	Amount (n mols / egg)			
	1	2	3	mean
L-Aspartate	36.6	27.4	25.6	29.9
L-Threonine	27.7	29.8	24.4	27.3
L-Serine	45.6	43.4	30.5	39.8
L-Glutamine	46.3	39.1	38.7	41.4
L-Proline	36.9	38.8	40.1	38.6
Glycine	27.0	27.0	18.3	24.1
L-Alanine	48.8	39.6	42.2	43.5
L-Cystine	8.3	9.0	8.5	8.6
L-Valine	17.9	31.4	30.6	26.6
L-Methionine	10.6	9.4	11.9	10.6
L-Isoleucine	34.6	22.6	24.9	27.4
L-Leucine	28.8	33.9	40.9	34.5
L-Tyrosine	9.9	12.9	15.1	12.6
L-Phenylalanine	18.4	18.4	9.9	15.6
L-Histidine	8.8	8.4	9.0	8.7
L-Lysine	20.1	21.5	30.4	24.0
L-Arginine	56.6	44.1	25.8	42.2

APPENDIX 3 : 14 Structural egg fatty acids

Fatty acid	Amount (percentage)		
	1	2	mean
C14-0	1.6	2.0	1.8
C16-0	22.4	11.2	16.8
C16-1	7.9	7.7	7.8
C18-0	5.6	5.0	5.3
C18-1	28.3	34.8	31.6
C18-2	6.4	5.3	5.9
C20-1	2.4	2.4	2.4
C20-3	0.6	1.0	0.8
(C22-5)	3.5	5.7	4.6
(C22-6)	23.7	14.7	19.2

Figures in brackets unknowns but assumed to be C22-5 and C22-6

APPENDIX 4 : 1 Bacterial Adherence To Hydrocarbons. Transformed data, mean percentage absorbance figures from 4 separate studies. Each study composed of 6 replicates (*P. fluorescens*).

Culture age :	Log phase			Stationary phase		
Volume of hexadecane (ml)	0.2	0.1	0.05	0.2	0.1	0.05
1)	71.8	73.6	76.7	40.0	68.5	55.6
2)	63.3	57.6	63.9	47.9	43.6	32.5
3)	49.2	61.8	69.6	26.7	36.2	58.2
4)	53.1	58.0	55.6	29.5	31.8	39.4

APPENDIX 4 : 1 (continued) Bacterial Adherence To Hydrocarbons. Transformed data, analysis of variance (*P. fluorescens*).

Variable (VAR)	Sums of Squares (SS)	Degrees of Freedom (DF)	Mean Square (MS)
A	2486.77045	1	2486.77045
B	324.013336	2	324.013336
AXB	31.6932983	2	15.8466492
Error	2277.93253	18	126.551807
F ratio (A) =	19.6502168	*	
F ratio (B) =	1.28016084	N.S.	
F ratio (AXB) =	0.125218672	N.S.	

A = major factor = Growth stage of cells (e.g. log / stationary)

B = minor factor = volume of added hydrocarbon (e.g. 0.2 / 0.1 / 0.05 ml)

AXB = Interaction between the two.

APPENDIX 4 : 2 Bacterial Adherence To Hydrocarbons. Transformed data, mean percentage absorbance figures from 4 separate studies. Each study composed of 6 replicates (*Cytophaga* sp.).

Culture age :	Log phase			Stationary phase		
Volume of hexadecane (ml)	0.2	0.1	0.05	0.2	0.1	0.05
1)	66.4	68.6	73.2	46.4	47.6	56.4
2)	57.9	63.2	66.0	62.7	60.3	63.8
3)	62.6	65.9	75.0	63.3	56.8	61.2
4)	64.9	68.1	70.1	43.5	66.0	49.4

APPENDIX 4 : 2 (continued) Bacterial Adherence To Hydrocarbons. Transformed data, analysis of variance (*Cytophaga* sp.).

Variable (VAR)	Sums of Squares (SS)	Degrees of Freedom (DF)	Mean Square (MS)
A	645.84375	1	645.84375
B	142.589935	2	71.2949677
AXB	27.0400391	2	13.5200195
Error	734.862488	18	40.8256938
F ratio (A) =	15.8195413	*	
F ratio (B) =	1.74632593	N.S.	
F ratio (AXB) =	0.331164477	N.S.	

A = major factor = Growth stage of cells (e.g. log / stationary)

B = minor factor = volume of added hydrocarbon (e.g. 0.2 / 0.1 / 0.05 ml)

AXB = Interaction between the two.

APPENDIX 4 : 3 Bacterial Adherence To Hydrocarbons. Transformed data, mean percentage absorbance figures from 4 separate studies. Each study composed of 6 replicates (*Serratia* sp.).

Culture age :	Log phase			Stationary phase		
Volume of hexadecane (ml)	0.2	0.1	0.05	0.2	0.1	0.05
1)	63.3	60.7	59.3	36.4	40.9	47.5
2)	59.7	59.4	58.0	26.5	33.7	41.8
3)	69.1	69.5	68.0	28.4	31.7	36.8
4)	70.7	72.9	69.5	21.7	35.2	53.2

APPENDIX 4 : 3 (continued) Bacterial Adherence To Hydrocarbons. Transformed data, analysis of variance (*Serratia* sp.).

Variable (VAR)	Sums of Squares (SS)	Degrees of Freedom (DF)	Mean Square (MS)
A	4996.82039	1	4996.82039
B	212.505829	2	106.252914
AXB	350.840851	2	175.420425
Error	623.372498	18	34.6318054
F ratio (A) =	144.284144	**	
F ratio (B) =	3.06807321	*	
F ratio (AXB) =	5.06529831	*	

A = major factor = Growth stage of cells (e.g. log / stationary)

B = minor factor = volume of added hydrocarbon (e.g. 0.2 / 0.1 / 0.05 ml)

AXB = Interaction between the two.

APPENDIX 4 : 4 Hydrophobic Interaction Chromatography. Percentage of cells adhering to column after addition of 1 M ammonium sulphate, 0.5 M ammonium sulphate and ethanol (6 replicates). Data transformed by arcsine. (Octyl sepharose).

P. fluorescens

	1M	0.5 M	Ethanol
1)	86.6	86.3	73.9
2)	87.9	86.6	75.7
3)	87.3	85.9	76.0
4)	87.8	86.4	77.4
5)	86.8	85.2	75.6
6)	86.9	85.3	74.0

Cytophaga sp.

	1M	0.5 M	Ethanol
1)	72.4	71.9	65.8
2)	71.2	70.5	66.0
3)	72.5	71.5	65.5
4)	72.9	72.0	65.2
5)	73.5	72.8	67.0
6)	73.8	72.9	68.3

Serratia sp.

	1M	0.5 M	Ethanol
1)	90.0	85.6	69.3
2)	88.4	85.6	75.6
3)	88.6	86.9	69.4
4)	89.2	85.8	75.0
5)	90.0	84.6	73.2
6)	88.9	84.6	74.8

APPENDIX 4 : 4 (continued) Hydrophobic Interaction Chromatography. Transformed data, analysis of variance of cells adhering to octyl sepharose.

Variable (VAR)	Sums of Squares (SS)	Degrees of Freedom (DF)	Mean Square (MS)
A	1840.37109	2	920.185547
B	1367.60693	2	683.803467
AXB	159.010864	4	39.7527161
Error	73.4434814	45	1.63207737
F ratio (A) =	563.812455	***	
F ratio (B) =	418.97736	***	
F ratio (AXB) =	24.3571272	***	

A = major factor = Bacterial species (e.g. *P. fluorescens* / *Cytophaga* sp. / *Serratia* sp.)

B = minor factor = Elutant (e.g. 1 M ammonium sulphate / 0.5 M ammonium sulphate / ethanol)

AXB = Interaction between the two.

APPENDIX 4 : 5 Hydrophobic Interaction Chromatography. Percentage of cells adhering to column after addition of 1 M ammonium sulphate, 0.5 M ammonium sulphate and ethanol (6 replicates). Data transformed by arcsine. (Phenyl sepharose).

P. fluorescens

	1M	0.5 M	Ethanol
1)	86.6	85.7	74.9
2)	87.0	85.9	74.8
3)	86.6	85.5	76.7
4)	87.4	86.4	75.6
5)	86.9	86.0	75.4
6)	86.7	85.6	74.7

Cytophaga sp.

	1M	0.5 M	Ethanol
1)	73.9	72.3	71.5
2)	73.3	71.6	70.5
3)	76.0	74.0	73.0
4)	74.5	73.0	72.0
5)	74.1	72.7	71.8
6)	73.8	72.5	71.6

Serratia sp.

	1M	0.5 M	Ethanol
1)	88.9	84.5	76.2
2)	88.9	85.6	77.6
3)	88.7	84.8	76.8
4)	88.6	85.2	78.1
5)	89.2	86.0	76.9
6)	89.2	85.8	76.8

APPENDIX 4 : 5 (continued) Hydrophobic Interaction Chromatography. Transformed data, analysis of variance of cells adhering to phenyl sepharose.

Variable (VAR)	Sums of Squares (SS)	Degrees of Freedom (DF)	Mean Square (MS)
A	1291.78784	2	645.893921
B	731.559937	2	365.779968
AXB	218.818848	4	54.7047119
Error	18.9683838	45	0.42151964
F ratio (A) =	1532.29852	***	
F ratio (B) =	867.764948	***	
F ratio (AXB) =	129.779746	***	

A = major factor = Bacterial species (e.g. *P. fluorescens* / *Cytophaga* sp. / *Serratia* sp.)

B = minor factor = Elutant (e.g. 1 M ammonium sulphate / 0.5 M ammonium sulphate / ethanol)

AXB = Interaction between the two.

APPENDIX 4 : 6 *In situ* Detachment From Eggs. Transformed data (3 replicates). (*P. fluorescens*)

Hours :	0	2	4	6	8	10	12	14	16	18
1)	24.2	27.0	22.0	30.5	27.2	31.8	21.3	21.2	29.4	33.7
2)	18.7	24.5	30.5	19.8	25.6	25.5	29.4	25.0	27.0	45.8
3)	13.1	24.5	32.1	29.9	18.2	36.9	24.1	22.7	34.5	34.5

Hours :	20	22	24
1)	37.6	48.7	67.8
2)	39.9	57.8	79.5
3)	44.6	54.2	73.7

APPENDIX 4 : 6 *In situ* Detachment From Eggs (continued). Transformed data (3 replicates). (*Cytophaga* sp.)

Hours :	0	2	4	6	8	10	12	14	16	18
1)	29.4	29.4	26.1	29.4	23.3	30.6	27.8	32.6	36.0	32.5
2)	27.2	27.8	33.1	32.2	30.2	32.6	31.0	30.0	36.5	58.4
3)	26.1	30.2	34.6	32.1	22.4	25.6	34.6	28.3	34.6	37.9

APPENDIX 4 : 6 *In situ* Detachment From Eggs (continued). Transformed data (3 replicates). (*Cytophaga* sp.)

Hours :	20	22	24
1)	38.4	37.9	44.9
2)	46.8	41.0	39.9
3)	36.7	65.4	46.5

APPENDIX 4 : 6 *In situ* Detachment From Eggs (continued). Transformed data (3 replicates).(*Serratia* sp.)

Hours :	0	2	4	6	8	10	12	14	16	18
1)	26.9	28.7	30.7	26.7	29.5	30.4	25.7	24.3	24.0	23.8
2)	35.2	27.8	26.3	37.5	33.6	28.7	29.3	30.9	23.1	28.7
3)	21.5	39.8	28.4	31.1	23.0	31.2	43.3	27.8	43.9	31.2

Hours :	20	22	24
1)	26.0	28.7	34.5
2)	24.6	39.1	32.9
3)	34.3	38.3	35.2

APPENDIX 4 : 6 *In situ* Detachment From Eggs (continued). Transformed data compared by 2 way split-plot analysis of variance.

Variable (VAR)	Sums of Squares (SS)	Degrees of Freedom (DF)	Mean Square (MS)
Ma. F	346.803711	2	173.401855
MP. Error	312.79483	6	52.1324717
MI.F	6361.18808	12	530.099007
A X B	3962.13196	24	165.088832
SP Error	2292.11838	72	31.8349775

F Ratio (Ma. F) =	3.32617752	*
F Ratio (Mi. F) =	16.6514648	***
F Ratio (A X B) =	5.18576876	***

Ma = major factor = Bacterial group (e.g. *P. fluorescens* / *Cytophaga* sp. / *Serratia* sp.)

Mi = minor factor = Time

A X B = Interaction of the two factors.

APPENDIX 5 : 1 Hatching success between egg groups

(A = rainbow trout eggs, source 1, B = brown trout eggs, source 1 and C = rainbow trout eggs, source 2).

	Rainbow trout (Source 1) (A)	Brown trout (Source 1) (B)	Rainbow trout (Source 2) (C)
Number of hatched eggs	1268	882	2467
Number of unhatched eggs	68	38	222

Statistical Analysis by χ^2 of hatching success between egg groups ;

Egg groups (A,B,C)	χ^2	d.f.	Significance
A v B v C	25.8764504	2	***
A v B	1.11989015	1	N.S.
A v C	13.3815515	1	***
B v C	17.4507406	1	***

APPENDIX 5 : 2 Number of egg deaths between groups at 7 day periods.

Days	Eggs	Rainbow trout (Source 1)	Brown trout (Source 1)	Rainbow trout (Source 2)
1-7	Live	1336	920	2689
	Dead	36	12	70
7-14	Live	1300	908	2619
	Dead	44	14	111
14-21	Live	1292	906	2578
	Dead	52	16	155
21-28	Live	1240	890	2423
	Dead	58	17	194

APPENDIX 5 : 2 (continued) Statistical analysis of egg mortality between groups at 7 day periods. (A = rainbow trout eggs, source 1, B = brown trout eggs, source 1 and C = rainbow trout eggs, source 2).

Days	Trays	χ^2	d.f.	Significance
1-7	A v B v C	5.49003508	2	*
7-14	A v B v C	13.7525257	2	**
14-21	A v B v C	26.5721758	2	***
21-28	A v B v C	43.3845977	2	***
1-7	A v B	4.85863852	1	N.S.
7-14	A v B	6.75617737	1	*
14-21	A v B	8.55295164	1	*
21-28	A v B	10.9351265	1	**
1-7	A v C	0.002758570	1	N.S.
7-14	A v C	1.54405554	1	N.S.
14-21	A v C	6.07279975	1	N.S.
21-28	A v C	12.4922796	1	**
1-7	B v C	5.00784887	1	*
7-14	B v C	13.5311855	1	**
14-21	B v C	23.9501532	1	***
21-28	B v C	36.7085913	1	***

APPENDIX 5 : 3 Analysis of variance of mean colony forming units (cfu's) / sq mm egg surface.

	Rainbow trout (Source 1)				Brown trout (Source 1)			
Days :	7	14	21	28	7	14	21	28
1)	1.7	25.8	32.7	108.8	0.3	16.8	61.6	33.2
2)	0.9	18	62.4	102.1	1.1	53.7	48.6	32.2
3)	4	52.5	241	93.8	1.4	31.9	103.3	93.6
4)	3.7	42.8	133	140	1.7	83.6	29.1	86.2

	Glass beads (Source 1)				Rainbow trout (Source 2)			
Days :	7	14	21	28	7	14	21	28
1)	2.4	2.2	1.2	3.9	4	39.8	201.2	419.9
2)	2.4	1.3	3.6	5.2	1.6	34.4	187	410.6
3)	0.8	7.6	2	26.2	2.6	29	320	503.9
4)	2.1	8.6	1.3	5.1	3.9	48.8	184.8	627.9

APPENDIX 5 : 3 (continued) Analysis of mean cfu's / sq mm egg surface by 2 way split-plot analysis of variance in a randomized design.

Variable (VAR)	Sums of Squares (SS)	Degrees of Freedom (DF)	Mean Square (MS)
Ma. F	304100.117	3	101366.706
MP. Error	22372.091	12	1864.34159
MI.F	265752.981	3	88584.3269
A X B	380772.481	9	42308.0532
SP Error	57767.5908	36	1604.6553

F Ratio (Ma. F) =	54.3713159	***
F Ratio (Mi. F) =	55.2045831	***
F Ratio (A X B) =	26.3658204	***

Ma = major factor = Egg group (e.g. rainbow trout, source 1 ; rainbow trout, source 2 ; brown trout, glass beads)

Mi = minor factor = Time

A X B = Interaction of the two factors.

APPENDIX 5 : 4 Correlation of egg surface bacterial numbers with egg mortality by multiple regression.

	Rainbow trout (Source 1)				Brown trout (Source 1)			
Days	7	14	21	28	7	14	21	28
Mean cfu's	2.6	34.8	117.3	111.2	1.1	46.5	60.7	61.3
Egg Deaths	36	44	52	58	12	14	16	17

	Rainbow trout (Source 2)			
Days	7	14	21	28
Mean cfu's	3	38	223.2	490.6
Egg deaths	70	111	155	194

Var.	SS.	D.F.	MS
Reg	30289.3561	2	15144.678
Error	10842.3362	10	1084.23362
F ratio =		13.9680948	
Multiple R-SQ =		0.736399462	

Var.	COEFF	S.E.	t
INTER.	58.8678777		
1	-2.1194188	1.19854209	-1.76833072
2	0.431383335	0.0083145362	5.18830306

APPENDIX 6 : 1 Hatching success between egg groups

(A = control eggs, B = eggs exposed to autoclaved *P. fluorescens*, C = eggs exposed to autoclaved *Cytophaga* sp., D = eggs exposed to live *P. fluorescens* and E = eggs exposed to live *Cytophaga* sp.)

	A	B	C	D	E
Number of hatched eggs	849	1058	723	907	769
Number of unhatched eggs	242	158	147	305	228

Statistical Analysis by χ^2 of hatching success between egg groups ;

Egg groups	χ^2	d.f.	Significance
A v B v C v D v E	70.9804664	4	***
A v B	33.8721873	1	***
A v C	8.5015566	1	**
A v D	2.82208936	1	N.S.
A v E	0.14100691	1	N.S.
B v C	6.1891293	1	*
B v D	58.268837	1	***
C v E	90.3207929	1	***
D v E	1.57576477	1	N.S.

APPENDIX 6 : 2 Number of egg deaths between groups at 7 day periods.

(A = control eggs, B = eggs exposed to autoclaved *P. fluorescens*, C = eggs exposed to autoclaved *Cytophaga* sp., D = eggs exposed to live *P. fluorescens* and E = eggs exposed to live *Cytophaga* sp.)

Days	Eggs	A	B	C	D	E
1-7	Live	1029	1175	826	1035	947
	Dead	62	41	44	177	50
7-14	Live	981	1163	815	1020	880
	Dead	110	53	55	192	117
14-21	Live	959	1157	802	1000	836
	Dead	132	59	68	212	161
21-28	Live	903	1110	765	949	800
	Dead	188	106	105	263	197

APPENDIX 6 : 2 (continued) Statistical analysis of egg mortality between groups at 7 day periods. (A = control eggs, B = eggs exposed to autoclaved *P. fluorescens*, C = eggs exposed to autoclaved *Cytophaga* sp., D = eggs exposed to live *P. fluorescens* and E = eggs exposed to live *Cytophaga* sp.)

Days	Trays	χ^2	d.f.	Significance
1-7	A v B v C v D v E	147.286801	4	***
7-14	A v B v C v D v E	107.155105	4	***
14-21	A v B v C v D v E	126.193104	4	***
21-28	A v B v C v D v E	99.2501343	4	***
1-7	A v B	7.20133616	1	**
7-14	A v B	28.6935151	1	***
14-21	A v B	39.7718303	1	***
21-28	A v B	37.4940979	1	***
1-7	A v C	0.370229991	1	N.S.
7-14	A v C	8.88296777	1	**
14-21	A v C	9.69411287	1	**
21-28	A v C	10.151979	1	**
1-7	A v D	49.130433	1	***
7-14	A v D	16.7137986	1	***
14-21	A v D	13.1416592	1	***
21-28	A v D	7.27756192	1	**
1-7	A v E	0.45767511	1	N.S.
7-14	A v E	1.46849608	1	N.S.
14-21	A v E	7.08127043	1	*
21-28	A v E	2.21266815	1	N.S.

APPENDIX 6 : 3 Analysis of variance of mean colony forming units (cfu's) / sq mm egg surface.

(A = control eggs, B = eggs exposed to autoclaved *P. fluorescens*, C = eggs exposed to autoclaved *Cytophaga* sp., D = eggs exposed to live *P. fluorescens* and E = eggs exposed to live *Cytophaga* sp.)

	A					B				
Time :	(12)	7	14	21	28	(12)	7	14	21	28
	(hours)		DAYS			(hours)		DAYS		
1)	1.1	3.2	124.7	307.9	306	1.2	3.8	30.3	495.6	565.7
2)	1.1	3.8	85.6	273.2	246	10.9	4.6	41.5	699.8	807.4
3)	0.8	6.4	38.2	629.8	783	1.4	21.6	24.4	960.6	390

	C					D				
Time :	(12)	7	14	21	28	(12)	7	14	21	28
	(hours)		DAYS			(hours)		DAYS		
1)	0.5	6.7	27.7	306.7	730.3	27	24.5	74.8	14445	915.4
2)	1.7	3.1	27.5	850.1	517.5	12.6	36	91.4	2426	1100.6
3)	0.6	8.9	15.8	634.4	817.7	46.2	23.8	137.8	234.3	1192.5

APPENDIX 6 : 3 (continued) Analysis of variance of mean cfu's / sq mm egg surface. (A = control eggs, B = eggs exposed to autoclaved *P. fluorescens*, C = eggs exposed to autoclaved *Cytophaga* sp., D = eggs exposed to live *P. fluorescens* and E = eggs exposed to live *Cytophaga* sp.)

			E		
Time :	(12)	7	14	21	28
	(hours)		DAYS		
1)	49.2	105.1	356.3	977.8	472.5
2)	59.4	31.9	156.5	509.1	612
3)	37.8	187.9	142.3	203.3	302.4

APPENDIX 6 : 3 (continued) Analysis of mean cfu's / sq mm egg surface by two split-plot analysis of variance.

Variable (VAR)	Sums of Squares (SS)	Degrees of Freedom (DF)	Mean Square (MS)
Ma. F	948950.66	4	237237.665
MP. Error	659080.965	10	65908.0965
MI.F	7556005.12	4	1889001.28
A X B	1608221.86	16	100513.866
SP Error	2835317.87	40	70882.9468

F Ratio (Ma. F) =	3.59952233	**
F Ratio (Mi. F) =	26.6495873	***
F Ratio (A X B) =	1.41802607	N.S.

Ma = major factor = Egg group (e.g. control eggs, eggs exposed to autoclaved *P. fluorescens*, eggs exposed to live *P. fluorescens*, eggs exposed to autoclaved *Cytophaga* sp. and eggs exposed to live *Cytophaga* sp.)

Mi = minor factor = Time

A X B = Interaction of the two factors.

APPENDIX 6 : 4 Correlation of egg surface bacterial numbers with egg mortality by multiple regression. (A = control eggs, B = eggs exposed to autoclaved *P. fluorescens*, C = eggs exposed to autoclaved *Cytophaga* sp., D = eggs exposed to live *P. fluorescens* and E = eggs exposed to live *Cytophaga* sp.)

	A				B			
Days	7	14	21	28	7	14	21	28
Mean cfu's	4.5	89.2	406.4	478.8	9	32.8	706.2	597.6
Egg Deaths	62	110	132	188	41	53	59	106

	C				D			
Days	7	14	21	28	7	14	21	28
Mean cfu's	6.2	24.1	600.3	680.7	28.6	106.5	1316	1075.1
Egg Deaths	44	55	68	105	177	192	212	263

	E			
Days	7	14	21	28
Mean cfu's	109.9	216.7	528	485.1
Egg Deaths	50	117	161	197

APPENDIX 6 : 4 Correlation of egg surface bacterial numbers with egg mortality by multiple regression.

Var.	SS.	D.F.	MS
Reg	64363.0493	2	32181.5246
Error	58696.3907	22	2668.01776
F ratio =		12.0619604	
Multiple R-SQ =		0.523024071	

Var.	COEFF	S.E.	t
INTER.	34.5846346		
1	3.50752364	1.67742518	2.09101645
2	0.005363703	0.0045356976	1.18253851

APPENDIX 7 : 1 Minimum inhibitory concentrations (M.I.C.)

Oxolinic acid

P.fluorescens : mg / litre	80	40	20	10	5	2.5	1.25	0.63
1)	-	-	+	+	+	+	+	+
2)	-	-	-	-	-	+	+	+
3)	-	-	+	+	+	+	+	+
4)	-	-	-	+	+	+	+	+
Cytophaga sp.								
1)	-	-	-	-	-	-	+	+
2)	-	-	-	-	-	+	+	+
3)	-	-	-	-	-	-	+	+
4)	-	-	-	-	-	+	+	+

+ = Bacterial growth

- = No bacterial growth

APPENDIX 7 : 1 (continued) Minimum inhibitory concentrations (M.I.C.)

Furazolidone

P fluorescens : mg / litre	80	40	20	10	5	2.5	1.25	0.63
1)	+	+	+	+	+	+	+	+
2)	+	+	+	+	+	+	+	+
3)	+	+	+	+	+	+	+	+
4)	+	+	+	+	+	+	+	+
Cytophaga sp.								
1)	-	-	-	-	+	+	+	+
2)	-	-	-	-	+	+	+	+
3)	-	-	-	-	-	+	+	+
4)	-	-	-	-	+	+	+	+

+ = Bacterial growth

- = No bacterial growth

APPENDIX 7 : 1 (continued) Minimum inhibitory concentrations (M.I.C.)

Oxytetracycline

P fluorescens : mg / litre	24	12	6	3	1.5	0.75	0.38	0.2
1)	-	-	-	+	+	+	+	+
2)	-	+	+	+	+	+	+	+
3)	-	-	+	+	+	+	+	+
4)	-	+	+	+	+	+	+	+
Cytophaga sp.								
1)	-	-	+	+	+	+	+	+
2)	-	+	+	+	+	+	+	+
3)	-	+	+	+	-	+	+	+
4)	+	+	+	+	+	+	+	+

+ = Bacterial growth

- = No bacterial growth

APPENDIX 7 : 1 (continued) Minimum inhibitory concentrations (M.I.C.)

Chloramphenicol

P fluorescens : mg / litre	32	16	8	4	2	1	0.5	0.25
1)	-	+	+	+	+	+	+	+
2)	-	+	+	+	+	+	+	+
3)	-	-	+	+	+	+	+	+
4)	-	+	+	+	+	+	+	+
Cytophaga sp.								
1)	-	+	+	+	+	+	+	+
2)	-	+	+	+	+	+	+	+
3)	-	-	+	+	+	+	+	+
4)	-	-	+	+	+	+	+	+

+ = Bacterial growth

- = No bacterial growth

APPENDIX 7 : 1 (continued) Minimum inhibitory concentrations (M.I.C.)

Chlortetracycline

P.fluorescens : mg / litre	16	8	4	2	1	0.5	0.25	0.13
1)	-	-	-	+	+	+	+	+
2)	-	-	-	+	+	+	+	+
3)	-	-	-	+	+	+	+	+
4)	-	-	-	-	+	+	+	+
Cytophaga sp.								
1)	-	-	+	+	+	+	+	+
2)	-	-	+	+	+	+	+	+
3)	-	-	+	+	+	+	+	+
4)	-	-	+	+	+	+	+	+

+ = Bacterial growth

- = No bacterial growth

APPENDIX 7 : 1 (continued) Minimum inhibitory concentrations (M.I.C.)

Erythromycin

P.fluorescens : mg / litre	12	6	3	1.5	0.75	0.38	0.19	0.01
1)	+	+	+	+	+	+	+	+
2)	+	+	+	+	+	+	+	+
3)	+	+	+	+	+	+	+	+
4)	+	+	+	+	+	+	+	+
Cytophaga sp.								
1)	+	+	+	+	+	+	+	+
2)	+	+	+	+	+	+	+	+
3)	+	+	+	+	+	+	+	+
4)	+	+	+	+	+	+	+	+

+ = Bacterial growth

- = No bacterial grow

APPENDIX 7 : 1 (continued) Minimum inhibitory concentrations (M.I.C.)

Gentamicin

P.fluorescens : mg / litre	32	16	8	4	2	1	0.5	0.25
1)	-	-	+	+	+	+	+	+
2)	-	-	+	+	+	+	+	+
3)	-	-	-	+	+	+	+	+
4)	-	-	+	+	+	+	+	+
Cytophaga sp.								
1)	-	+	+	+	+	+	+	+
2)	-	+	+	+	+	+	+	+
3)	-	+	+	+	+	+	+	+
4)	-	+	+	+	+	+	+	+

+ = Bacterial growth

- = No bacterial growth

APPENDIX 7 : 1 (continued) Minimum inhibitory concentrations (M.I.C.)

Carbenicillin

P fluorescens : mg / litre	320	160	80	40	20	10	5	2.5
1)	-	-	-	+	+	+	+	+
2)	-	-	-	+	+	+	+	+
3)	-	-	-	+	+	+	+	+
4)	-	-	-	+	+	+	+	+
Cytophaga sp.								
1)	-	+	+	+	+	+	+	+
2)	-	+	+	+	+	+	+	+
3)	-	+	+	+	+	+	+	+
4)	-	+	+	+	+	+	+	+

+ = Bacterial growth

- = No bacterial growth

APPENDIX 7 : 1 (continued) Minimum inhibitory concentrations (M.I.C.)

Polymyxin (P) and Streptomycin (S)

P.fluorescens : mg / litre	160	80	40	20	10	5	2.5	1.25	(P)
	128	64	32	16	8	4	2	1	(S)
1)	+	+	+	+	+	+	+	+	
2)	-	+	+	+	+	+	+	+	
3)	-	+	-	+	+	+	+	+	
4)	-	+	+	+	+	+	+	+	
Cytophaga sp.									
1)	-	+	+	+	+	+	+	+	
2)	+	+	+	+	+	+	+	+	
3)	-	+	+	+	+	+	+	+	
4)	+	+	+	+	+	+	+	+	

+ = Bacterial growth

- = No bacterial growth

APPENDIX 7 : 1 (continued) Minimum inhibitory concentrations (M.I.C.)

Malachite Green

P.fluorescens : ppm	100	50	25	12.5	6.3	3.2	1.6	0.8
1)	+	+	+	+	+	+	+	+
2)	+	+	+	+	+	+	+	+
3)	+	+	+	+	+	+	+	+
4)	+	+	+	+	+	+	+	+
Cytophaga sp.								
1)	-	-	-	-	+	+	+	+
2)	-	-	-	-	+	+	+	+
3)	-	-	-	-	+	+	+	+
4)	-	-	-	+	+	+	+	+

+ = Bacterial growth

- = No bacterial growth

APPENDIX 7 : 1 (continued) Minimum inhibitory concentrations (M.I.C.)

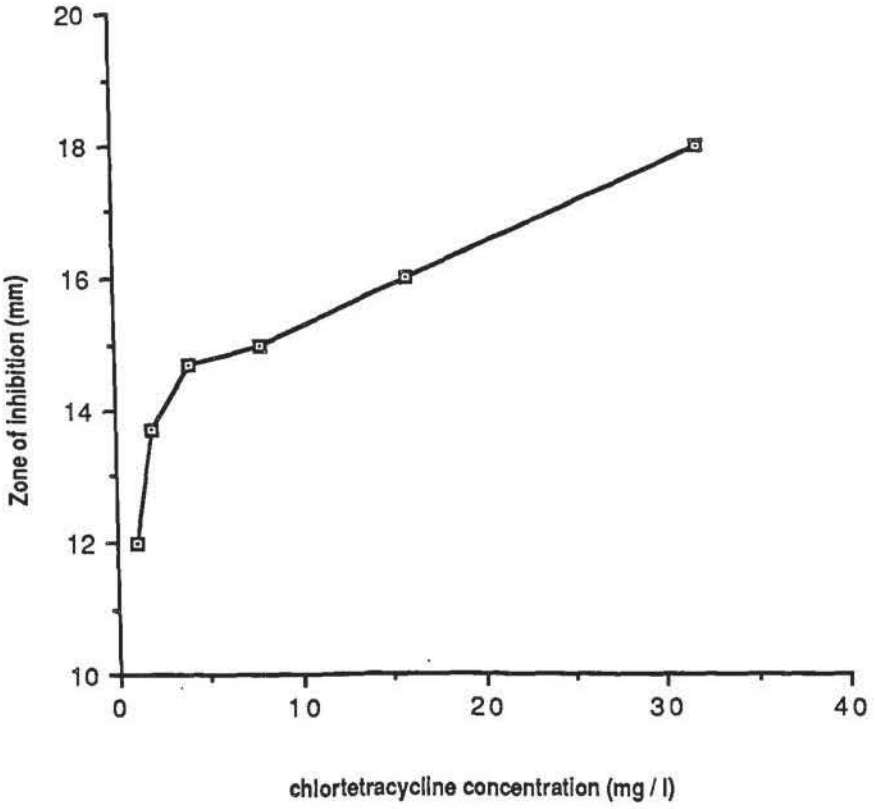
Buffodyne (Iodine disinfectant)

P.fluorescens : mg / litre	500	250	125	63	31	16	8	4
1)	-	-	-	+	+	+	+	+
2)	-	-	-	+	+	+	+	+
3)	-	-	-	+	+	+	+	+
4)	-	-	-	+	+	+	+	+
Cytophaga sp.								
1)	-	-	-	-	-	-	-	-
2)	-	-	-	-	-	-	+	+
3)	-	-	-	-	-	-	-	+
4)	-	-	-	-	-	+	+	+

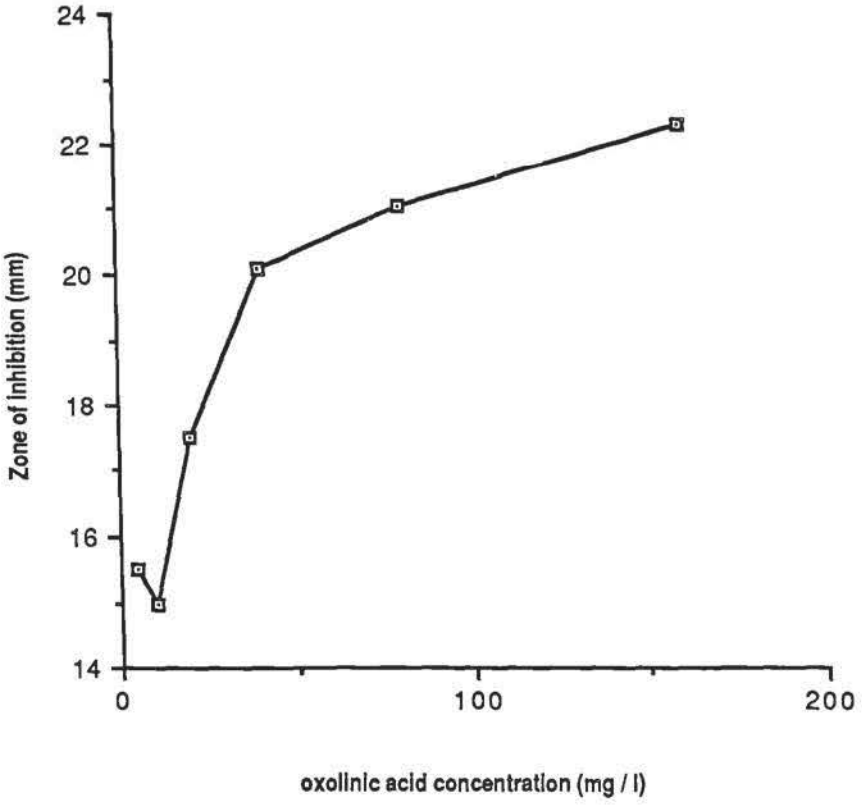
+ = Bacterial growth

- = No bacterial growth

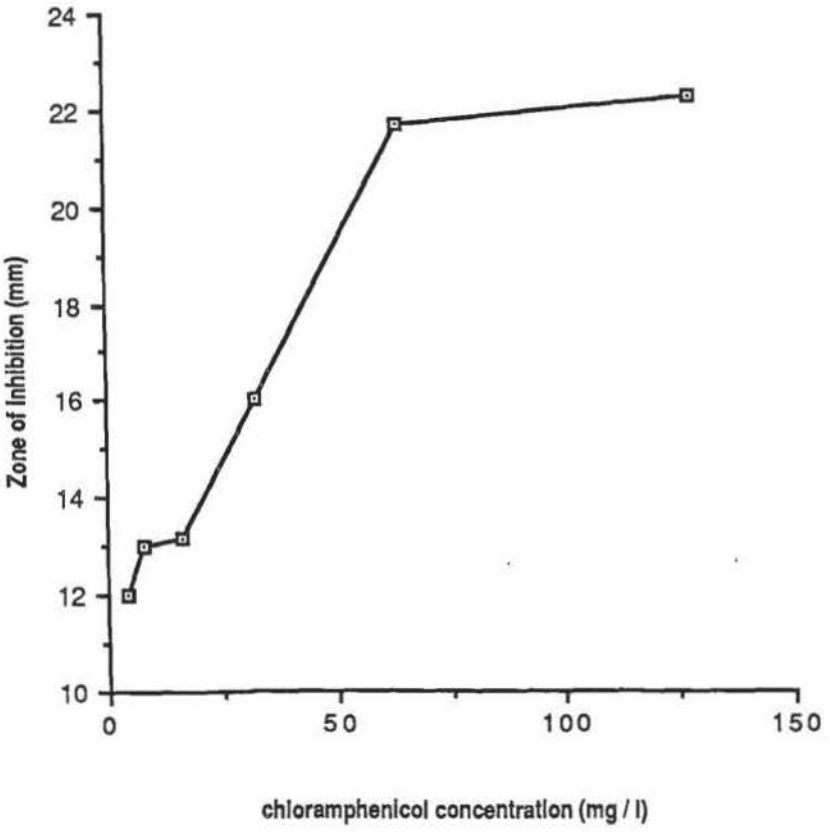
APPENDIX 7 : 2 Standard curve chlortetracycline



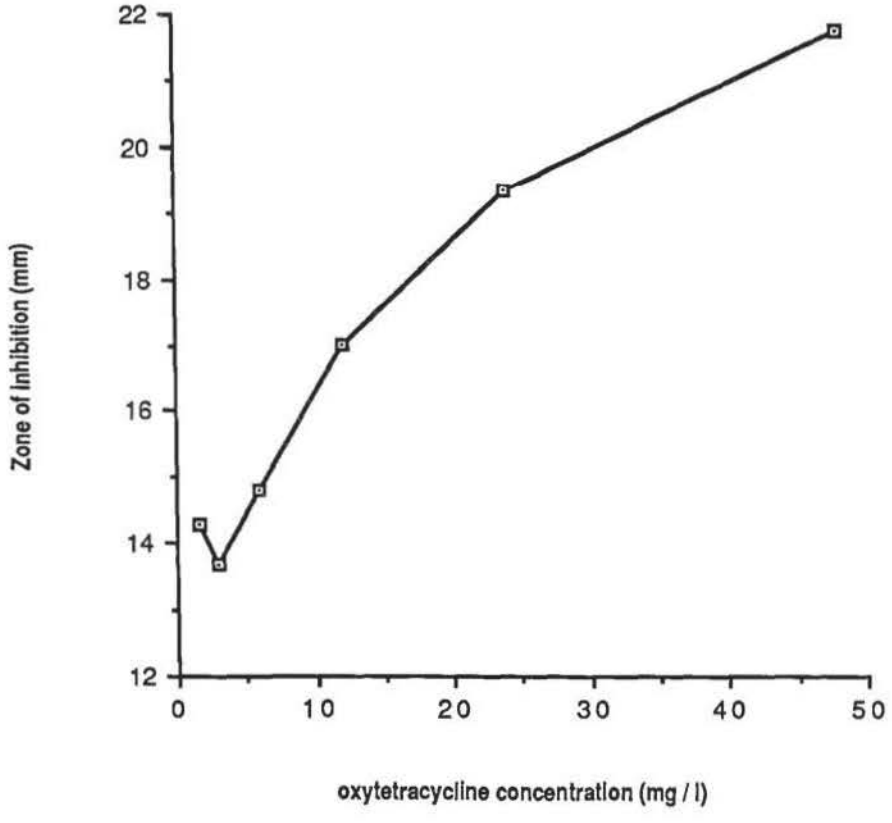
APPENDIX 7 : 2 (continued) Standard curve oxolinic acid



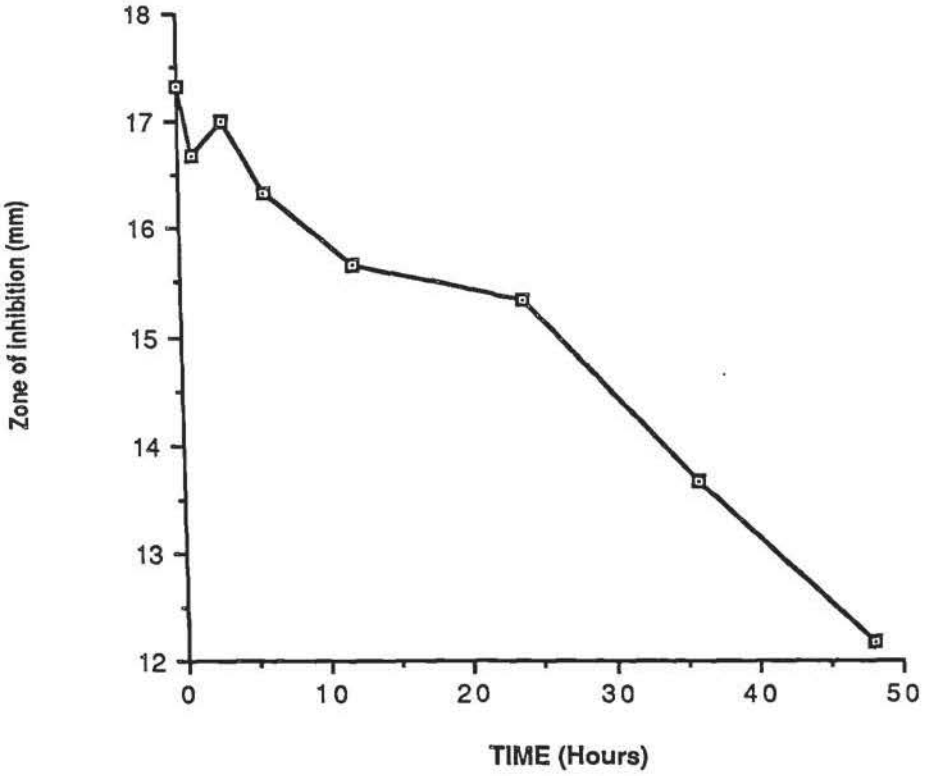
APPENDIX 7 : 2 (continued) Standard curve chloramphenicol



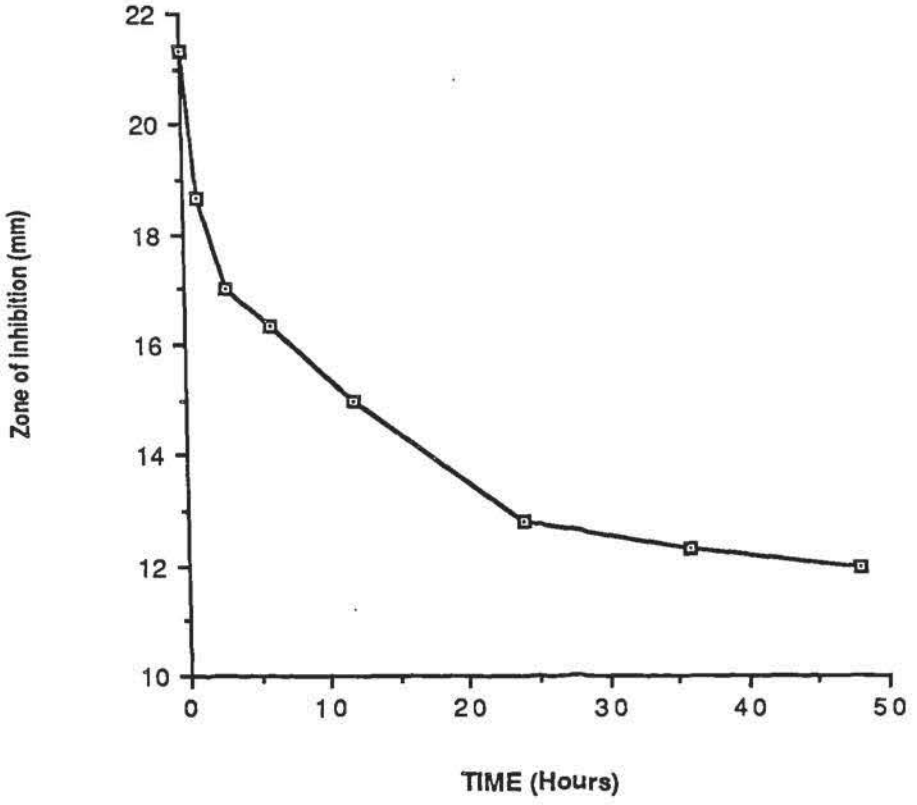
APPENDIX 7 : 2 (continued) Standard curve oxytetracycline



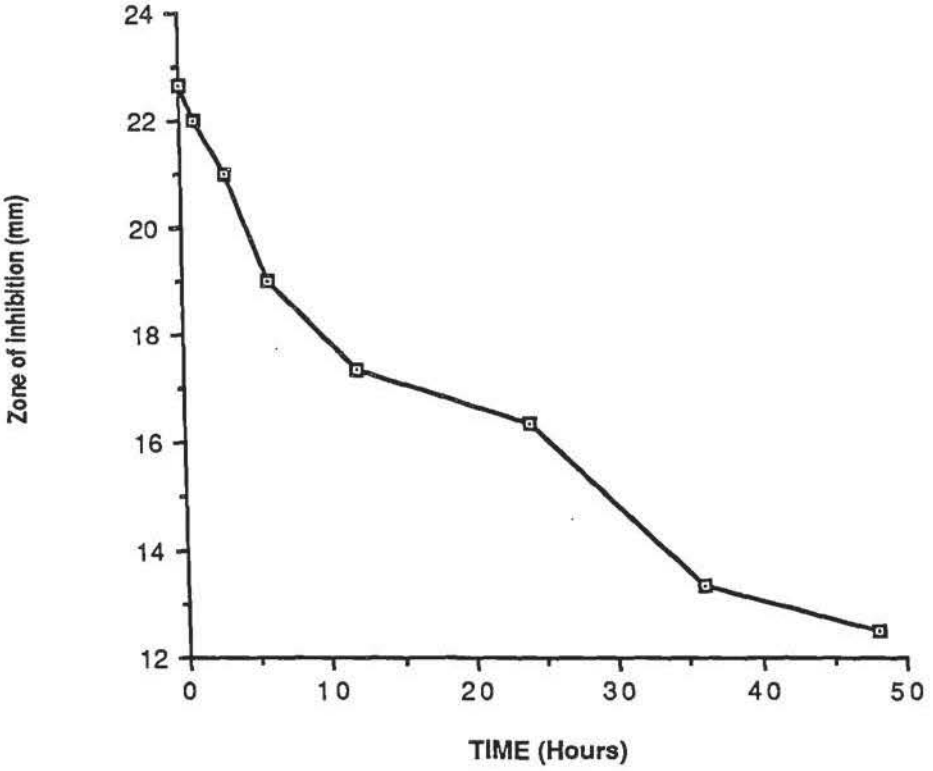
APPENDIX 7 : 3 Deterioration of chlortetracycline in incubation systems (starting concentration : 16 mg/L)



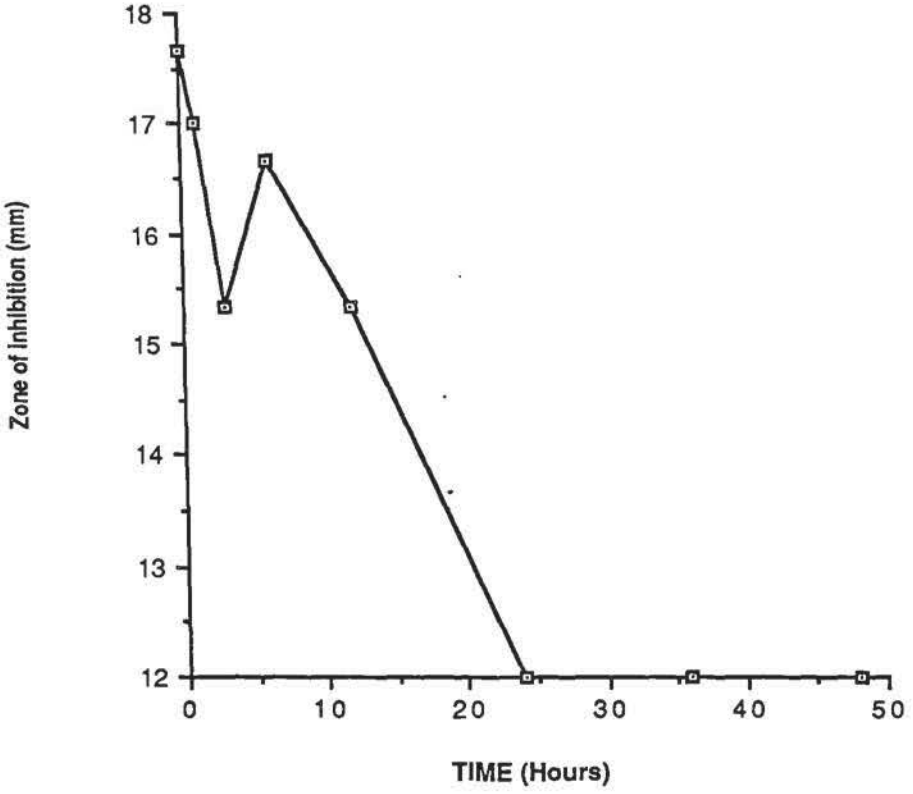
Appendix 7 : 3 (continued) Deterioration of oxolinic acid in incubation systems (starting concentration : 80 mg/L)



APPENDIX 7 : 3 (continued) Deterioration of chloramphenicol in incubation systems (starting concentration : 64 mg/L)



APPENDIX 7 : 3 (continued) Deterioration of oxytetracycline in incubation systems (starting concentration : 24 mg/L)



APPENDIX 7 : 4 Hatching success between egg groups treated with oxolinic acid.

(Egg groups assigned randomly to trays)

	Tray 1 (Control)	Tray 2 (Control)	Tray 3 (x2 M.I.C.)	Tray 4 (x1 M.I.C.)
Number of hatched eggs	55	54	49	58
Number of unhatched eggs	45	46	51	42

Statistical Analysis by χ^2 of hatching success between egg trays ;

Trays	χ^2	d.f.	Significance
1 v 2 v 3 v 4	1.69082126	3	N.S.
1 v 2	0.00201633	1	N.S.
1 v 3	0.721153846	1	N.S.
1 v 4	0.183094294	1	N.S.
2 v 3	0.500450406	1	N.S.
2 v 4	0.324675325	1	N.S.
3 v 4	1.62797709	1	N.S.

APPENDIX 7 : 5 Number of egg deaths between groups at 7 day periods.

Days	Eggs	Tray 1 (Control)	Tray 2 (Control)	Tray 3 (x2 M.I.C.)	Tray 4 (x1 M.I.C.)
1-7	Live	100	100	100	100
	Dead	6	7	4	8
7-14	Live	94	93	96	92
	Dead	23	29	17	14
14-21	Live	77	71	83	86
	Dead	29	36	21	24
21-28	Live	71	64	79	76
	Dead	31	38	31	27

APPENDIX 7 : 5 (continued) Statistical analysis of egg mortality between groups at 7 day periods.

Days	Trays	χ^2	d.f.	Significance
1-7	1 v 2 v 3 v 4	1.32609597	3	N.S.
7-14	1 v 2 v 3 v 4	5.25609045	3	*
14-21	1 v 2 v 3 v 4	6.19941352	3	*
21-28	1 v 2 v 3 v 4	3.37016919	3	N.S.
1-7	1 v 2	0.07229833	1	N.S.
7-14	1 v 2	0.593312447	1	N.S.
14-21	1 v 2	0.992416437	1	N.S.
21-28	1 v 2	1.07310789	1	N.S.
1-7	1 v 3	0.380986937	1	N.S.
7-14	1 v 3	0.851745031	1	N.S.
14-21	1 v 3	1.48608718	1	N.S.
21-28	1 v 3	0.124957813	1	N.S.
1-7	1 v 4	0.267046022	1	N.S.
7-14	1 v 4	1.67216236	1	N.S.
14-21	1 v 4	0.894863435	1	N.S.
21-28	1 v 4	0.441062544	1	N.S.
1-7	2 v 3	0.775684596	1	N.S.
7-14	2 v 3	2.83753487	1	N.S.
14-21	2 v 3	4.8407579	1	*
21-28	2 v 3	1.98451064	1	N.S.

APPENDIX 7 : 5 (continued) Statistical analysis of egg mortality between groups at 7 day periods.

Days	Trays	χ^2	d.f.	Significance
1-7	2 v 4	0.006201685	1	N.S.
7-14	2 v 4	4.13552229	1	*
14-21	2 v 4	3.7923712	1	N.S.
21-28	2 v 4	2.8853005	1	N.S.
1-7	3 v 4	1.25830959	1	N.S.
7-14	3 v 4	0.151839801	1	N.S.
14-21	3 v 4	0.008509703	1	N.S.
21-28	3 v 4	0.103991951	1	N.S.

APPENDIX 7 : 6 Analysis of variance of mean colony forming units (cfu's) / sq mm egg surface with oxolinic acid treatment.

	TRAY 1 (control)				TRAY 2 (control)			
Days :	7	14	21	28	7	14	21	28
1)	154.7	21.6	918.8	62.2	649.4	39.2	1264.2	29.9
2)	114.6	22.5	569.9	180.6	92.3	828.9	89.1	14.7
3)	6.9	56.9	264.5	54.6	155.4	179.0	275.6	552.6
4)	244.4	124.4	63.9	107.1	131.8	8.1	1367.8	302.2
5)	449.0	103.6	393.8	112.0	49.5	12.1	699.4	32.8
6)	76.0	65.6	431.8	255.6	25.0	3.5	388.6	241.8
7)	621.7	136.4	435.2	310.9	162.8	7.8	38.0	877.3
8)	621.7	146.8	225.0	101.2	168.7	95.0	6.9	569.9
9)	93.8	147.8	580.3	183.5	267.7	281.3	42.1	33.7
10)	282.7	435.2	50.1	5.2	55.3	18.4	835.9	95.6

APPENDIX 7 : 6 (continued) Analysis of variance of mean cfu's / sq mm egg surface with oxolinic acid treatment.

	TRAY 3 (x2 M.I.C.)				TRAY 4 (x1 M.I.C.)			
Days :	7	14	21	28	7	14	21	28
1)	4.6	26.3	10.4	17.3	25.9	39.2	253.3	4.6
2)	24.2	287.8	5.2	27.6	42.1	106.4	205.9	14.7
3)	4.3	22.8	635.5	38.7	81.2	19.0	170.7	22.1
4)	46.1	4.6	129.9	47.8	8.6	196.9	112.5	23.5
5)	3.5	25.3	849.7	39.7	74.3	3.5	37.3	269.4
6)	34.5	19.9	6.9	24.2	113.4	7.8	126.7	6.9
7)	3.5	167.8	3.5	6.9	54.6	56.7	414.5	587.2
8)	79.4	322.5	1533.6	70.8	62.8	26.5	716.7	345.4
9)	4.6	153.7	62.8	86.4	362.7	29.9	230.3	442.1
10)	8.6	241.8	96.7	56.4	129.0	36.3	393.8	8.1

APPENDIX 7 : 6 (continued) Analysis of mean cfu's / sq mm egg surface with oxolinic acid treatment by two way split-plot analysis of variance in a randomized design.

Variable (VAR)	Sums of Squares (SS)	Degrees of Freedom (DF)	Mean Square (MS)
Ma. F	564558.426	3	188186.142
MP. Error	1782792.89	36	49522.0248
MI.F	1721138.37	3	573712.788
A X B	397974.246	9	44219.3607
SP Error	7032340.94	108	65114.268

F Ratio (Ma. F) =	3.80004943	***
F Ratio (Mi. F) =	8.81086137	***
F Ratio (A X B) =	0.679104013	N.S.

Ma = major factor = Tray 1 / Tray 2 / Tray 3 / Tray 4

Mi = minor factor = Time

A X B = Interaction of the two factors.

APPENDIX 7 : 7 Correlation of egg surface bacterial numbers with egg mortality by multiple regression (oxolinic acid).

	TRAY 1 (control)				TRAY 2 (control)			
Days	7	14	21	28	7	14	21	28
Mean cfu's	271.5	139.0	378.0	140.6	163.4	199.9	520.4	289.2
Egg Deaths	6	23	29	31	7	29	36	38

	TRAY 3 (x2 M.I.C.)				TRAY 4 (x1 M.I.C.)			
Days	7	14	21	28	7	14	21	28
Mean cfu's	29.1	149.9	388.0	46.5	93.0	65.8	251.1	195.5
Egg deaths	4	17	21	31	8	14	24	27

Var.	SS.	D.F.	MS
Reg	1480.10942	2	740.05471
Error	349.828081	13	26.9098523
F ratio =		27.5012549	
Multiple R-SQ =		0.808830585	

Var.	COEFF	S.E.	t
INTER.	-1.63855157		
1	1.09841218	0.17269386	6.36045881
2	0.00191714868	0.00102578098	1.86896493

APPENDIX 7 : 8 Hatching success between groups treated with chlortetracycline.

(Egg groups assigned randomly to trays)

	Tray 1 (x1 M.I.C)	Tray 2 (Control)	Tray 3 (Control)	Tray 4 (x2 M.I.C.)
Number of hatched eggs	41	39	19	27
Number of unhatched eggs	59	61	81	73

Statistical Analysis by χ^2 of hatching success between egg trays ;

Trays	χ^2	d.f.	Significance
1 v 2 v 3 v 4	14.9692967	3	***
1 v 2	0.008333333	1	N.S.
1 v 3	11.5238095	1	***
1 v 4	4.36720143	1	N.S.
2 v 3	9.71345313	1	**
2 v 4	3.25644505	1	N.S.
3 v 4	1.8068876	1	N.S.

APPENDIX 7 : 9 Number of egg deaths between groups at 7 day periods.

Days	Eggs	Tray 1 (x1 M.I.C.)	Tray 2 (Control)	Tray 3 (Control)	Tray 4 (x2 M.I.C.)
1-7	Live	100	100	100	100
	Dead	1	2	3	0
7-14	Live	99	98	97	100
	Dead	6	10	13	7
14-21	Live	94	90	87	93
	Dead	8	15	33	8
21-28	Live	92	85	67	92
	Dead	11	16	51	18

APPENDIX 7 : 9 (continued) Statistical analysis of egg mortality between groups at 7 day periods.

Days	Trays	χ^2	d.f.	Significance
1-7	1 v 2 v 3 v 4	3.28472588	3	N.S.
7-14	1 v 2 v 3 v 4	3.24770569	3	N.S.
14-21	1 v 2 v 3 v 4	16.5924104	3	***
21-28	1 v 2 v 3 v 4	42.6054494	3	***
1-7	1 v 2	0.328415194	1	N.S.
7-14	1 v 2	0.963013657	1	N.S.
14-21	1 v 2	2.17436975	1	N.S.
21-28	1 v 2	1.18326797	1	N.S.
1-7	1 v 3	0.980486398	1	N.S.
7-14	1 v 3	2.48442012	1	N.S.
14-21	1 v 3	14.1481733	1	***
21-28	1 v 3	28.8520851	1	***
1-7	1 v 4	0.995049505	1	N.S.
7-14	1 v 4	0.006308589	1	N.S.
14-21	1 v 4	0.004214954	1	N.S.
21-28	1 v 4	1.46118635	1	N.S.
1-7	2 v 3	0.195126595	1	N.S.
7-14	2 v 3	0.378115754	1	N.S.
14-21	2 v 3	5.8267441	1	*
21-28	2 v 3	19.2112884	1	***

APPENDIX 7 : 9 (continued) Statistical analysis of egg mortality between groups at 7 day periods.

Days	Trays	χ^2	d.f.	Significance
1-7	2 v 4	1.98039216	1	N.S.
7-14	2 v 4	0.54497441	1	N.S.
14-21	2 v 4	2.10273802	1	N.S.
21-28	2 v 4	0.00106162	1	N.S.
1-7	3 v 4	2.95631068	1	N.S.
7-14	3 v 4	1.80455552	1	N.S.
14-21	3 v 4	13.9132558	1	***
21-28	3 v 4	19.4566786	1	***

APPENDIX 7 : 10 Analysis of variance of mean cfu's / sq mm egg surface with chlortetracycline treatment.

	TRAY 1 (x1 M.I.C.)				TRAY 2 (control)			
Days :	7	14	21	28	7	14	21	28
1)	68.3	165.2	44.9	80.8	416.7	395.1	71.8	251.4
2)	46.2	22.8	35.9	30.5	832.1	11943.3	95.8	1436.8
3)	89.8	359.2	143.7	45.5	471.5	395.1	35.9	2514.4
4)	66.0	134.7	1587.6	89.8	928.8	7902.3	1534.3	7686.8
5)	55.7	24.0	59.9	179.6	188.6	835.1	287.4	10919.5
6)	33.7	18.0	35.9	71.8	628.6	1423.3	1029.7	3376.4
7)	86.7	35.9	251.4	179.6	672.2	942.9	478.9	5567.5
8)	52.4	160.0	215.5	274.8	287.4	4489.9	5603.5	1885.8
9)	89.8	35.9	44.9	583.7	1221.3	8171.7	6537.4	7423.4
10)	73.2	35.9	53.9	395.1	330.5	1885.8	413.1	6824.7

APPENDIX 7 : 10 (continued) Analysis of variance of mean cfu's / sq mm egg surface with chlortetracycline treatment.

	TRAY 3 (Control)				TRAY 4 (x2 M.I.C.)			
Days :	7	14	21	28	7	14	21	28
1)	933.9	2873.6	1293.1	116.7	35.9	35.9	461.8	158.1
2)	1113.5	9069.7	30531.6	263.4	21.6	62.9	256.6	35.9
3)	632.2	201.2	2753.8	161.6	51.7	143.7	1185.4	273.0
4)	449.0	1234.7	8117.8	206.5	21.6	251.4	461.0	215.5
5)	790.2	2563.8	19827.6	826.2	58.4	71.8	143.7	53.9
6)	951.9	2604.2	660.9	89.8	68.3	229.8	251.4	53.9
7)	763.3	871.1	359.2	1041.7	106.6	80.8	116.7	71.8
8)	416.7	9788.1	22629.3	409.5	44.3	143.7	467.0	2514.4
9)	763.3	525.3	484.9	1796.0	18.9	71.8	188.6	1257.2
10)	467.0	1153.9	933.9	688.5	57.9	53.9	222.7	898.0

APPENDIX 7 : 10 (continued) Analysis of variance of mean cfu's / sq mm egg surface with chlortetracycline treatment.

F Ratio (Ma. F) =	6.78598372	***
F Ratio (Mi. F) =	4.09661673	***
F Ratio (A X B) =	5.17107138	***

Ma = Major factor = Tray 1 / Tray 2 / Tray 3 / Tray 4

Mi = Minor factor = Time

A X B = Interaction of the two factors

APPENDIX 7 : 11 Correlation of egg surface bacterial numbers with egg mortality by multiple regression (chlortetracycline).

	TRAY 1 (x1 M.I.C.)				TRAY 2 (Control)			
Days	7	14	21	28	7	14	21	28
Mean cfu's	66.2	132.6	338.1	218.2	627.0	3198.9	2187.1	4915.7
Egg Deaths	1	6	8	11	2	10	15	16

	TRAY 3 (Control)				TRAY 4 (x2 M.I.C.)			
Days	7	14	21	28	7	14	21	28
Mean cfu's	762.1	2444.9	7118.5	645.7	51.5	135.0	432.3	524.6
Egg deaths	3	13	33	51	0	7	8	18

Var.	SS.	D.F.	MS
Reg	1271.35305	2	635.676527
Error	1290.39695	13	99.2613036
F ratio =		6.40407192	
Multiple R-SQ =		0.496283031	

Var.	COEFF	S.E.	t
INTER.	-6.5642601		
1	0.954730926	0.329655712	2.89614556
2	0.0016651778	0.00130625909	1.27476845

APPENDIX 7 : 12 Hatching success between groups treated with chloramphenicol.

(Egg groups assigned randomly to trays)

	Tray 1 (Control)	Tray 2 (x1 M.I.C.)	Tray 3 (x2 M.I.C.)	Tray 4 (Control)
Number of hatched eggs	44	47	40	39
Number of unhatched eggs	56	53	60	61

Statistical Analysis by χ^2 of hatching success between egg trays ;

Trays	χ^2	d.f.	Significance
1 v 2 v 3 v 4	1.67774936	3	N.S.
1 v 2	0.181469906	1	N.S.
1 v 3	0.328407225	1	N.S.
1 v 4	0.514880033	1	N.S.
2 v 3	0.99684671	1	N.S.
2 v 4	1.30558956	1	N.S.
3 v 4	0.000292269	1	N.S.

APPENDIX 7 : 13 Statistical analysis of egg mortality between groups at 7 day periods.

Days	Eggs	Tray 1 (Control)	Tray 2 (x1 M.I.C.)	Tray 3 (x2 M.I.C.)	Tray 4 (Control)
1-7	Live	100	100	100	100
	Dead	8	5	8	14
7-14	Live	87	77	69	69
	Dead	13	23	31	31
14-21	Live	77	72	61	63
	Dead	23	28	39	37
21-28	Live	73	67	60	60
	Dead	27	33	40	40

APPENDIX 7 : 13 (continued) Statistical analysis of egg mortality between groups at 7 day periods.

Days	Trays	χ^2	d.f.	Significance
1-7	1 v 2 v 3 v 4	4.41281776	3	*
7-14	1 v 2 v 3 v 4	11.8394378	3	**
14-21	1 v 2 v 3 v 4	7.87978426	3	**
21-28	1 v 2 v 3 v 4	5.18681319	3	*
1-7	1 v 2	0.65018315	1	N.S.
7-14	1 v 2	3.38753388	1	N.S.
14-21	1 v 2	0.65798131	1	N.S.
21-28	1 v 2	0.85714286	1	N.S.
1-7	1 v 3	0	1	N.S.
7-14	1 v 3	9.44055944	1	**
14-21	1 v 3	5.98410472	1	*
21-28	1 v 3	3.79306475	1	N.S.
1-7	1 v 4	1.47527911	1	N.S.
7-14	1 v 4	9.44055944	1	**
14-21	1 v 4	4.66666667	1	*
21-28	1 v 4	3.79306475	1	N.S.
1-7	2 v 3	0.65018315	1	N.S.
7-14	2 v 3	1.62354135	1	N.S.
14-21	2 v 3	2.71574459	1	N.S.
21-28	2 v 3	1.05705965	1	N.S.

APPENDIX 7 : 13 (continued) Statistical analysis of egg mortality between groups at 7 day periods.

Days	Trays	χ^2	d.f.	Significance
1-7	2 v 4	3.89988128	1	N.S.
7-14	2 v 4	1.62354135	1	N.S.
14-21	2 v 4	1.84615385	1	N.S.
21-28	2 v 4	1.05705965	1	N.S.
1-7	3 v 4	1.47527911	1	N.S.
7-14	3 v 4	0	1	N.S.
14-21	3 v 4	0.00848896	1	N.S.
21-28	3 v 4	0	1	N.S.

APPENDIX 7 : 14 Analysis of variance of mean colony forming units (cfu's) / sq mm egg surface with chloramphenicol treatment.

	TRAY 1 (control)				TRAY 2 (x1 M.I.C.)			
Days :	7	14	21	28	7	14	21	28
1)	690.4	643.9	21613.8	7114.5	18.0	474.3	1368.9	2782.8
2)	10.8	818.2	6844.3	144.1	94.9	2101.4	746.2	2003.8
3)	257.0	215.2	7654.9	351.2	41.1	281.0	11347.3	344.8
4)	57.6	606.4	243.2	126.1	85.3	56.2	7204.6	3782.4
5)	59.4	126.1	492.3	405.3	3.6	842.9	4412.8	553.9
6)	159.2	25.2	3152.0	743.0	100.9	20.4	15219.7	1260.8
7)	27.9	474.3	4502.9	1017.7	69.2	702.5	1038.7	842.0
8)	154.2	677.2	770.9	3692.4	351.2	87.7	7925.1	3692.4
9)	162.1	72.7	2161.4	1232.0	8.4	1193.9	9276.0	1891.2
10)	54.0	612.4	3890.5	655.6	132.4	1621.0	3140.0	438.3

APPENDIX 7 : 14 (continued) Analysis of variance of mean cfu's / sq mm egg surface with chloramphenicol treatment.

	TRAY 3 (x2 M.I.C.)				TRAY 4 (Control)			
Days :	7	14	21	28	7	14	21	28
1)	92.2	2281.5	8105.2	792.5	2161.4	15850.2	6364.1	1013.2
2)	54.0	7805.0	13238.5	522.3	747.5	4773.1	58837.7	690.4
3)	87.2	1298.3	21523.8	36.0	2761.8	16390.5	126.1	840.5
4)	324.2	257.1	819.5	3332.1	1278.8	9366.0	55235.4	894.6
5)	662.8	804.5	7953.2	252.2	1203.2	4593.0	135.1	670.9
6)	864.6	6484.2	5853.8	1044.7	749.3	11167.2	7204.6	591.8
7)	47.6	1260.8	1955.5	726.5	408.3	4923.2	7624.9	880.0
8)	907.8	1531.0	14679.4	1697.6	774.5	11257.2	28818.5	360.2
9)	137.5	2440.6	8195.3	427.8	1561.0	1711.1	2983.9	589.9
10)	75.7	1999.3	13058.4	387.3	621.4	12848.2	33321.3	588.4

APPENDIX 7 : 14 (continued) Analysis of variance of mean cfu's / sq mm egg surface with chloramphenicol treatment.

F Ratio (Ma. F) =	6.32048264	**
F Ratio (Mi. F) =	19.8276343	***
F Ratio (A X B) =	2.65680599	*

Ma = Major factor = 4 trays

Mi = Minor factor = Time

A X B = Interaction of the two factors

APPENDIX 7 : 15 Correlation of egg surface bacterial numbers with egg mortality by multiple regression (chloramphenicol).

	TRAY 1 (Control)				TRAY 2 (x1 M.I.C.)			
Days	7	14	21	28	7	14	21	28
Mean cfu's	191.9	456.8	5014.6	1481.0	98.9	773.5	5152.7	1590.3
Egg Deaths	8	13	23	27	5	23	28	33

	TRAY 3 (x2 M.I.C.)				TRAY 4 (Control)			
Days	7	14	21	28	7	14	21	28
Mean cfu's	316.8	2918.8	7953.2	941.0	1148.5	9309.1	16963.5	716.5
Egg deaths	8	31	39	40	14	31	37	40

Var.	SS.	D.F.	MS
Reg	1722.58591	2	861.292956
Error	467.414088	13	35.9549298
F ratio =		23.9547945	
Multiple R-SQ =		0.78656891	

Var.	COEFF	S.E.	t
INTER.	2.1339133		
1	1.13118433	0.195166066	5.79600929
2	0.0000892755	0.00003431342	2.60176757

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