EFFECT OF YEAST GLUCAN ON IMMUNOSTIMULATION OF CELLULAR NON-SPECIFIC DEFENCES, GROWTH AND SURVIVAL OF ARCTIC CHARR (SALVELINUS ALPINUS L.)

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by

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ABSTRACT

Arctic charr is a promising alternative aquaculture species in Atlantic Canada. Although one of the factors which limit the productivity of this species is microbial diseases, it has been given very little research attention.

The use of vaccines and antibiotics are the two methods available for protecting farmed fish against microbial diseases. However, both methods suffer some drawbacks, leading researchers to search for alternative methods for controlling diseases.

Immunostimulants, a group of biological and synthetic compounds, have been shown to increase non-specific activity in fish. The immunostimulatory effect of β-glucan from the yeast, *Saccharomyces cerevisiae* on non-specific defences, resistance to bacterial pathogen, *Yersinia ruckeri* infection, and growth in Arctic charr, were investigated.

The effect on neutrophils and macrophages was monitored after treatment by injection with either 1 or 10 mg/kg and/or by immersion in 1 or 10mg/L of glucan suspension. Neutrophil activity was evaluated by their ability to stick to glass and staining by Nitro Blue Tetrazolium (NBT). Maximum neutrophil activation was observed 14 days after treatment by immersion in 1mg/L of glucan suspension.

Phagocytic activity, which was assessed by Phagocytic Ratio (PR) and Phagocytic Index (PI), was determined by ingestion of heat treated yeast cells by

Phagocytic activity, which was assessed by Phagocytic Ratio (PR) and Phagocytic Index (PI), was determined by ingestion of heat treated yeast cells by head kidney macrophages from glucan treated fish. Both PR and PI were significantly elevated by immersion in 10mg/L between Days 2-14 as compared to Day 0 and Days 21-35 after treatment.

Effect on disease resistance was monitored after infection of fish by intraperitoneal injection with *Yersinia ruckeri*. Glucan treated fish were challenged on Days 1, 7 or 14 following immersion in 1mg/L of glucan suspension. Treatment on 14 days prior to challenge resulted in higher survival than on Days 1 and 7.

Maximum neutrophil and macrophage activation coincided with the day of maximum survival. This suggests that disease resistance in Arctic charr may be due to neutrophil and macrophage activation, which is stimulated by glucan treatment.

Effect of glucan on the growth of the Arctic charr was also investigated in this study . However, glucan treatment of fish did not show any enhancement or reduction of growth.

This study has demonstrated the potential of yeast glucan as a method for control of microbial diseases in Arctic charr.

A special dedication to my late father

who always encouraged hard

work and perseverance.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND SYMBOLS USED

Alternative Complement Pathway.....ACP

Analysis of Variance.....ANOVA

Bacterial Kidney Disease.....BKD

Colony Forming Units.....CFU

Colony Stimulating Activity .....CSA

Enteric Redmouth.....ERM

Infectious Pancreatic Necrosis.....IPN

Least Significant Difference.....LSD

Nitro Blue Tetrazolium.....NBT

Optical Density.....OD

Phagocytic Index.....PI

Phagocytic Ratio.....PR

Phosphate Buffered Saline.....PBS

Proliferative Kidney Disease.....PKD

Rotations Per Minute.....RPM

Relative Percent Protection.....RPP

Standard Deviation..... SD

Standard Error of Mean.....SEM

Specific Growth Rate.....SGR

Trypticase Soy Agar.....TSA

Trypticase Soy Broth.....TSB

Vita-Stim Taito.....VST

Beta (Greek).....β

Chi (Greek).....χ

CHAPTER 1 - INTRODUCTION

1.1 GENERAL BACKGROUND

The world population is expected to reach close to seven billion by the turn of this century, and estimates suggest that the capture fisheries will continue to stagnate at its current level of about one hundred million tonnes annually. This gives us every reason to believe that aquaculture will play an ever-increasing role in providing resources for which the capture fishery has traditionally been relied upon (Boghen, 1995). As food demand continues to increase, so must production be intensified. Intensive methods of aquaculture production have been therefore adopted to provide a reliable and sufficient supply of fish protein. These methods are characterized by highly mechanized land and water based systems combined with high stocking densities in many culture facilities. Inevitably, this has often caused degradation of water quality due to accumulation of food and metabolic wastes in the environment, coupled with the fast spread of pathogens from host to host (Reichenbach-Klinke, 1973). These two factors have caused wide spread diseases in the aquaculture industry, resulting in devastating losses

in terms of fish, time and money. To avoid such losses, effective methods of controlling infectious diseases have been sought.

There is also stiff competition for quality products within the industry, with only those of highest qualities reaching the international market. For aquaculture to grow, many products need to be developed to increase variety and reduce competition. In Atlantic Canada, fish farming mainly concentrates on three salmonid species: Atlantic salmon (*Salmo salar*), Rainbow trout (*Oncorhynchus mykiss*) and Steelhead trout, a variety of Rainbow trout which can be grown in salt water at some stages of its life-cycle. The high international competition for marketing these species has resulted in the Canadian Atlantic salmon industry receiving lower prices for it's products. Furthermore, one needs only look at the recent decline of the northern Atlantic cod fishery to see what can happen when an industry depends on one species. Introduction of new aquaculture species to the region will add diversity as difficulties of production or marketing of one species will be offset by the success of another. The development of new products and job opportunities will also be beneficial to the economy of a region that has been severely depressed by decline of the capture fisheries (Brown, 1994; Brown et al., 1995). The introduction of species that will adapt well to culture conditions, grow rapidly and tolerate the cold water conditions of the region will expand the potential number of suitable sites for aquaculture and provide avenues for future economic growth.

Arctic charr, *Salvelinus alpinus* is the most northern salmonid and the world's most northerly spawning fresh-water fish. It is well adapted to extreme cold water temperatures and demonstrates better growth at low temperatures than other salmonids. Under culture conditions, juvenile Arctic charr have an advantage over Atlantic salmon at water temperatures below 10°C because they grow three to six times faster than Atlantic salmon of similar age (McGeathy and Delabbio, 1989).

Arctic charr has the added advantage of its ability to achieve high growth performance under high stocking densities in intensive culture conditions, with low densities showing significantly lower growth and slightly higher mortalities than higher ones. This has been associated with reduced antagonistic behavior and stimulation of schooling behavior at high stocking density (Wallace et al., 1988). In comparison to other farmed salmonids, charr have smaller heads relative to their body size, therefore produce a fillet yield of at least 7-8% higher than Rainbow trout or Atlantic salmon (Delabbio, 1995). Fresh, farmed Arctic charr is a new product in the market and is still considered a speciality item, warranting higher prices than other commercialized salmonids. For these reasons, Arctic charr is a suitable alternative candidate for cold water aquaculture.

Although interest in charr culture is high, commercial production is currently low. The reasons for this include biological constraints such as fry survival, poor egg viability and the lack of broodstock development programs (Glebe and Turner,

1993). Research is currently focused on increasing egg viability, nutrition, environmental aspects, fish husbandry and egg rearing.

Disease control is another area that has received little research attention. Prior to development of resistance, Arctic charr are more susceptible to Proliferative Kidney Disease (PKD) than Rainbow trout and Atlantic salmon especially at temperatures above 12 ° C (Brown et al., 1991). The species is also susceptible to other bacterial diseases such as Furunculosis, Vibriosis, Bacterial Kidney Disease (BKD), Infectious Pancreatic Necrosis (IPN), Enteric Redmouth (ERM) disease, and other diseases that infect salmonids especially at sexual maturation (Souter et., 1987; Hjeltnes, 1988; Erikson et., 1993; Groman, 1993). Metacercariae of the digenean, *Cryptocotyle lingua,* have also been reported to infest land-locked Arctic charr in Norway (Kristofferson, 1988). Metazoan parasitic infections (Bouillon, and Dempsons, 1989) and nematode infection of the swimbladder by *Cysticola farionis* (Giaever, 1991) have also been found to occur.

Diseases such as Furunculosis, BKD, IPN, ERM and PKD have been reported to affect freshwater and marine fish in Newfoundland, posing problems for aquaculture (Shaw and Hart, 1990). There is, therefore, a need to develop effective methods for controlling infections in Arctic charr so that production of this potentially viable species may be increased in the region.

1.2 DISEASE CONTROL METHODS

In aquaculture, it is important to take preventive measures to control losses through microbial diseases. This can be achieved by maintenance of good water quality and the use of vaccines. When prevention fails, therapeutic treatment is given. However, extensive use of antibiotics has raised much concern for consumer liability and for the development of antibiotic-resistant strains of bacteria in the aquatic environment, thus making further treatment difficult (Ward, 1982). There is also a need to develop additional methods of disease control since antibiotics are only effective in the treatment of some diseases such as Furunculosis, ERM and Bacterial Septicemias (Anderson, 1992). Furthermore, some antibiotics such as oxytetracyclines have been found to suppress the immune responses in fish (Rijkers et al., 1981).

The use of chemicals for disease control has also raised some environmental concerns. Use of chemicals, including salt (NaCl), results in release of large quantities into the aquatic environment. Use of formalin, which is used against parasitic and fungal infections of salmonid eggs, has been found to be carcinogenic (Reichenbach-Klinke and Landolt, 1973). The success of chemical treatment is usually achieved at levels that result in accumulation of toxic chemicals in the environment. A major disadvantage to the use of both antibiotics and chemicals is that treatment is usually started after the outbreak of disease has

been confirmed, at which time a significant proportion of fish have been infected, thus increasing costs of treatment.

Promotion of disease resistance and maintenance of individually acquired immunity, which depends on production of antibodies, can be stimulated by vaccination (Robertson et al., 1990a). However, this has some disadvantages. Vaccines have been developed for some bacterial diseases such as Hitra disease but not for others of commercial importance such as Bacterial Kidney Disease (BKD) and for viral diseases. In cases where fish are susceptible to more than one disease, multivalent vaccines are usually required. This makes vaccination an expensive exercise. In addition, the method cannot be used when the pathogenic agent is unknown. Therefore, researchers continue to seek other ways for controlling microbial diseases in farmed fish, including Arctic charr.

1.3 DISEASE DEFENCE MECHANISMS IN FISH

In identifying effective methods for disease control in aquaculture, an understanding of the natural defence mechanisms of fish is necessary. While these can be categorized into specific and non-specific factors, it is important to note that *in vivo*, the two are interdependent in many ways and the sum total of defence factors responsible for immunity to a particular disease is a highly

complex interrelationship of specific and non-specific defences (Roberts, 1989). Specific factors, also referred to as acquired or adapted, include T-cells, B-cells and immunoglobulins, and are characterized by an ability to acquire memory and specificity towards a pathogen (Engstad, 1994). They require adaptive changes in the lymphoid system and form the second line of defence (Roberts, 1989).

Non-specific defences include surface barriers, a variety of humoral defense mechanisms and cellular aspects such as phagocytic cells which include macrophages, monocytes, neutrophils and cytotoxic cells (Robertson et al., 1990; Anderson et al., 1992; Engstad 1994). They lack the ability to acquire memory and specificity towards pathogens and form the first line of defence since they are part of the normal body constituents (Ward, 1982). Due to the complex nature of antibody synthesis combined with dependence on temperature, specific defences take longer to develop (Anderson and Jeney, 1992). Furthermore, antibodies have a restricted diversity and lack the ability to mature in fish (Wilson and Warr, 1992). Non-specific defences are therefore relatively more important to fish compared to higher vertebrates, especially for short-lived cool or cold water fish.

1.3.1 Surface barriers and humoral factors

The skin and gills of fish are covered by a mucous layer which contains lytic enzymes that are toxic to certain micro-organisms. They also have epithelial cells which are highly responsive to chemical or physical damage; resulting in thickening of the cuticle, or hyperplasia. Mucous membranes lining the gastrointestinal tract act as a barrier which combine with the digestive enzymes and low pH of the gut, to provide an unsuitable environment for potential pathogens (Roberts, 1989).

Non-specific humoral factors consist of proteins found in body fluids, whose protective role is achieved either by reacting with microbes and microbial products to inhibit their growth, or by neutralizing enzymes on which the pathogens depend. They include enzyme inhibitors found in tissue fluids and blood serum, growth inhibitors, lysins, precipitins and agglutinins (Anderson, 1992).

1.3.2 Cellular factors

In fish, cellular responses consist of phagocytes and non-specific cytotoxic cells. Phagocytes include mononuclear cells and granulocytes, the former

consisting of monocytes and macrophages. Monocytes are found in the blood and kidney where they form the precursors of macrophages. Macrophages are mononucleated tissue cells with undulating membranes and are highly phagocytic or pinocytic, and have adherence abilities to glass and plastic surfaces. Some fish macrophages, known as melanomacrophages, contain melanosomes and are believed to have a bactericidal role (Ellis, 1976; 1977). Monocytes have the ability to migrate from the blood to inflammatory sites where they give rise to macrophages, which are phagocytic for foreign substances such as yeast (Pulsford et al., 1994; Yuwaraj et al., 1994), sheep red blood cells (Sovenyi and Kusuda, 1987; Anderson and Jeney, 1992), and bacteria (Kitao and Yoshida, 1986).

Fish granulocytes include neutrophils, eosinophils and basophils. Neutrophils are associated with defence of fish from fungal and bacterial infections (Wood et al., 1986; Lamas and Ellis, 1994). A Criterion for provisional identification of the neutrophils are the presence of a grey cytoplasm in cells stained with Wright's stain, a nucleus with 2-5 lobes and peroxidase positive cytoplasmic granules. They derive their name from the inability of their granules to stain markedly with either acid or basic dyes (Finn and Nielson 1971). Although the major granulopoietic organ is the kidney, the spleen may also play a role. Anderson et al. (1992) reported phagocytic ability, diapedesis and oxidative radical production

of neutrophils. Phagocytosis can be detected by the engulfment of particles. Diapedesis, or active migration from the circulatory system towards an injury site, can be assayed by the cells' ability to adhere to glass. Oxidative radical production, which is directed at the destruction of invaders, can be detected by chemiluminescence or Nitro Blue Tetrazolium (NBT) staining.

The glass adherent ability of activated circulatory neutrophils combined with their production of reactive oxygen species (ROS) such as superoxide anion, (O_2^-), hydrogen peroxide (H_2O_2), activated or singlet oxygen (O^-), and free hydroxyl ions (OH^-) can be monitored by the glass adherent Nitro Blue Tetrazolium (NBT) staining method as described by Anderson et al. (1992).

1.4 IMMUNOSTIMULANTS

Unlike vaccines which function in controlling disease by triggering production of specific antibodies towards particular organisms, a group of biological and synthetic compounds have been found to enhance the non-specific defence mechanisms in fish and other animals by their immuno-stimulatory action. These compounds are known as immunostimulants. They provide an important method, which may be the answer to the future of disease control in aquaculture.

An immunostimulant has been defined as a chemical, drug, stressor, or action that elevates the non-specific defence mechanisms or the specific immune response. By themselves, immunostimulants activate nonspecific defenses. They can also be administered with a vaccine to elevate specific immune responses. They are given by injection, immersion or orally in feed components. Immunostimulants have sometimes been classified as biological response modifiers as they cause immuno-modulation by stimulation or suppression (Anderson, 1992).

Immunostimulants for fish have been obtained from various biological and synthetic sources. Important biological sources include bacteria (*Alcaligenes faecalis; A. levanicum*), yeast (*Saccharomyces cerevisiae*) and filamentous fungi (*Sclerotium glucanicum, Schizophyllum commune*) (Yano et al., 1991). Synthetic

immunostimulants of importance in fish include the synthetic peptide, FK-565 (hepatonoyl-y-D-glutamyl-(L) mesodiamiopimelyl-(D)-alanine), ISK (a short-chain polypeptide) derived from fish products, CFA (Complete Freund's Adjuvant) a paraffin oil and killed tubercle bacilli, QAC (Quaternary Ammonium Compounds), levamisole (levo-isomer of tetramisole), and glucans (Anderson, 1992).

1.5 YEAST GLUCANS

These are insoluble, long chain polysaccharides, which form important structural components in the cell wall of most yeasts and fungi. Their ability to stimulate non-specific defences in fish has been investigated by several authors including, Robertsen et al. (1990), Yano et al. (1991), Engstad et al. (1992), Jorgensen et al. (1993), Jeney and Anderson (1993), and Brattgjerd et al. (1994).

Glucans are extracted from whole pressed cells of bakers yeast by a series of processes that involve removal of mannan, and precipitation and removal of glycogen. Structural analysis by methylation, periodate oxidation and enzymolysis has shown them to have a branched structure made of (1⌧6)-linked β-glucose residues to which are attached linear side chains of (1⌧3)-linked β-glucose

residues (Figure 1). The (1⌫3)-linked side chains are a major component, forming about 85% of the linkages (Duffus et al., 1982).

Glucan is known to stimulate defence mechanisms in vertebrates and invertebrates. Fish have developed a mechanism for recognition of glucan, which has evolved as a mechanism for defence against fungal pathogens (Onarheim, 1992). Robertsen et al. (1990a) have demonstrated enhanced resistance to infection by *Vibrio salmonicida*, *Vibrio anguillarium* and *Yersinia ruckeri* in glucan injected Atlantic salmon. Bactericidal activity by macrophages of glucan-treated Rainbow trout has also been demonstrated (Jorgensen et al., 1993b). Nikl et al. (1992; 1993) have shown its ability to improve effectiveness of furunculosis vaccines while Engstad et al. (1992) have demonstrated it's ability to stimulate an increase in lysozyme and complement-mediated haemolytic activity. Stimulatory effects of glucan have been attributed to its activation on the phenoloxidase (proPO) system in shrimp (*Penaeus monodon*), which represents the terminal components of a complex cascade of enzymes that function in non-self recognition and host defence (Sung et al., 1994).

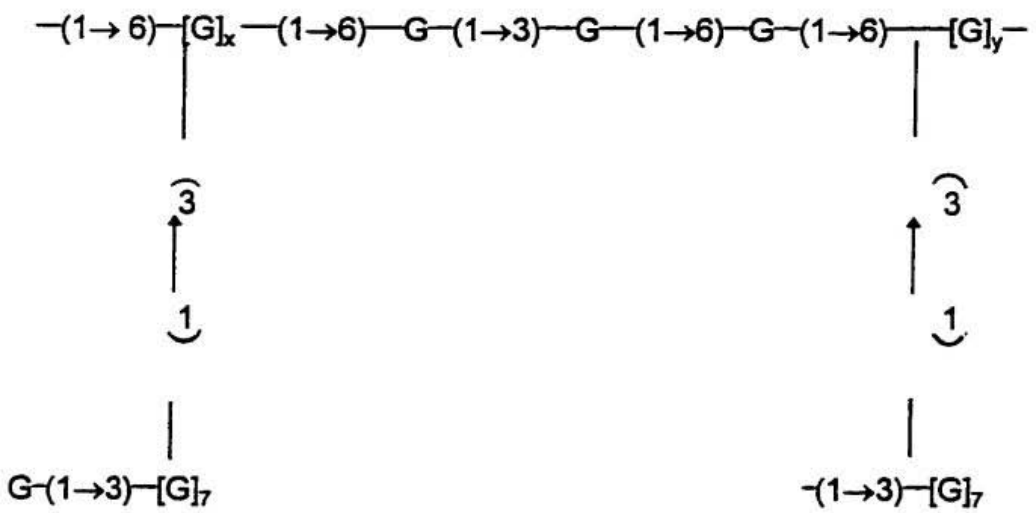

Figure 1. Partial Structure of yeast glucan. G denotes a β-D-glucopyranose residue, and x + y = 40-50 (adapted from Duffus et al., 1982).

Fish macrophages are able to recognize β-1,3- glucan structures in yeast cell walls either directly through a specific receptor, or indirectly by binding with complement. The branched structure of glucan is of importance in eliciting stimulatory effects in macrophages which have receptors that can recognize the side chains of glucan molecules. This has been demonstrated by the low stimulatory effects of glucans with few or no side chains (Engstad and Robertsen, (1993).

Glucan has also been shown to stimulate growth. The effect on growth is based on the assumption that glucan treated fish are healthier and thus grow faster. However, Matsuo and Miyazono (1993) found that peptidoglycan, a type of glucan derived from cell walls of gram-negative bacteria, enhances resistance of juvenile Rainbow trout against *Vibrio anguillarium* infection, but does not influence growth. It has nonetheless been reported to be effective in enhancing growth of shrimp, whose mechanism for better growth was attributed to higher disease resistance (Sung et al., 1994).

Much of the research work done on immuno-stimulation by glucan in fish has focused on it's ability to increase protection from microbial infection. Rainbow trout and Atlantic salmon fed with diets containing glucan have shown reduced losses from disease (Onarheim and Roberts, 1992). Commercialization of β-glucans is currently in the form of feed supplements (Lall and Olivier, 1995). Stimulation of

immune response by oral administration is associated with the presence of lymphoid tissue in the gastrointestinal tract, which processes β-glucan particles using the mucosal immune system's (MIS) phagocyte system. Trials with diets containing β-glucans have shown increased non-specific activity such as number of lymphocytes and phagocytic activity in macrophages of Channel catfish (*Ictalurus punctatus*) (Duncan and Klesius, 1996).

Studies with fish have shown that glucan improves the efficiency of vaccines compared to the vaccine alone. The survival of Atlantic salmon (*Salmo salar*) challenged with large numbers of pathogens of diseases such as Hitra, Vibriosis and ERM disease has been shown to improve after glucan treatment (Mitchell, 1992).

1.6 RATIONALE AND AIM OF STUDY

To meet the current demand for aquaculture products, the Canadian aquaculture industry needs to diversify its product lines and to reduce losses in culture facilities. Arctic charr has been found to be well adapted to the cold waters of the region, demonstrating better growth than Rainbow trout or Atlantic salmon at water temperatures below 10 ° C (McGeathy and Dellabio, 1989; Wallace, 1990). It performs well under high stocking densities (Delabbio, 1995), has higher

yields, and fetches higher prices than other salmonids. It is, therefore, a promising candidate for culture and global production of char will most likely continue to increase.

Although interest in culture of Arctic charr is high, commercial production is low. This is attributed to biological constraints such as poor fry survival and egg viability and lack of broodstock development programs (Glebe and Turner, 1993). Another potential setback to production is the financial loss due to infectious diseases. Disease control has received little attention even though all diseases common to other salmonids also occur in Arctic charr (Vieira, 1989).

The aim of this study was to investigate the effect of an immunostimulant, Macrogard®, a β-1,3/1,6 -glucan, which is derived from the cell walls of a yeast, *Saccharomyces cerevisiae*, on non-specific defences, growth and survival in Arctic charr. Survival was determined by challenge with the bacterial pathogen, *Yersinia ruckeri*, the aetiogical agent for ERM disease, which has been partly responsible for financial loss suffered by the aquaculture industry (Newman and Majnarich, 1992).

CHAPTER 2 - MATERIALS AND METHODS

2.1 FISH

Juvenile Arctic charr, weighing approximately 95g, were obtained from the Arctic Charr Hatchery, Daniel's Harbour, Newfoundland, Canada. They were maintained in a flow through system in $1m^3$ circular fiberglass tanks at the Marine Institute of Memorial University. Water flow was regulated at a rate of 2L per minute. The water was aerated with supplemental oxygen and its temperature maintained at $12 \pm 1^{\circ}C$. Fish were held at a stocking density of 60 kg/m^3 and were fed to satiation twice a day on Alpine feed (Moore Clarke Co. Canada Inc.) unless otherwise stated.

2.2 GLUCAN

Macrogard®, the β-glucan (2.5%) used in this study, was provided through the courtesy of Biotec Mackzymal, Trømso, Norway. Glucan was prepared from the cell walls of yeast, *Saccharomyces cerevisiae* (2.5%) and preserved in formalin

(0.2%). For preparation of required doses, it was diluted with Phosphate Buffered Saline (PBS, pH 7.4).

2.3 ADMINISTRATION OF GLUCAN

Glucan was administered by injection and immersion methods. Feeding was withheld a day before and after each time fish were treated with glucan. Fish were anaesthetized with MS-222, Finquel (100mg/L) before injection with glucan.

Fish were treated with glucan by either injection or immersion. For injection of glucan, the ventral surface of the fish was swabbed with 70% alcohol prior to treatment. Two groups of fish were injected with 2ml of either 1 or 10 mg/kg glucan suspension. A third group was injected with 2ml PBS (0 mg/kg) and served as the injection control. For immersions, two groups of fish were removed from their respective tanks and placed into buckets containing 1 or 10mg/L glucan suspension supplied with supplemental oxygen and held for 30 minutes. A third group was immersed in fresh system water (0 mg/L) supplied with supplemental oxygen for 30 minutes and served as the immersion control. A fresh glucan immersion was prepared for every group of ten fish. Fish were held in recovery buckets containing 12°C system water and supplied with supplemental oxygen for thirty minutes before being returned to their respective tanks.

2.4 NITRO BLUE TETRAZOLIUM (NBT) ASSAY

Five fish from each glucan treated and non-treated control group were randomly sampled for the presence of glass-adherent, NBT-positive neutrophil cells. The fish were sacrificed by using MS-222 (200mg/L) one day prior to treatment (Day 0) and 14 and 28 days after treatment with glucan thereafter. Approximately 0.1 ml of blood was withdrawn from the caudal peduncle of each fish into a syringe containing lithium salt heparin from porcine intestinal mucosa (Sigma Chemical Company, St. Louis), and immediately placed on two glass cover slips. The cover slips were placed in 60 mm petri dishes lined with wet paper towels to maintain humidity (Figure 2). They were incubated for 30 minutes at room temperature (22 ± 1°C). The cover slips were washed gently with PBS then transferred upside down to a microscope slide containing a drop of 0.2% filtered NBT solution (2-2'-Di-p-nitrophenyl-5-5'-diphenyl-3,3'-[3, 3'- dimethoxy-4,4'-diphenylene] ditetrazolium chloride), $C_{40}H_{30}Cl_2N_{10}O_6$, (Sigma chemicals Co., St. Louis, USA). After 30 minutes, the number of glass-adherent neutrophils were determined from three random fields for each slide.

2.5 PREPARATION OF YEAST CELLS

Yeast cells , for the phagocytic assay, were prepared by the method adopted from Namaware et al. (1994). Twenty grams of bakers yeast (*S. cerevisiae*) was mixed with 20 ml 0.15 M PBS and inactivated by heating in a water bath at 80 ° C for 15 minutes. The cells were washed three times in 5 ml PBS by centrifuging at 15, 300 × g for 10 minutes (Sorvall centrifuge, Du Pont instruments, USA). The supernatant was decanted, the yeast cell pellet resuspended in PBS and then mixed using a vortex mixer (Fisher Scientific, Fair Haven, New Jersey). Cells were counted on a Naubauer improved haemocytometer (Dynatech, West Germany) and diluted to 1.2×10^6 cells/ml with PBS. After the final supernatant was decanted, cells were resuspended in sterile L-15 medium (Sigma Co.) containing 4% fetal calf serum, 10 units/ml of heparin lithium salt from porcine intestine mucosa (Sigma Chemical Company, St. Louis) and 100units /ml lyophilized, γ-irradiated and sterile penicillin-streptomycin (Sigma Chemical Company, St. Louis, USA).

2.6 PHAGOCYTIC ASSAY

Five fish from each group were sacrificed by immersion in 200mg/L of MS-222 (Finquel) on Days 0, 2, 7, 14, 21, 28, and 35. About 2 cm of the posterior portion of the kidney was excised and placed in a petri dish containing L-15 complete medium with 2% fetal calf serum, 100 units/ml of penicillin/streptomycin and 10 units/ml heparin. Macrophages were isolated by the method described by Narnaware et al. (1994). Kidney samples from each treatment were pooled as it was not technically practical to perform macrophage activation assays on individual fish due to the time-consuming nature of the assays. Samples were pressed through a 100μm wire mesh with 4 ml of complete L-15 medium to make a pronephric cell suspension. One ml of the kidney cell suspension from each treatment was flooded onto individual pre-washed microscope slides and phagocytic cells allowed to adhere for 60 minutes at room temperature (22 ± 1°C).

Non-adherent cells were washed off with PBS and the attached macrophages were flooded with 1 ml of yeast cells in L-15 medium containing 4% fetal calf serum. Sixty minutes later, the slides were rinsed with PBS. Cells were stained with Wrights stain modified (0.3 % w/v, pH 6.9) (Sigma Diagnostics, St. Louis, USA) then examined under oil immersion. Phagocytic activity of macrophages

was determined by Phagocytic Index (PI) and Phagocytic Ratio (PR) (Yano et al., 1989).

For each slide, two hundred macrophages were examined to determine the PI as:

$$PI = \frac{\text{no. of macrophages ingesting yeast cells}}{200} \times \frac{\text{no. of yeast cells ingested}}{200} \times 100$$

From the examined cells, the percentage of macrophages which engulfed one or more yeast cells was determined to give percent (%) phagocytosis, also referred to as Phagocytic Ratio (PR) as:

$$PR = \frac{\text{no. of macrophages ingesting at least one yeast cell}}{200} \times 100$$

2.7 GROWTH STUDIES

Fish were held at a stocking density of 60 kg/m^3 . Individual fish were identified by using Carling tags. Wet weight(g) measurements were taken using an electronic balance (A & D company, Tokyo, Japan) one day prior to treatment

with glucan (Day 0) and on 30, 56, and 78 days thereafter. Length measurements were taken using a measuring board on the first and last days of weight measurements. Fish were fed two times a day on Alpine feed (Moore Clarke Co. Canada Inc.) at a 2% body weight ration per day. Specific Growth Rate (SGR) was determined according to Efthimiou (1996):

$$\text{Specific Growth Rate} = \frac{\ln \text{final weight (g)} - \ln \text{initial weight (g)}}{\text{time (days)}} \times 100$$

2.8 CHALLENGE WITH BACTERIA

2.8.1 Culture of bacteria, confirmation of identity and virulence

Yersinia ruckeri, originally isolated during the summer of 1992 from a dead salmon at Conne River, Bay d'Espoir, were obtained from the Department of Fisheries and Oceans (DFO), St. John's, Newfoundland, Canada.

The pathogen was maintained on Trypticase Soy Agar (TSA) then characterized by using conventional biochemical methods. Virulence of the pathogen was enhanced by intraperitoneal (IP) injection of 10^5 to 10^6 CFU of viable bacteria into each of *x* fish. The bacteria were re-isolated on TSA from

infected fish and similarity with the original isolate was established biochemically by the API 20E® system (BioMérieux, France). The cultures were maintained on TSA slants under sterile mineral oil for use as the starting inoculum for all studies with the pathogen.

2.8.2. Determination of growth characteristics of *Yersinia ruckeri*

A loopful of *Y. ruckeri* was thawed then aseptically transferred from a vial to Trypticase Soy Broth (TSB) and incubated at 20 ± 1 ° C for 24 hours. One tenth of a ml of this culture was used to inoculate a 250 ml Erlenmeyer flask containing 100 ml of sterile TSB. The flask was incubated at 20 ± 1 ° C with gentle agitation on a gyrotory shaker (Scientific Industries, NY, USA). Samples were aseptically removed from the flask for determination of cell concentration. The first sample of 1ml was removed thirty minutes after inoculation and every thirty minutes thereafter until the onset of the stationary phase of growth. Absorbence of bacterial suspensions was measured by a photoelectric colorimeter (Klett Manufacturing Incorporated., USA) using the TSB as the reference solution for calculation of Optical Density (OD) of the culture as:

O.D = colorimeter reading x 0.02

Optical density values were used to determine the logarithmic phase of growth of *Y. ruckeri.* Bacteria from the mid-log phase of growth was used for infecting fish in the challenge experiment (section 2.8.5).

2.8.3 Determination of concentration of *Yersinia ruckeri* for challenge experiment

Cultures of *Y. ruckeri* were harvested at the mid-log phase of growth (section 2.8.2) by centrifugation at 15,300 × g and 10,000 rotations per minute (Du Pont Instruments, USA). This was done at 4 ° C for 20 minutes. The supernatant medium was decanted and autoclaved before it was discarded. The bacterial pellet was resuspended by manual agitation in sterile distilled water. Ten-fold dilutions of the suspended bacteria were prepared in sterile disposable screw cap test-tubes with sterile distilled water.

One ml of each of the 10^7 and 10^8 bacterial suspensions was pipetted onto triplicate, TSA plates. Twenty ml of molten (45-48°C) agar was poured onto the suspensions and agitated to ensure uniform mixing of the suspension and molten agar. After solidification of the agar, the petri plates were inverted, labeled and incubated at room temperature (21 ± 1°C) for approximately 48 hours. Plates

having between 30-300 colonies were counted using a dark-field colony counter (American Optical Company, Buffalo, N.Y.). The concentration was calculated from the number of colonies counted, which corresponds to the number of Colony Forming Units per ml (CFU/ml) of bacterial suspension and the dilution as:

$$CFU/ml = Dilution \times number\ of\ colonies\ counted$$

2.8.4 Determination of route and dose of bacteria for artificial infection

A preliminary experiment was performed to determine the appropriate concentration of bacteria and route of administration for challenge (section 2.8.5). A randomized design of six groups each with eight fish was set up. Two groups were exposed to 2.4×10^{7} and 2.4×10^{8} CFU/ml immersion and four groups were injected with 2.4×10^{6}, 2.4×10^{7}, 2.4×10^{8}, and 2.4×10^{9} CFU of bacteria in 0.1 ml of 0.85% NaCl. Before injection with bacteria, fish were anaesthetized with 100mg/L MS-222 then swabbed with 70% alcohol on the ventral surface. Fish were moved into the experimental facility two weeks before the study to permit recovery from handling and to allow acclimation to the new environment. Feeding was withheld during the 24 hour period immediately prior to infection.

Bacterial suspensions for the preliminary challenge study was prepared as follows: *Y. ruckeri* previously isolated from Arctic charr artificially infected by intraperitoneal injection was re-plated from TSA slants onto TSA plates for 24 hours. A well isolated colony from the plate was aseptically inoculated into a test tube containing 10 ml of TSB and incubated at room temperature (20 ± 1°C) for 8 hours with gentle agitation on a shaker (Scientific Industries, NY). One tenth of a ml of the culture was aseptically inoculated into a flask containing 100 ml sterile TSB and incubated as above. The culture was transferred to a 250 ml sterile centrifuge bottle and centrifuged at 5000 x g at 10 ± 1°C for 30 minutes. The supernatant medium was decanted, autoclaved and discarded.

The pellet was resuspended in 0.1 ml of 0.85 % saline and mixed gently on a shaker to make a bacterial suspension of 2.4×10^{10} CFU in 0.1 ml of injectable bacterial suspension as obtained in section 2.8.3, then serially diluted to make bacterial suspensions for infection by the injection route. The same procedure was followed for infection by immersion, however, the pellet was resuspended in 100 ml saline, the original volume of the culture solution and diluted ten-fold to give two bacterial concentrations for infection by immersion.

Water temperature was gradually raised from 10 to 15°C before infection with bacteria. Each immersion bucket had 4950 ml of vigorously aerated flow-through system water to which 50 ml of the bacterial suspension was introduced to make

the required final concentration. Fish were introduced into respective buckets, which were supplied with supplemental oxygen for thirty minutes, with buckets covered to reduce excitation and stress. Clinical signs of oxygen inadequacy and other stressors were checked periodically. Before being returned to their respective tanks, fish were held in recovery buckets containing fresh system water and supplied with supplemental oxygen.

Observations were made twice daily. Mortalities were removed and recorded daily. External and internal signs of infection and other possible causes of death were recorded. Bacterial pathogen was isolated from dead fish and identified using API 20E biochemical system (Biomerieux France).

2.8.5 Challenge of Arctic charr with *Yersinia ruckeri*

Challenge experiments were carried out at the Marine Institute's quarantine facility. Four-hundred and twenty fish of 30-40 g average weight were maintained at 10°C in 0.5 m^3 fiberglass tanks. Temperatures were raised to 15°C before bacterial infection. Fish were randomly distributed into six groups of 70, each representing a treatment or control group for challenge on Days 1, 14 and 21 after glucan treatment. Treatment was done by immersion in 1mg/L of glucan

suspension for thirty minutes, which was found to be most effective in activating neutrophils (sections 2.4 and 3.11).

Fish were injected with 2.4×10^7 CFU/ml of *Y. ruckeri*, which was the most effective for artificial infection (sections 2.8.4 and 3.3.4). Mortalities were recorded on a daily basis and isolates from kidneys obtained for pathogen identification (section 2.8.1) to verify the cause of death. Resistance to infection was reported as Relative Percent Protection (RPP), which was calculated by the following method adopted from Robertsen et al. (1990):

$$RPP = \left[1 - \frac{(\%\ \text{mortality in glucan treated group})}{(\%\ \text{mortality in control group})} \right] \times 100\%$$

2.9 STATISTICAL ANALYSIS

Mean and Standard Error of Mean (SEM) were calculated for activated neutrophils. For PI and PR, means were calculated for each group. Statistical analysis of data was performed using one way Analysis of Variance (ANOVA) for determination of significant differences between control and experimental groups ($P<0.05$). The Least Significant Difference (LSD) method of multiple comparisons was used to identify responses that were significantly different. Mortalities in

different groups of fish infected with bacteria were compared by Chi-square (χ^2) tests ($P<0.05$) (Zar, 1996). All data was analyzed with the of Microsoft© Office Excel statistical program Version 5.0.

CHAPTER 3 - RESULTS

3.1 EFFECT OF GLUCAN ON NON-SPECIFIC CELLULAR RESPONSES

3.1.1 Neutrophil activation

Activated neutrophils were identified according to descriptions by Schreck and Moyle (1990) by the presence of a greyish-blue cytoplasm and presence of many small granules which stained dark-blue with NBT with 0.2% NBT stain in PBS. Nuclei were frequently bilobed and eccentrically positioned, with a spherical or horse-shoe shape. They were relatively large, forming about 30-50 % of the total cell size. The shape of the cells varied from spherical to ovoid and sometimes showed pseudopodia.

It was observed that immersion in 1 and 10mg/L of glucan resulted in neutrophil activation ($F=12.91$, $df=5$, $P<0.05$) 14 days following treatment. (Figure 3 and Table). However, among the groups treated by injection, only the group that received 10 mg/kg resulted in significant activation of circulatory

neutrophils (Figure 4 and Table 1). This indicates that immersion is more effective than injection as a route of glucan administration in Arctic charr.

Neutrophil activation on Days 1 and 28 after glucan injection or immersion was not significant ($P>0.05$). Overall, the stimulatory effect of glucan on neutrophils was highest on Day 14 following immersion in 1 mg/L (Table 1 and Figure 3).

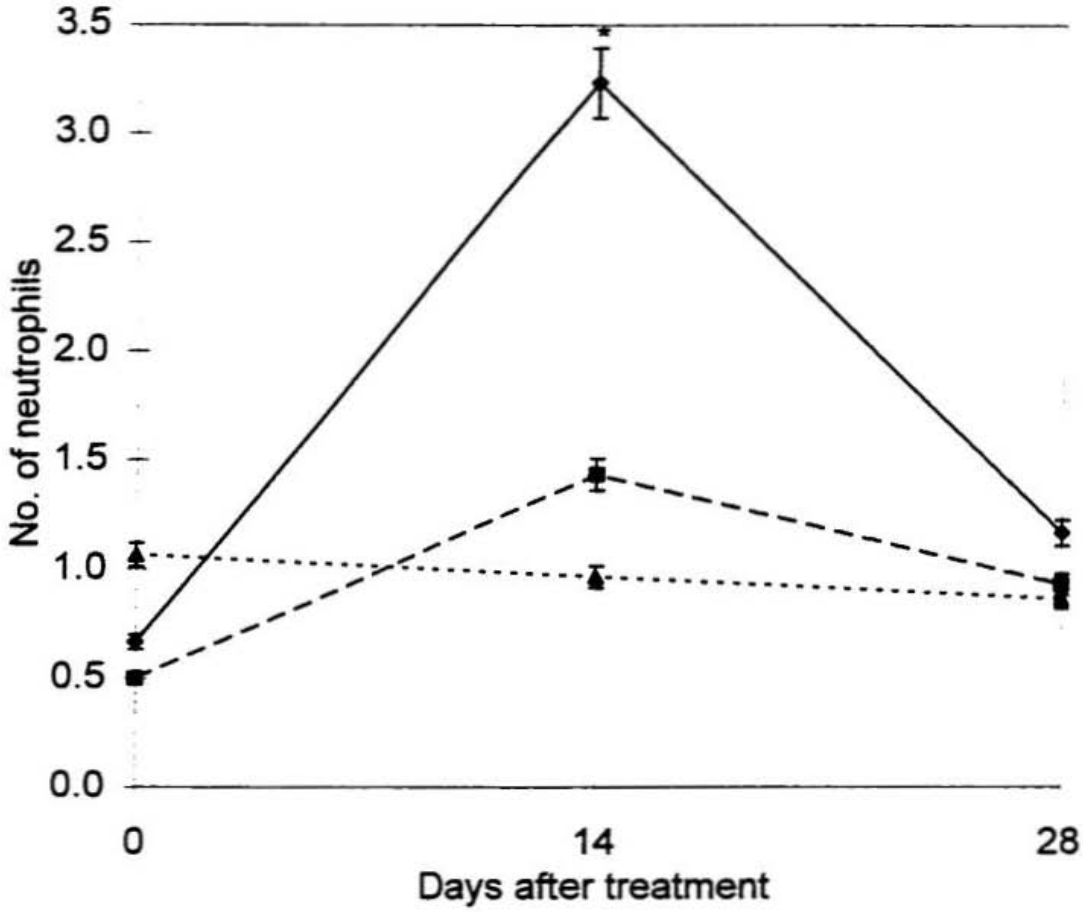

Figure 3. Activated neutrophils in Arctic charr (*S. alpinus*) at 0, 14 and 28 days after treatment by immersion in 0 mg/L (·····◆·····), 1 mg/L (——◆——) or 10mg/L (---■-----) glucan suspension. Results are expressed as means. Vertical bars are standard error of mean. N=3 for each group.

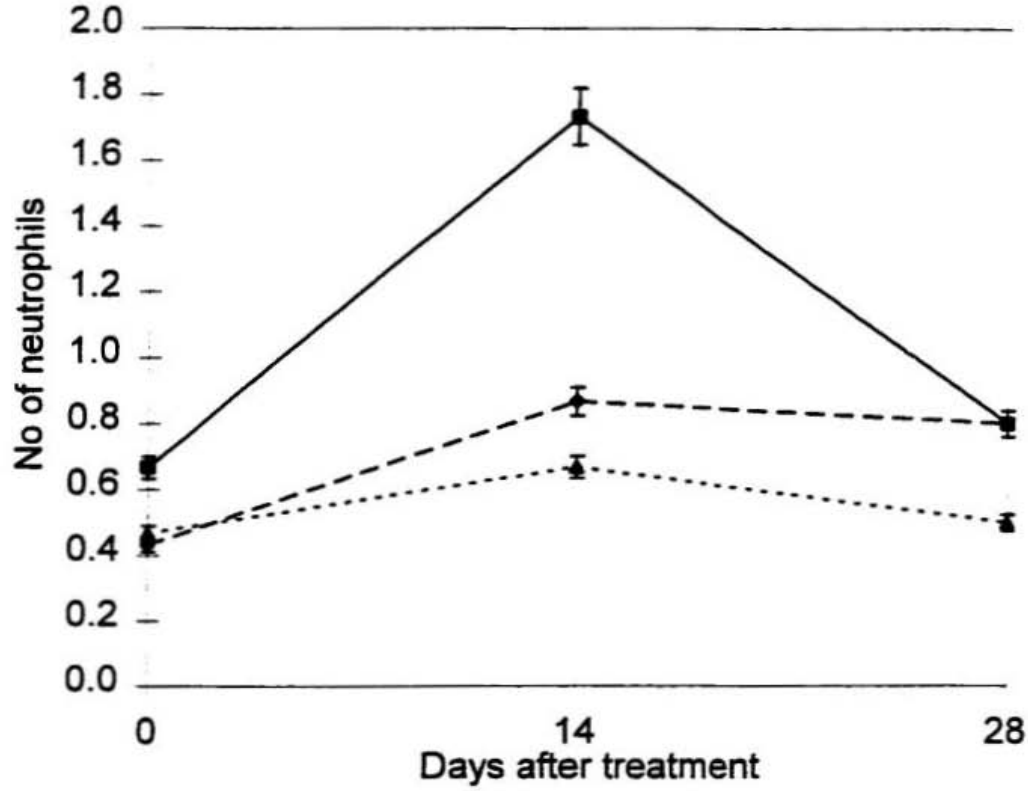

Figure 4. Activated neutrophils in Arctic charr (*S. alpinus*) at 0, 14 and 28 days after treatment by injection with 0 mg/kg (·····•···), 1 mg/kg(----◆---) or 10mg/kg ←—■—) glucan suspension. Results are expressed as means. Vertical bars are standard error of mean. N=3 for each group.

Table 1. Activated neutrophils in blood of glucan treated Arctic charr (*S. alpinus*). Means and standard deviation are indicated. Significant activation is indicated by * ($P < 0.05$).

Method of administration	Dose	Days after treatment		
		0	14	28
Injection	0mg/kg	0.47 ± 0.97	0.67 ± 0.07	0.50 ± 0.19
	1mg/kg	0.43 ± 0.10	0.87 ± 0.19	0.80 ± 0.36
	10mg/kg	0.67 ± 0.16	1.73 ± 0.16	0.80 ± 0.36
Immersion	0mg/L	1.07 ± 0.25	0.97 ± 0.10	0.87 ± 0.23
	1mg/L	0.67 ± 0.12	3.23 ± 0.50*	1.13 ± 0.31
	10mg/L	0.50 ± 0.19	1.43 ± 0.28	0.87 ± 0.06

3.1.2 Phagocytic activity by macrophages

Phagocytic activity by macrophages isolated from Arctic charr was expressed as Phagocytic Ratio (PR) and Phagocytic Index (PI). Both macrophages and neutrophils were observed under the microscope, but for purposes of this study, only macrophages were counted.

Macrophages were identified by the possession of numerous membrane bound cytoplasmic granules which stain dark blue with NBT and have many vacuoles and pinocytic vesicles. They also have nuclei which were often eccentric and varied from horse-shoe to brain shaped. The cytoplasm is greenish-blue, and characterized by many colourless cytoplasmic vacuoles possibly containing accumulations of ingested material. The cells have irregular membranes and vary in size and shape from oval to spherical (Schreck and Moyle, 1990).

Phagocytized yeast cells were quantified by washing the slides with PBS to remove non-phagocytized yeast cells and then counting the remaining yeast cells.

There was no activation prior to glucan treatment (Day 0) and on Days 21-35 after treatment. PR was significantly increased between 2-14 days by

immersion in 10mg/L (ANOVA, $F=53.91$, $P<0.05$). There was no increase in PR by other treatments (Figure 5, Table 2).

When measured by PI, macrophage activity was significantly higher (ANOVA, $F=154.98$, $P<0.05$) during the 2-14 days in the groups treated with 10 mg/L of glucan by immersion. PI for other groups was not significantly increased during this period) (Figure 6, Table 3).

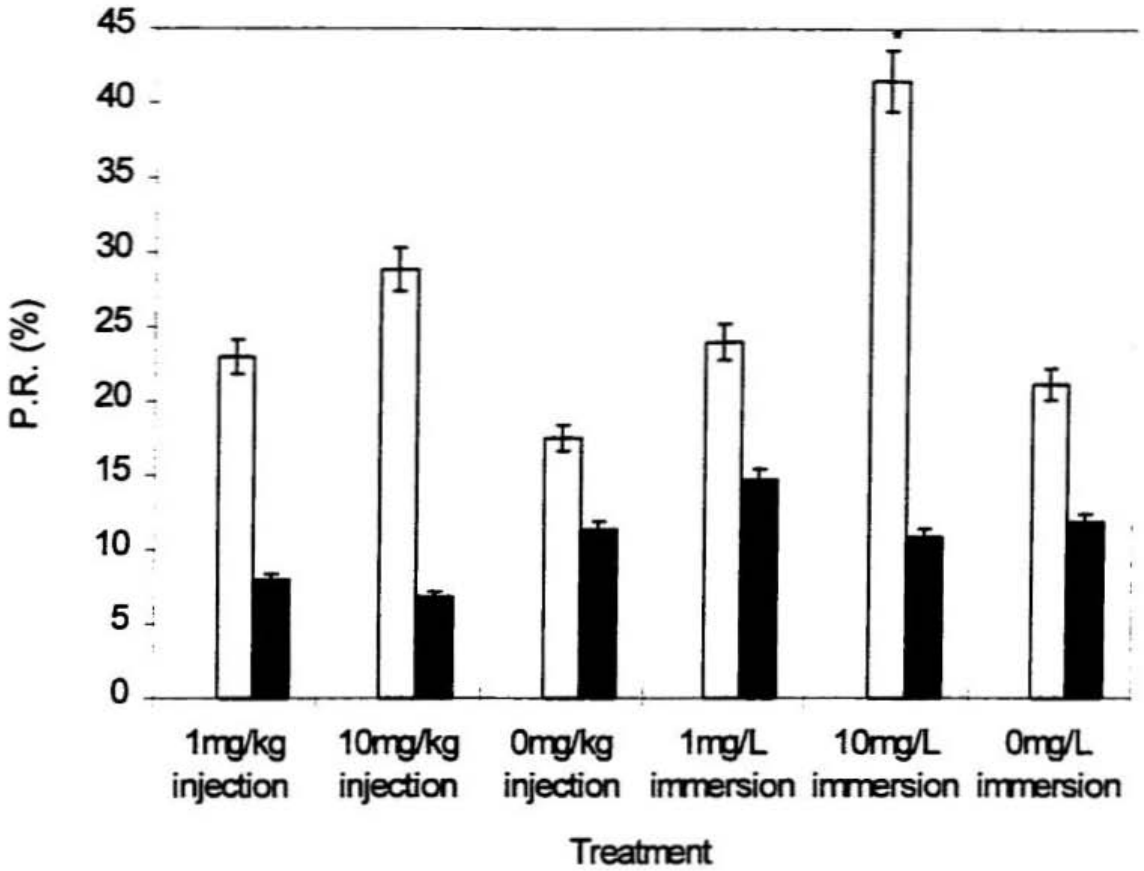

Figure 5. Phagocytic Ratio (PR) of head kidney macrophages isolated from glucan treated Arctic charr (*S. alpinus*) on Days 2-14 (□) or Days 21-35 (□) after glucan treatment. * indicates significantly activated macrophages ($P < 0.05$).

Table 2. Phagocytic Ratio (PR) of macrophages isolated from glucan treated Arctic charr (*S. alpinus*). Phagocytic assays were performed on Days 0, 2, 7, 14, 21, 28 and 35 after glucan treatment. Samples for Days 2-14 and 21-35 were pooled. * indicates significantly activated macrophages ($P < 0.05$).

Method of administration	Dose	Days after treatment		
		0	2-14	21-35
Injection	0mg/kg	0.00	17.50	11.33
	1mg/kg	0.00	23.00	6.83
	10mg/kg	0.00	28.83	6.83
Immersion	0mg/L	0.00	21.17	11.83
	1mg/L	0.00	24.00	14.67
	10mg/L	0.00	41.50*	10.83

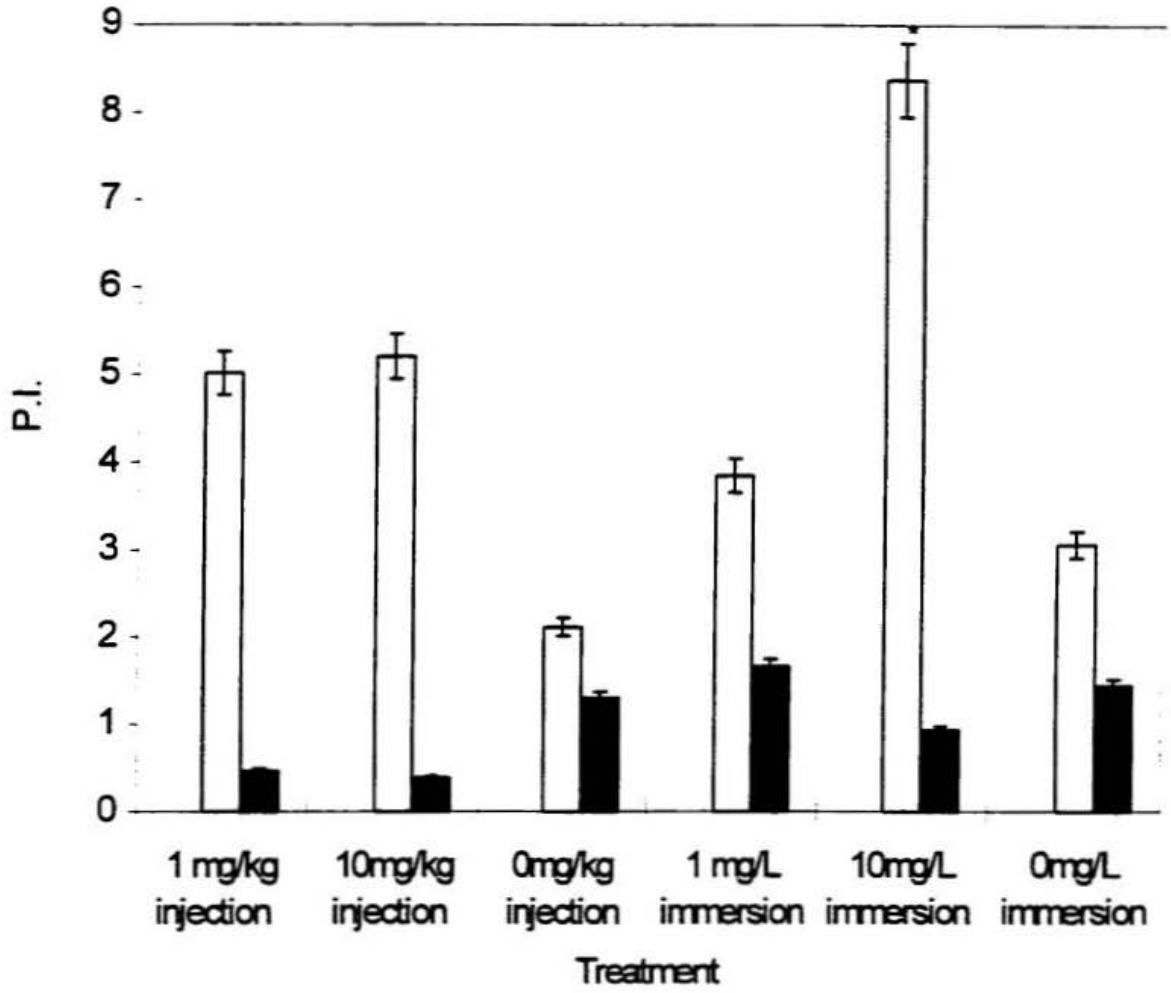

Figure 6. Phagocytic index of head kidney macrophages isolated from glucan treated Arctic charr (*S. alpinus*) on Days 2-14 (□) or Days 21-35 (□) after glucan treatment. * indicates significantly activated macrophages ($P < 0.05$).

Table 3. Phagocytic Index (PI) of macrophages isolated from glucan treated Arctic charr (*S. alpinus*). Phagocytic assays were performed on Days 0, 2, 7, 14, 21, 28 and 35 after glucan treatment. Samples for Days 2-14 and 21-35 were pooled. * indicates significantly activated macrophages ($P < 0.05$).

Method of administration	Dose	Days after treatment		
		0	2-14	21-35
Injection	0mg/kg	0.00	2.12	1.31
	1 mg/kg	0.00	5.02	0.47
	10mg/kg	0.00	5.20*	0.39
Immersion	0mg/L	0.00	3.05	1.44
	1mg/L	0.00	3.84	1.66
	10mg/L	0.00	8.37*	0.93

3.2 EFFECT OF GLUCAN ON GROWTH

All treatment groups exhibited positive growth throughout the trial as shown by weight measurements (Figure 7 and 8; Table 4) and specific growth rate (SGR) (Table 5). There was no statistical difference observed in growth between glucan treated and non-treated fish.

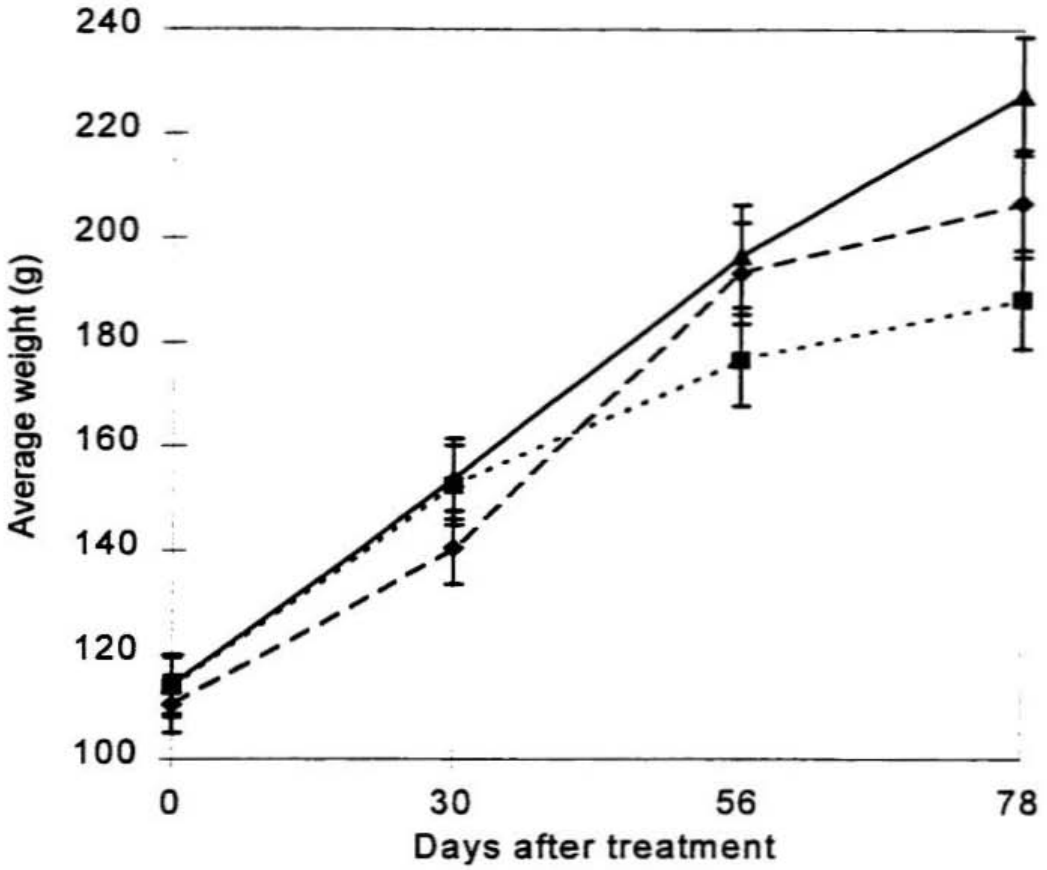

Figure 7. Changes in weight (g) at various times in Arctic charr (*S. Alpinus*) following treatment by immersion in glucan at 0mg/L (—•—), 1mg/L (----◆----) or 10mg/L (····■····). Vertical bars are standard error of mean. N=10 for each group.

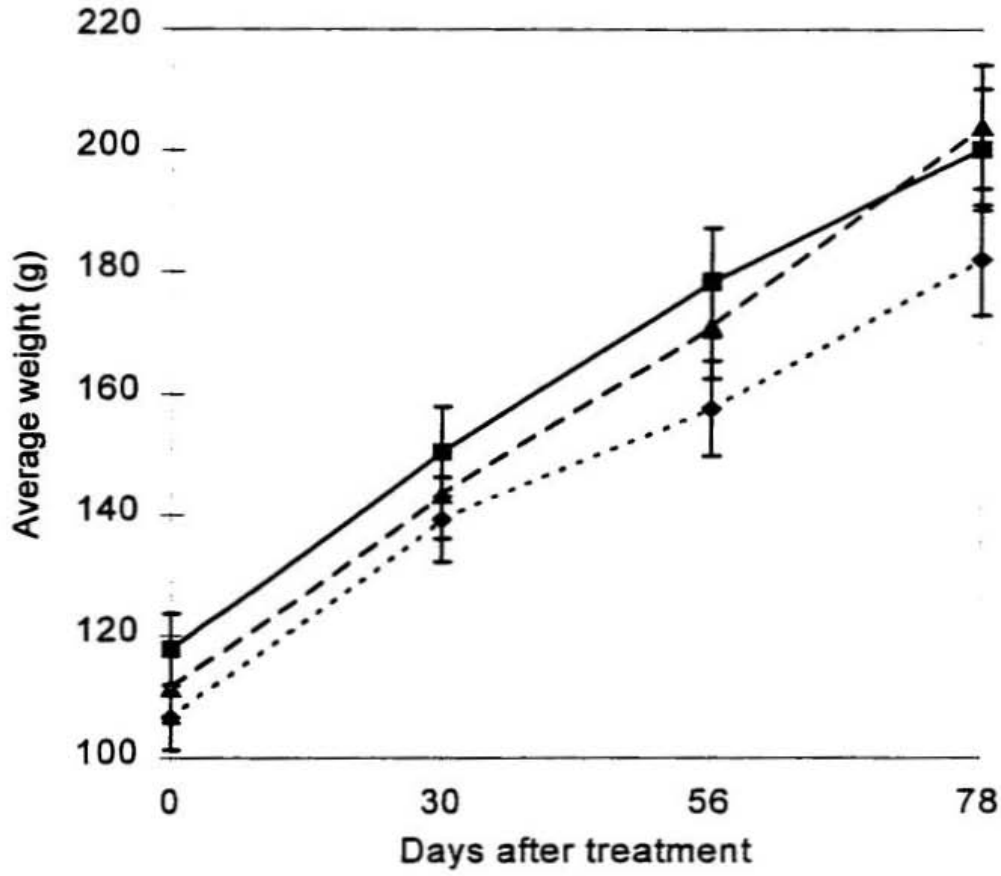

Figure 8. Changes in weight (g) at various times in Arctic charr (*S. alpinus*) following treatment by injection with glucan at 0mg/kg (♦-----), 1mg/kg (◆·····) or 10mg/kg (—■—). Vertical bars are standard error of mean. N=10 for each group.

Table 4. Changes in weight and length of Arctic charr following treatment by glucan injection with 1 and 10 mg/kg or immersion in 1 and 10 mg/L. * indicates measurements taken from only one fish due to loss of identification tags.

Method of administration	Dose	Average weight (g)				Average length (cm)	
		Day 0	Day 30	Day 56	Day 78	Day 0	Day 78
Injection	0mg/kg	111.5 ± 12.93	143.2 ± 13.88	171.0 ± 25.64	203.9 ±18.36	19.8 ± 0.95	23.1 ± 1.25
	1mg/kg	106.7 ± 13.77	139.2 ± 21.67	157.5 *	181.9 *	19.8 ± 1.01	22.5 *
	10mg/kg	120.7 ± 11.67	150.4 ± 38.22	178.2 ± 29.67	200.2 ± 5.06	20.5 ± 0.69	23.8 ± 1.57
Immersion	0mg/L	114.4 ± 17.14	153.6 ± 28.44	196.4 ± 42.62	227.4 ± 7.77	19.7 ± 1.46	23.6 ± 2.25
	1mg/L	110.6 ±17.37	140.5 ± 30.50	193.1 ± 30.69	206.6 +28.28	19.6 ± 0.90	23.3 ± 1.06
	10mg/L	114.0 ± 15.22	152.5 ± 18.50	176.5 ± 26.65	188.2 ± 7.14	19.9 ± 0.94	23.5 ± 1.32

Table 5. Specific growth rates of Arctic charr following treatment by glucan injection with 1 and 10 mg/kg or immersion in 1 and 10 mg/L.

Method of administration	Dose	Specific growth rate (SGR)		
		Day 30	Day 56	Day 78
Injection	0mg/kg	0.84	0.69	0.80
	1mg/kg	0.89	0.48	0.65
	10mg/kg	0.73	0.65	0.33
Immersion	0mg/L	0.98	0.95	0.66
	1mg/L	0.80	1.22	0.31
	10mg/L	0.97	0.56	0.29

3.3 EFFECT OF GLUCAN ON DISEASE RESISTANCE

3.3.1 Growth characteristics of *Yersinia ruckeri*

Y. ruckeri exhibited logarithmic growth between 6-12 hours after inoculation into fresh medium (Figure 9). Therefore, bacterial cultures at nine hours after inoculation were used for challenge.

3.3.2 Concentration of bacterial suspension

Cultures of *Y. ruckeri* in the mid phase of growth were harvested and diluted to determine concentration. Bacterial concentration was 2.4×10^{10} Colony Forming Units (CFU)/ml. This was diluted to make concentrations for dose determination in challenge experiments.

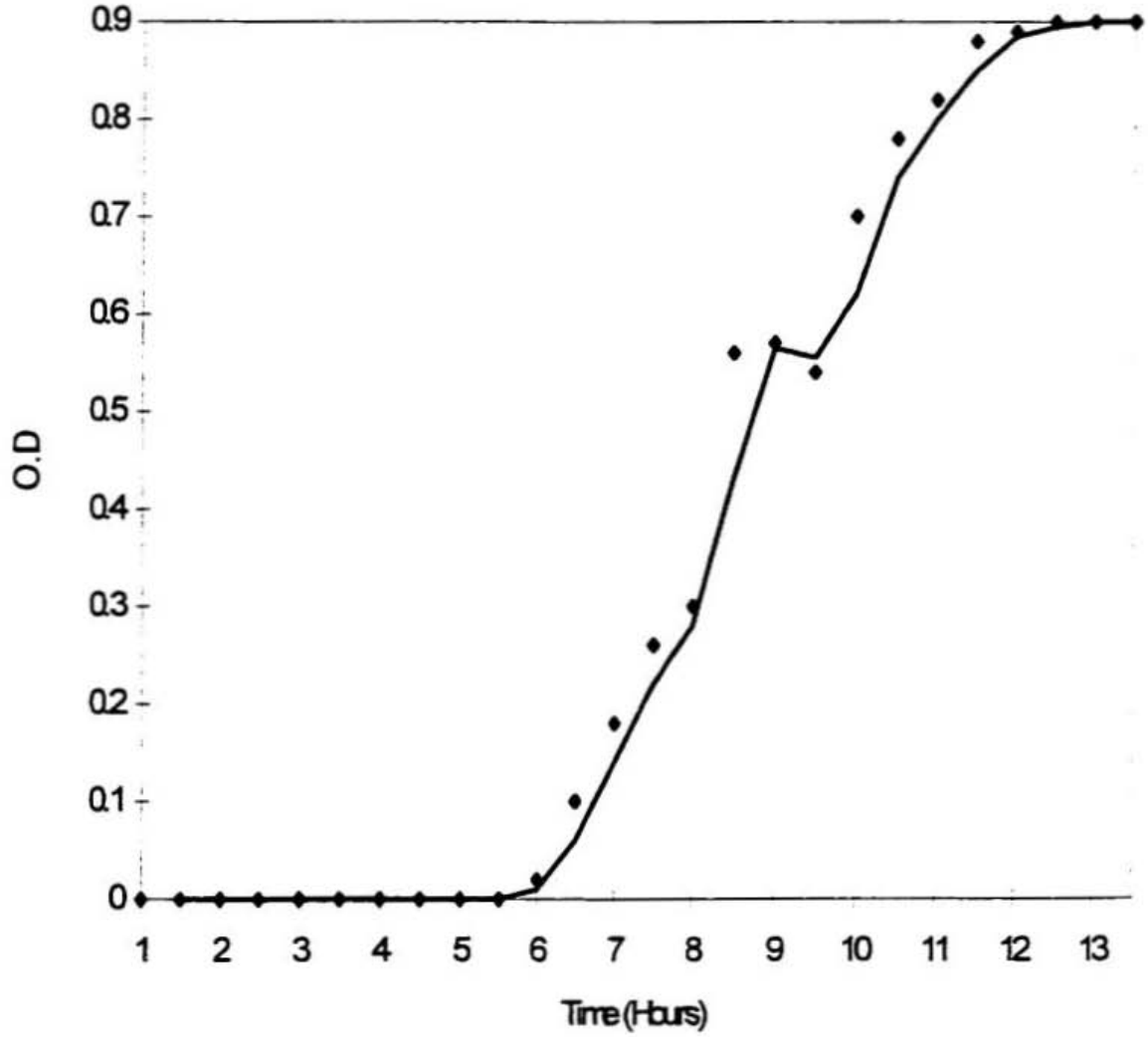

Figure 9. Growth curve showing the logarithmic phase of growth of *Y. ruckeri* .

3.3.3 Route and dose of infection

To determine the effect of glucan on disease resistance in Arctic charr, fish were challenged with *Y. ruckeri*. Prior to infection, the concentration and appropriate route of infection resulting in moderate mortalities were determined.

It was observed that infection by immersion in *Y. ruckeri* suspension did not induce mortality. Injection of fish with either 2.4×10^9 and 2.4×10^{10} CFU/fish resulted in mortalities on Day 1 and on Days 1 and 2 respectively (Table 6). Injection with 2.4×10^7 CFU/fish was found to be the most effective dose for infection because it induced evenly spread mortalites over five days and was therefore used as the route and concentration of bacterial infection of fish in challenge in section 2.8.5.

Table 6. Infection in Arctic charr (*S. alpinus*) with *Yersinia ruckeri*.

* Indicates the most appropriate route and dose of infection.

Method of administration	CFU/ml/fish	No. of mortalities on days after challenge					Total mortality (/10)
		1	2	3	4	5	
Injection	2.4×10^6	0	0	0	0	0	0
	2.4×10^7	1	0	2	0	1	4*
	2.4×10^8	2	4	0	0	0	6
	2.4×10^9	8	0	0	0	0	8
Immersion	2.4×10^7	0	0	0	0	0	0
	2.4×10^8	0	0	0	0	0	0

3.3.4 Resistance to infection

For determination of the effect of glucan on disease resistance, fish were treated with glucan and challenged with *Y. ruckeri* on Days 1, 7 and 14 after treatment. Mortalities were recorded twice daily. All dead and severely moribund fish were counted and collected for internal and external examination. Fish that died from jumping out of their tanks onto the floor were excluded in the mortality counts and initial numbers, since it was assumed they did not die from experimental infection.

Fish were monitored for at least three weeks, except for those challenged on Day 7 after glucan treatment. This was because all the fish in the control for this group died due to mechanical problems. Mortalities in the Day 7 glucan treated group were monitored for 20 days. However, only the first five days were taken into account in calculating % mortality and Relative Percent Protection (RPP) for these groups.

In all groups, there was no mortality observed on the day of challenge (Day 0). The earliest mortalities recorded were a day after challenge in both the treatment group challenged on Day 7 after immersion in glucan, and the non-treated control for fish challenged on Day 14 after immersion in glucan. Infection was typified by severe congestion and petechial haemorrhage on the

head and by flaccid, inflamed intestines containing mucus, combined with swelling of internal organs.

The haemorrhage was especially obvious around the edge of the eye where a red ring was observed. Application of pressure on the abdomen produced a discharge of blood-stained mucous from the anus. The region around the mouth, especially the lower jaw, and the region between the mouth and the base of pectoral fins was also blood stained. In some cases, this condition was extended to the gills and base of the pectoral and ventral fins. Internally, the stomach was virtually empty except for blood-stained mucous. In severe cases, most internal organs such as kidney, liver, and spleen were dark and appeared to be swollen. Most of the other organs were covered with a thick layer of the blood-stained mucous.

Bacteria obtained from the infected fish showed yellowish-beige colonies which had glistening surfaces. The colonies were 1-1.5 mm in diameter and characterized by convex elevations, entire margins and a butter-like consistency. Isolates were gram-negative and were shaped as straight, short rods without spores. Identification by API 20E® biochemical system confirmed the presence of *Y. ruckeri*.

Cumulative mortality was highest in the Day 14 non-treated control and lowest in the Day 14 treatment group (Table 7). Treatment by immersion in 1mg/L of glucan suspension on 1 and 7 days before challenge resulted in higher,

but not statistically significant percent mortalities ($P>0.05$). Relative Percent Protection (RPP) was highest in the group that was challenged 14 days after glucan treatment, an indication that glucan treatment 14 days in advance of infection was more effective than treatment on 1 and 7 days prior to infection.

Chi-square (χ^2) tests used to compare survival of glucan-treated fish against non-treated controls showed that treatment with glucan 14 days prior to infection resulted in a significant increase in survival ($P<0.05$).

Table 7. Mortality (%) and Relative Percent Protection (RPP) of fish challenged 1, 7 and 14 days after treatment with glucan. * indicates significant χ^2 distribution probability value ($P<0.05$).

No. of days from immersion treatment to challenge	No. of fish per group (glucan/control)	Total mortalities (glucan/control)	Mortality (%) (glucan/control)	Relative Percent Protection (RPP)	P value
1	64/65	28/27	43.8/41.5	-5.3	0.79
7	70/76	27/29	38.6/38.2	-1.1	0.95
14	69/62	21/29	30.4/46.8	34.9	0.05*

CHAPTER 4 - DISCUSSION

4.1 ACTIVATION OF NEUTROPHILS

Administration of some immunstimulants has been reported to increase the resistance of fish to microbial infection. This has been attributed to enhanced non-specific defences, particularly the phagocyte system. This study has demonstrated the ability of β-glucans to enhance non-specific defences of Arctic charr.

Activated neutrophils were recognized by their ability to stain dark blue with Nitro Blue Tetrazolium (NBT). Maximum neutrophil activation was observed on Day 14 following glucan administration by immersion in 1mg/L of glucan suspension.

Neutrophils of fish injected with glucan did not exhibit significant activation. This may be related to stress induced by handling of fish during injection and by anaesthesia. Un-neutralized MS-222, the anaesthetic used in this study, has been found to induce metabolic disturbances which, when combined with stress, may cause suppression of neutrophils. It also causes blood chemistry changes,

which are attributed to the low pK of MS-222 (Black and Connor, 1964; Wedemeyer, 1970; Pickering, 1993).

Studies with other fish species have shown that activation of circulatory neutrophils may be achieved by application of certain immunostimulants such as Levamisole, Quaternary Ammonium Compound (QAC) and short-chain polypeptides (Anderson and Jeney, 1992; Jeney and Anderson, 1993). In these studies, relatively low doses of about 5 μg per fish have been found effective; with much higher or lower doses having adverse effects such as increased mortality (Robertsen et al., 1990a).

Although the precise mechanisms involved in glucan induced neutrophilia in fish are not well understood, in mammals they have been associated with enhanced production of Colony Stimulating Activity (CSA), which results in increased production, recruitment, and/or release of neutrophils (Williams et al., 1988). Neutrophils have been associated with tissue damage through production of Reactive Oxygen Species (ROS) such as O_2^-, H_2O_2, and OH, which are toxic to pathogens entering the host's body and play an important role in their destruction (Chung and Secombes, 1987).

4.2 ACTIVATION OF MACROPHAGES

Fish macrophages have a wide range of functions including phagocytosis, presentation of antigens, release of antimicrobicidal and antitumour agents, and production of cytokines (MacArthur and Fletcher, 1985; Narnaware et al., 1994). Activity of these cells can be modulated to increase immunocompetence (Pulsford et al., 1994). The biochemical pathways involved in microbicidal activity of macrophages are not well known, but are dependent on two mechanisms; an oxygen dependent system which involves production of Reactive Oxygen Species (ROS) and an oxygen independent system. Macrophage activation can be achieved directly by soluble factors such as plant lectins and bacterial cell wall proteins as well as by lymphokines secreted from antigen or mitogen stimulated T-lymphocytes (Chung and Secombes, 1987).

Macrophage activation was monitored by Phagocytic Ratio (PR) and by Phagocytic Index (PI). PR is a percentage of phagocytic cells and is a less sensitive measure than PI, which is a measure of the number of engulfed particles (yeast cells in this case) by each macrophage (Narnaware et al., 1994). PR was enhanced on Days 2-14 by immersion in 10 mg/L of glucan suspension, with 41.5% of the cells ingesting ingest yeast cells. This ratio is relatively low compared to 78-97 % phagocytosis obtained by Narnaware et al.

(1994). It is possible that this low phagocytic ratio may be related to the stress induced by handling and anaesthesia.

PI values were also increased by on Days 2-14 by immersion in 10mg/L and were in general agreement with those obtained by Narnaware et al., (1994), who obtained values between 1.8 and 4.2. Therefore, we concluded that immersion in 10mg/L was the most effective treatment for stimulation of phagocytic activity of macrophages in Arctic charr. Inability of the injection method to increase the phagocytic activity of Arctic charr macrophages in this study further emphasizes it's stressful nature.

Increased activation between Days 2-14 may be explained by reduced levels of glucan particles during this period, therefore facilitating phagocytosis (Robertsen et al., 1990a). In the period between Days 21-35, glucan levels may have become reduced to sub-optimal levels for significant stimulation of macrophages.

This study has revealed that high doses of glucan (10 mg/L) activated macrophages while lower levels were more effective in neutrophil activation. This may be because macrophages are larger and can ingest more glucan particles than neutrophils. In plaice, *Pleuronectes platessa*, for example, macrophages have a diameter of 12-20μm compared to 8-10μm for neutrophils (Ellis, 1976).

Effects of β-glucans on host defences have attracted much attention and have been investigated by several authors in various cultured fish species (Yano and Mangidaan, 1989; Yano et al., 1989, 1991; Chen and Ainsworth, 1992; Engstad et al., 1992; Matsuyama et al., 1992; Brattgjerd et al., 1994; Jeney and Anderson, 1993; Engstad, 1994; Efthimiou, 1996). The stimulatory effect of glucan on disease resistance is associated with their structure and is believed to have developed early in the evolution of plants and animals as a mechanism for defence against fungal pathogens.

Glucan is a polysaccharride made up of glucose units (Section1.5). The way these glucose units are linked, and the number and length of side branches, are important (Onarheim, 1992). The side chains are recognized by macrophage receptors which they stimulate, thus triggering a series of microbicidal activities in preparation for ingestion of the glucan particles (Mitchell, 1992). Therefore, treatment of fish with yeast glucan prepares their non-specific defences in readiness to fight against potentially dangerous micro-organisms (Efthimiou, 1996; Engstad and Robertsen, 1993, Engstad et al., 1993).

4.3 EFFECT ON GROWTH

Arctic charr treated with glucan did not show evidence of an increased or inhibitory growth effect. This observation is similar to that of other studies in which glucan did not stimulate growth. Juvenile dentex (*Dentex dentex*) did not increase growth when fed with glucan-incorporated diets (Efthimiou, 1996), and studies with Rainbow trout (*Oncorhynchus mykiss*) also showed lack of increased growth following glucan treatment (Matsuo and Miyazono, 1993).

However, several other immunostimulants have shown an ability to increase growth. Levamisole, a levo-isomer of tetramisole and is used as a drug for treating helminth infections in ruminants, has been found to be an effective growth stimulant for carp (*Cyprinus carpio*) larvae. This has been attributed to stimulation of metabolism, thus increasing immune functions (Siwicki and Kossakowski, 1988). Immuno-stimulation of Tiger shrimp (*Penaeus monodon*) with glucan has been shown to be effective in enhancing their growth (Sung et al., 1994).

It appears, therefore, that results obtained on the effects of glucan on growth are inconsistent. However, it should be noted that effects may be compromised by acute forms of handling stress, such as those associated with the routine procedures of grading, transportation and artificial stripping. These result in physiological stress responses and an elevation of plasma

catecholamines and corticosteroids coupled with changes in osmotic and ionic regulation. Chronic stresses associated with intensive fish farming include such phenomena as deterioration of water quality and the less obvious but equally important stresses of social dominance, with submissive fish showing enhanced interrenal activity, increased susceptibility to infections and reduced growth (Pickering, 1981; 1993; Pickering et al., 1982; Pulsford et al., 1994).

4.4 RESISTANCE TO INFECTION

The results of the present study have demonstrated the ability of yeast glucan to induce protection in Arctic charr against Enteric Redmouth (ERM) disease. Fish treated with glucan by immersion in 1mg/L of glucan suspension and challenged by injection with *Y. ruckeri*, the aetiological agent for ERM, showed significant, increased resistance.

Artificial infection of Arctic charr with *Y. ruckeri* resulted in internal and external clinical signs similar to those described by Newman and Majnarich (1982) and Bullock and Stuckey (1976).

Fish that were challenged 14 days after treatment with glucan displayed the highest survival. This is in general agreement with other studies (Robertsen et al., 1990a; Yano et al., 1991, 1992; Anderson and Siwicki, 1994; Sung et al., 1994), and confirms the ability of yeast glucan to induce protection from disease

infection in Arctic charr. Resistance to *Y. ruckeri* infection after glucan treatment followed similar kinetics as those for neutrophil and macrophage activation, where maximum activation was observed on Day 14 after glucan treatment. Therefore, neutrophils and macrophages partly explain the mechanism by which glucan induces protection in Arctic charr. Stimulation of non-specific defences can thus be timed in anticipation of infection, especially when fish are expected to become exposed to a specific pathogen at a particular time such as before transfer from hatchery to culture facilities (Anderson and Siwicki, 1994).

β-glucans have been shown to increase the survival of carp, *Cyprinus carpio*, from *Edwardsiella tarda* and *Aeromonas hydrophilla* infections at concentrations of 2-10 and 5 mg/kg respectively when given by intraperitoneal injection (Yano et al., 1989; 1991). This increased survival was due to the activation of the Alternative Complement Pathway (ACP). Chen and Ainsworth (1992) have demonstrated the ability of β-1,3 glucans to reduce mortality as a result of experimental infection with *Edwardsiella ictaluri* in Channel catfish, *Ictalurus punctatus*, and have shown the important role played by these substances in stimulating protective responses against bacterial infection. They found that concentrations of 0.5 mg/kg were effective in conferring protection against infection at lower bacterial concentrations, while higher doses were required for higher bacterial concentrations.

Treatment of Brook trout, *Salvelinus fontinalis*, with a glucan extract from barley by single injections of 1 mg/kg or 30-minute immersions in 1000 mg/L resulted in high protection levels against infection with *Aeromonas salmonicida*, with injection giving more protection than immersion. Protection was induced in the injected group earlier than in the immersed, and had diminished in both injected and immersed fish 14 days after immuno-stimulation (Anderson and Siwicki, 1994).

Glucan has also been administered in the form of a feed component and has been found to increase protection of fish from disease infection. Peptidoglycan, a type of glucan which is derived from the bacterium, *Bifidobacterium thermophilum*, when fed to Rainbow trout at 6 μg/kg per day for 56 days resulted in increased survival after infection with *Vibrio anguillarium*. This increase diminished by day 56, suggesting negative impacts on disease resistance by prolonged oral administration (Matsuo and Miyazono, 1993). Studies have shown that feeding of glucan above a certain threshold does not improve the stimulatory effects (Onarheim,1992). Chinook salmon (*Oncorhynchus tswaytscha*) when fed on diets containing a type of β- glucan, VitaStim-Taito (VST), at rates of 0.1 and 1.0%, showed increased protection from *A. salmonicida* infection (Nikl et al., 1993). On the other hand, Lall and Olivier (1995) have found that incorporation of glucan in diets yields inconsistent levels of protection.

It seems that glucan can enhance protection against diseases within only certain concentration ranges. Sung et al. (1994) observed that immersion in high glucan concentrations (2000 mg/L) causes adverse gill tissue effects while concentrations of 500 and 1000 mg/L increase protection from infection by *Vibrio vulnificus* in Tiger shrimp. Robertsen et al. (1990a) suggested that injection at concentrations as high as 100mg/kg may cause adverse effects such as increased mortality.

The ability of glucan to enhance disease resistance in Arctic charr is related to its effect on non-specific defences, namely activation of neutrophils and macrophages. The results of this study have demonstrated that maximum activation of these cells correlates with the day that showed the highest survival.

The time and duration of protection depends on concentration and route of administration. High doses take a longer time to achieve protection than lower ones, probably because of overloading of phagocytic cells with glucan particles. It has been shown that 3-4 weeks are required to achieve maximal protection with 100 mg/kg (Robertsen et al., 1990a) and one week with 2-10 mg/kg (Matsuyama et al., 1992). In general, injection at concentrations of 0.5-10 mg/kg have been found to be effective at inducing protection, which lasts about 7-14 days (Yano et al., 1989, 1991; Chen and Ainsworth, 1992) while immersion in glucan suspensions of 500-1000 mg/L produce protection for about 14-18 days (Anderson and Siwicki, 1994; Sung et al., 1994).

Nonetheless, other factors such as the type of pathogen in the environment may contribute to determining the route of administration to be used. In cases where the disease is associated with skin damage, for example, immersion may be a better method of application (Efthimiou, 1996).

Most documented work on glucan has been based on artificially induced diseases in laboratory settings. However, it's effects on natural outbreaks have been observed recently in juvenile dentex, *Dentex dentex*. It was observed that glucan treated fish developed resistance to a protozoan infection (Efthimiou, 1996), thus showing the ability of the substance to enhance protection during natural epizootic infections.

Whilst the effectiveness of glucan at increasing protection against disease is well documented, some studies have reported increased mortality following administration. According to Robertsen et al. (1990a), this may be due to phagocytic overloading, with high concentrations of glucan particles lowering phagocytic activity. High concentrations of other types of immunostimulants have shown the same effect (Robertsen et al., 1990b).

The inability of glucan to induce protection has also been documented. Nikl et al. (1993) demonstrated that Chinook salmon *Oncorhynchus tshawytscha* immersed in VitaStim-Taito (VST), a commercial type of glucan, failed to yield a significant amount of protection. It was suggested that VST was not readily taken up through this route.

It has been shown that activated macrophages produced after glucan treatment may be responsible for increased antibacterial defences (Robertsen et al., 1990b). Yano et al. (1989) have shown that polysaccharrides derived from fungi such as *Schizophyllum commune*, *Sclerotium glucanium* and *Lentinus edodes* are able to induce increased survival and to reduce bacterial numbers in the blood of polysaccharride-treated carp (*Cyprinus carpio*). They attribute these activities to an increased phagocytic activity of carp pronephros cells induced by treatment with the polysaccharrides.

Appearance of macrophages in the peritoneum and presence of glucan particles in peritoneal macrophages after intraperitoneal injection of yeast glucan in Atlantic salmon further supports the idea that macrophages are the target cell for glucan action in fish and other vertebrates (Jorgensen et al., 1993).

It has been shown that glucans mediate their effect by activating a number of non-specific defence mechanisms such as increased production of lysozyme. Another possible mechanism is through the Alternative Complement Pathway (ACP), which has been described as partly responsible for increased protective effects of 1,6-branched -β-1,3-glucans (Yano et al., 1991).

CHAPTER 5 - CONCLUSION

The effects of β-glucan derived from yeast cells, *S. cerevisiae* on the non-specific defences and resistance to ERM disease in Arctic charr (*Salvelinus alpinus*) have been illustrated by the present study .

Neutrophil activation was monitored by the Nitro Blue Tetrazolium (NBT) assay and was highest at Day 14 following glucan treatment. Glucan administration by immersion was found to be more effective at stimulating neutrophil activation than when given by injection. Lower doses of 1mg/L had the greatest stimulatory effect.

Phagocytic activity was monitored by PR and PI and were increased by immersion in 10mg/L of glucan suspension between Days 2-14 after glucan treatment. Therefore, of the two methods that were used in this study, immersion at 10mg/L was more effective in elevating phagocytic activity of macrophages in Arctic charr.

Glucan did not affect the growth of Arctic charr. Survival of Arctic charr infected with *Y. ruckeri* showed that treatment by immersion in 1mg/L of glucan suspension at 14 days before infection increases survival.

RECOMMENDATIONS

From the observations on neutrophil and macrophage activation, this study has established that duration of this activation lasts for about 14 days, after which it declines. Therefore, it is recommended that booster doses should be given every 14 days. Multiple doses may be more effective at producing prolonged protection against natural outbreaks of microbial infections. However, since the prolonged effects by injection or immersion are not well understood, their long-term effect requires investigation.

Oral administration of glucan as a feed component may be a better alternative to administration of multiple doses by injection or immersion as it is less stressful and time consuming. Onarheim (1992) has suggested that fish should be on fed glucan diets for two weeks followed by six weeks of regular feeding since prolonged feeding with glucan diets causes immuno-suppression. However, a major disadvantage to this mode of administration is that fish must regularly feed on the glucan diets for immuno-stimulation to occur. Oral administration would therefore be of limited use, especially in sick fish whose appetite is usually diminished. In healthy fish, it provides a more attractive method of administration

Introduction of new culture species for warm and cold water culture is a major consideration in the aquaculture industry. The effect of glucans in some warm water aquaculture species such as Common carp (*C. carpio*) (Yano and Mangindaan, 1989) and Tiger shrimp (*P. monodon*), (Sung et al., 1994) have been studied. However, there has been no work done on some of the other cold water species of growing economic interest to Atlantic Canada's aquaculture industry, including Winter flounder (*Pleuronectes americanus*), Yellowtail flounder (*Pleuronectes ferruginea*), Atlantic halibut (*Hippoglossus hippoglossus*) and Wolffish (*Anarhichas spp.*) (Brown et al., 1995). Similar studies in these species would help to develop effective disease control programs for increased production in the region.

Currently, the centre of focus is on the effect of glucan on the development of resistance to artificially induced diseases (Efthimiou, 1996). There is also a need to study the effect of glucan on resistance to subclinical diseases and natural disease outbreaks.

The effect of glucan on growth is inconsistent and requires further investigation. Nonetheless, growth indices provide safety measures in glucan application schedules.

Glucan's ability to increase resistance provides a suitable prophylactic tool for controlling opportunistic infections which are notorious for manifesting themselves during stressful conditions, such as those induced by handling

during prophylactic treatment, grading, transportation, and in sub-optimal environments (Efthimiuo, 1996). Treatment with glucan prior to transfer to new environments can also boost non-specific defences in preparation for the unknown. Under culture, it can be used regularly to keep the non-specific defences alert in case of an outbreak, which would help to reduce loses before specialized forms of treatment, if required, are given.

Treatment with glucan holds promise to an effective, environmentally acceptable, and cost effective tool for control of infectious diseases. It is likely to be the method of choice in the near future for the aquaculture industry in Atlantic Canada, as well as other parts of the world.

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APPENDICES

Appendix A: One-way analysis of variance (ANOVA) for neutrophil activation by glucan treatment.

Day 0

1mg/kg	10mg/kg	0mg/kg	1mg/L	10mg/L	0mg/L
0.333	0.167	0.667	0.500	0.833	1.167
0.667	0.667	0.167	0.833	0.500	0.833
0.333	0.500	0.667	0.667	1.000	0.667
0.667	1.000	0.333	1.000	0.167	0.667
0.167	1.000	0.500	0.333	0.000	2.000

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	2.167	0.433	0.050
Column 2	5	3.333	0.667	0.125
Column 3	5	2.333	0.467	0.047
Column 4	5	3.333	0.667	0.069
Column 5	5	2.500	0.500	0.181
Column 6	5	5.333	1.067	0.314

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.378	5	0.276	2.1032	0.1	2.621
Within Groups	3.144	24	0.131			
Total	4.522	29				

Appendix A (Cont'd)

Day 14

1mg/kg	10mg/kg	0mg/kg	1mg/L	10mg/L	0mg/L
1.333	1.667	0.833	1.667	2.167	1.000
1.333	1.333	0.500	2.833	2.000	1.167
0.667	1.667	0.833	4.333	0.833	1.167
0.500	2.333	0.500	4.333	1.333	0.667
0.500	1.667	0.667	3.000	0.833	0.833

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	4.333	0.867	0.186
Column 2	5	8.667	1.733	0.133
Column 3	5	3.333	0.667	0.028
Column 4	5	16.167	3.233	1.272
Column 5	5	7.167	1.433	0.397
Column 6	5	4.833	0.967	0.047

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	22.208	5	4.442	12.913	4E-06	2.621
Within Groups	8.256	24	0.344			
Total	30.464	29				

Appendix A (Cont'd)

Day 28

1mg/kg	10mg/kg	0mg/kg	1mg/L	10mg/L	0mg/L
2.167	2.167	1.167	0.667	0.833	1.667
0.833	0.833	0.333	0.667	0.833	1.000
0.500	0.500	0.167	2.333	1.000	0.333
0.167	0.167	0.667	1.167	0.667	0.667
0.333	0.333	0.167	0.833	1.000	0.667

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	4.000	0.800	0.644
Column 2	5	4.000	0.800	0.644
Column 3	5	2.500	0.500	0.181
Column 4	5	5.667	1.133	0.492
Column 5	5	4.333	0.867	0.019
Column 6	5	4.333	0.867	0.256

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.027	5	0.205	0.5511	0.736	2.621
Within Groups	8.944	24	0.373			
Total	9.971	29				

Appendix B: One-way analysis of variance (ANOVA) for phagocytic index by glucan treatment.

1mg/kg

Day 2-14	Day 21-35
13.3	0.7475
0.495	0.33
1.25	0.325

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	3	15.045	5.015	51.623
Column 2	3	1.4025	0.4675	0.0588

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	31.02	1	31.02	1.2004	0.3348	7.7086
Within Groups	103.36	4	25.841			
Total	134.38	5				

Appendix B (Cont'd)

10 mg/kg

Day 2-14	Day 21-35
4.03	0.35
1.12	0.325
10.458	0.49

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	3	15.608	5.2025	22.828
Column 2	3	1.165	0.3883	0.0079

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	34.764	1	34.764	3.0447	0.1559	7.7086
Within Groups	45.672	4	11.418			
Total	80.437	5				

Appendix B (Cont'd)

0mg/kg

Day 2-14	Day 21-35
3.255	0.455
1.275	2.025
1.815	1.44

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	3	6.345	2.115	1.0476
Column 2	3	3.92	1.3067	0.6296

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.9801	1	0.9801	1.1688	0.3405	7.7086
Within Groups	3.3543	4	0.8386			
Total	4.3344	5				

Appendix B (Cont'd)

1mg/L

Day 2-14	Day 21-35
6.825	1.575
1.76	2.25
2.9375	1.15

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	3	11.523	3.8408	7.0256
Column 2	3	4.975	1.6583	0.3077

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	7.145	1	7.145	1.9486	0.2352	7.7086
Within Groups	14.667	4	3.6666			
Total	21.812	5				

Appendix C: One-way analysis of variance (ANOVA) for phagocytic ratio by glucan treatment

1mg/kg

Day 2-14	Day 21-35
47.5	11.5
9	6
12.5	6.5

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	3	69	23	453.25
Column 2	3	24	8	9.25

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	337.5	1	337.5	1.4595	0.2936	7.7086
Within Groups	925	4	231.25			
Total	1262.5	5				

Appendix C (Cont'd)

10mg/kg

Day 2-14	Day 21-35
26	7
16	6.5
44.5	7

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	3	86.5	28.833	209.08
Column 2	3	20.5	6.8333	0.0833

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	726	1	726	6.9418	0.0579	7.7086
Within Groups	418.33	4	104.58			
Total	1144.3	5				

Appendix C (Cont'd)

0mg/kg

Day 2-14	Day 21-35
21	7
15	15
16.5	12

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	3	52.5	17.5	9.75
Column 2	3	34	11.333	16.333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	57.042	1	57.042	4.3738	0.1047	7.7086
Within Groups	52.167	4	13.042			
Total	109.21	5				

Appendix C (Cont'd)

1 mg/L

Day 2-14	Day 21-35
32.5	17.5
16	15
23.5	11.5

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	3	72	24	68.25
Column 2	3	44	14.667	9.0833

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	130.67	1	130.67	3.3793	0.1399	7.7086
Within Groups	154.67	4	38.667			
Total	285.33	5				

Appendix C (Cont'd)

10mg/L

Day 2-14	Day 21-35
41	19
40.5	7.5
43	6

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	3	124.5	41.5	1.75
Column 2	3	32.5	10.833	50.583

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1410.7	1	1410.7	53.911	0.0018	7.7086
Within Groups	104.67	4	26.167			
Total	1515.3	5				

Appendix C (Cont'd)

0mg/L

Day 2-14	Day 21-35
15	16
35	10
13.5	9.5

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	3	63.5	21.167	144.08
Column 2	3	35.5	11.833	13.083

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	130.67	1	130.67	1.6628	0.2667	7.7086
Within Groups	314.33	4	78.583			
Total	445	5				

Appendix D: One-way analysis of variance (ANOVA) for weights by glucan treatment.

Day 0

1mg/kg	10mg/kg	0mg/kg	1mg/L	10mg/L	0mg/L
115.8	115.8	112.4	113.3	121.7	123.3
114.3	123.6	103.6	130.0	114.1	109.1
106.4	108.5	108.5	117.0	102.6	106.9
113.5	108.5	123.2	101.8	102.9	138.8
100.4	133.9	103.7	143.2	114.3	145.1
134.2	141.4	110.8	103.6	141.9	117.1
105.4	132.5	143.4	102.4	130.2	95.2
100.3	109.8	102.8	117.8	120.4	101.3
87.0	117.1	101.9	88.7	99.0	112.9
89.6	116.0	104.4	88.2	92.4	94.6

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	1066.9	106.69	189.55
Column 2	10	1207.1	120.71	136.21
Column 3	10	1114.7	111.47	167.37
Column 4	10	1106	110.60	301.58
Column 5	10	1139.5	113.95	231.77
Column 6	10	1144.3	114.43	293.69

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1103	5	220.61	1.003	0.425	2.386
Within Groups	11882	54	220.03			
Total	12985	59				

Appendix D (Cont'd)

Day 30

1mg/kg	10mg/kg	0mg/kg	1mg/L	10mg/L	0mg/L
134.9	93.8	145.5	129.9	142.6	124.2
163.3	136.7	131.5	135.5	176.2	200.6
151.5	174.3	163.5	207.4	157.2	165.0
157.1	216.0	128.9	144.9	133.9	124.7
117.5	161.8	146.8	123.2		151.8
110.8	149.8		154.4		155.5
	161.5		111.8		
	109.1		116.8		

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	835.1	139.18	469.69
Column 2	8	1203	150.38	1460.7
Column 3	5	716.2	143.24	192.93
Column 4	8	1123.9	140.49	930.03
Column 5	4	609.9	152.48	342.58
Column 6	6	921.8	153.63	809.07

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1228.7	5	245.75	0.306	0.906	2.523
Within Groups	24929	31	804.15			
Total	26157	36				

Appendix D (Cont'd)

Day 56

1mg/kg	10mg/kg	0mg/kg	1mg/L	10mg/L	0mg/L
157.5	135.4	173.7	199.1	163.5	147.3
	194.4	206.3	220.4	203.4	264.5
	190.3	151.5	159.9	193.5	185.4
	211.2	152.6		145.7	198.2
	206.3				186.8
	148.7				
	161.1				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	1	157.5	157.50	-
Column 2	7	1247.4	178.20	880.3
Column 3	4	684.1	171.03	657.4
Column 4	3	579.4	193.13	941.8
Column 5	4	706.1	176.53	710.1
Column 6	5	982.2	196.44	1816.2

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2712.1	5	542.43	0.527	0.753	2.773
Within Groups	18533	18	1029.6			
Total	21245	23				

Appendix D (Cont'd)

Day 78

1mg/kg	10mg/kg	0mg/kg	1mg/L	10mg/L	0mg/L
181.9	156.0	216.9	226.6	202.0	170.2
	222.9	222.4	186.6	205.6	299.0
	242.5	186.7		156.9	217.3
	232.5	189.6			242.8
	147.1				207.6

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	1	181.9	181.90	-
Column 2	5	1001	200.20	2030.3
Column 3	4	815.6	203.90	337.19
Column 4	2	413.2	206.60	800.00
Column 5	3	564.5	188.17	736.44
Column 6	5	1136.9	227.38	2282.4

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4004.6	5	800.9	0.546	0.739	2.958
Within Groups	20535	14	1466.8			
Total	24540	19				

Appendix E: One-way analysis of variance (ANOVA) for lengths by glucan treatment.

Day 0

1mg/kg	10mg/kg	0mg/kg	1mg/L	10mg/L	0mg/L
20.0	20.0	19.0	19.0	19.5	20.5
19.0	20.5	19.0	20.5	20.0	20.0
20.5	19.5	18.5	19.5	19.0	19.5
20.0	20.0	21.0	19.0	19.5	21.5
19.5	20.5	20.0	21.5	20.5	21.0
21.0	22.0	19.5	19.5	21.5	20.0
20.0	21.0	21.5	18.5	21.0	16.5
21.0	20.0	19.5	20.0	20.0	19.5
18.0	20.5	20.5	19.0	19.0	20.0
18.5	20.5	19.5	19.0	18.5	18.0

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	197.5	19.75	1.0139
Column 2	10	204.5	20.45	0.4694
Column 3	10	198.0	19.80	0.9000
Column 4	10	195.5	19.55	0.8028
Column 5	10	198.5	19.85	0.8917
Column 6	10	196.5	19.65	2.1139

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5.021	5	1.004	0.973	0.443	2.386
Within Groups	55.725	54	1.032			
Total	60.746	59				

Appendix E (Cont'd)

Day 78

1mg/kg	10mg/kg	0mg/kg	1mg/L	10mg/L	0mg/L
22.5	21.5	23.5	24.0	23.5	22.5
	24.5	24.5	22.5	24.0	26.5
	25.5	23.0		21.5	20.5
	24.5	21.5			24.5
	23.0				24.0

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	1	22.5	22.50	-
Column 2	5	119.0	23.80	2.45
Column 3	4	92.5	23.13	1.56
Column 4	2	46.5	23.25	1.13
Column 5	3	69.0	23.00	1.75
Column 6	5	118.0	23.60	5.05

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.63	5	0.53	0.187	0.963	2.958
Within Groups	39.31	14	2.81			
Total	41.94	19				

Appendix F: Chi-square (χ^2) distributions for survival of fish and glucan treatment.

Day 1

actual:

	treated	non-treated	TOTAL
survived	36	38	74
died	28	27	55
TOTAL	64	65	129

expected:

	treated	non-treated	TOTAL
survived	36.713	37.2868	74
died	27.287	27.7132	55
TOTAL	64	65	129

0.80

Day 14

Actual:

	treated	non-treated	TOTAL
survived	48	33	81
died	21	29	50
TOTAL	69	62	131

expected:

	treated	non-treated	TOTAL
survived	42.664	38.3359	81
died	26.336	23.6641	50
TOTAL	69	62	131

0.05