MORPHOLOGY AND BIOFILM STUDIES OF THE INTESTINES OF THE NILE TILAPIA (*Oreochromis niloticus*).

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ABSTRACT

Tilapia, a warm fresh-water herbivorous fish, forms a major component of the species used in tropical and subtropical aquaculture, mainly due to its fast growth, efficient use of natural feeds, resistance to diseases, and tolerance to a variety of environmental conditions.

The study investigated the morphology and mucus histochemistry of the intestines of Nile tilapia (*Oreochromis niloticus*), the biofilm on the microvillar surface of the intestines, and interaction of these biofilms with a microbe prevalent in tilapia habitats, *Aeromonas hydrophila*. The Nile tilapia intestines were processed for routine histology and electron microscopy. The use of antimucus antibodies in the fixative preserved the biofilm for light and electron microscopic evaluation. The relationship between time, location, numbers of *A. hydrophila*, and the biofilm was then investigated using an immunofluorescent test.

The intestines of Nile tilapia reflect adaptations for a herbivorous diet, in their length, and presence of mucosal foldings. The thin walled intestines are compacted in the abdominal cavity in complex convolutions bound by the mesentery and mesenteric fat. There are no regional distinctions besides progressive caudal tapering. The Nile tilapia intestines have no structural demarcation between the lamina propria and the submucosa, which borders the bi-layer muscularis and are wrapped in the serosal layer. The luminal surface of the intestines of Nile tilapia, maintained in both flow-through and recirculating fish holding facilities is lined with an adherent biofilm. The biofilm appeared in several forms and, was associated with a mucin matrix and numerous microbial morphotypes. Exposure to *A. hydrophila* revealed that it is associated with the biofilm, but did not establish autochthonity.

The preservation of the biofilm provides an opportunity to discern the intestinal microbial ecology and dynamics, and its role in herbivorous digestion, gut immunity and the relationship to the microbial population in the aquatic environment.

DEDICATION

'tû Waîthîra

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Abbreviations

AB	Alcian Blue
BSA	Bovine Serum Albumin
DF	Degrees of freedom
dPBS	Phosphate Buffered Saline
FT	Flow-Through fish holding system
Hr	Hours
IFAT	Indirect Fluorescent Antibody Test
IgG	Immunoglobulin G
LM	Light Microscopy
ml	millilitres
OMP	Outer membrane protein
PAS	Periodic Acid Schiff
RC	Recirculating fish holding system
RER	Rough endoplasmic reticulum
SAP	Surface array protein
SEM	Scanning Electron Microscopy
SD	Standard deviation
TEM	Transmission Electron Microscopy
TSA	Trypticase soy Agar
µl	microlitres
µm	micrometers

1. General introduction

1.1 Tilapia and tilapia culture

Fish numerically constitute a dominant component of the 41,000-plus species of vertebrates (Fange and Grove 1979). This large number of species has evolved a variety of structural and physiological adaptations allowing the fish to reside in diverse ecosystems (Bond 1979), utilizing a variety of habitats (Fange and Grove 1979, Thurmond 1979). Tilapia are warm, freshwater and mainly herbivorous fish belonging to the family *Cichlidae* (Trewavas 1982). They are indigenous to Africa, where more than a hundred species are distributed in numerous geographical zones (Balarin and Hatton 1979). Tilapia are versatile species that have evolved to inhabit both fast flowing and swampy rivers, deep, saline and desert lakes, and coastal brackish waters and lagoons (Fryer and Iles 1972, Balarin and Hatton 1979, Philippart and Ruwet 1982, Pruginin et al 1988). Tilapia are distributed widely, primarily due to transfers for aquaculture, but restricted by their preference for high water temperature. Their natural distribution is restricted to the 20°C winter isotherms (Balarin and Hatton 1979), with the temperature range for growth and reproduction ranging between 20-35°C (McBay 1961, Reite et al 1974). Tilapia tolerate low dissolved oxygen (DO), and high carbon dioxide, salinity and turbidity levels unlike salmonids (Balarin and Hatton 1979). Tilapia are mainly herbivorous fish that are non selective opportunistic feeders, and are secondary to primary food producers (plants) in the food chain (Steffens 1989). Their diets vary depending on species and habitat, from coarse vegetation including grasses

and water weed to phytoplankton, unicellular algae and bacteria (Bowen 1982). Tilapia adapt well to commercial pelletized feeds (Lovell 1989). Fryer and Iles (1972) attribute the high yields of tilapia in tropical water bodies to their ability to consume the abundant and highly nutritious blue-green algae.

The adaptability of tilapia has led to their widespread utilization in aquaculture, making them a species of major economic importance (Chamberlain 1993, Balarin and Hatton 1979). Tilapia are raised in diverse culture systems, using different husbandry methods (Lovell 1989). The pond system is the most common culture system producing tilapia at various stocking rates. In small scale enterprises where commercial diets are unavailable, use of organic and/or inorganic fertilizers are commonly practiced. Fertilizers stimulate food chains that are favourable to tilapia (Hepher and Pruginin 1982), and have been useful in integrated semi-intensive aquaculture. The integrated culture incorporates tilapia production in other various agricultural activities, commonly practised at subsistence production levels (Gopalakrishnan 1988, Mang-umphan and Arce 1988). The efficient conversion of diverse foods to high quality protein make tilapia a valuable food source in many tropical countries (Jauncey and Ross 1982). In non intensive culture systems, the stocking rates remain low as the fish rely on the natural feed for complete nutrition. Though the level of production in these systems is low, they remain appropriate due to their sustainability primarily associated with the limited initial capital outlay. However, as the natural feed is replaced with high quality supplemental feed, the stocking rates increase substantially.

Tilapia polyculture systems, where different species are raised together to exploit

feeding synergisms, have been successful, particularly in combination with carp (Hepher and Pruginin 1982). However, intensive polyculture is limited by requirement for high quality feed instead of natural feeds which are cheaper and more appropriate in many rural locations (Hepher and Pruginin 1982, Coche 1982).

Tilapia are fast growing fish that resist many diseases. Low levels of parasitic infections such as trichodinids, *Ichthyophthirius*, and immature stages of flukes have been identified in cultured tilapia without significant mortalities (Nguenga 1988, Roberts and Sommerville 1982). Bacterial pathogens mainly, *Aeromonas hydrophila*, myxobacteria and *Edwardsiella tarda*, have been associated with tilapia morbidity and mortality, especially in stressed fish (Lightner et al 1988).

1.2 Biofilms

Current studies in microbiology show that in natural populations most microbes grow in colonies that develop as biofilms (Costerton et al 1981, 1987, Khoury and Costerton 1991, Marshall 1992). Biofilms are composed of microorganisms organized in hydrated, viscous exopolysaccharide matrices (Cheng et al 1981), that promote bacterial survival by facilitating nutrient and free radical trapping, and by protecting bacteria against antimicrobial insults such as antibodies, antibiotics and bacteriophages (Costerton et al 1987). The tilapia's warm habitat is conducive to microbial growth (McMillan and Santucci 1990) which constitutes a major component of eutrophic water bodies and plays an important role in the formation of the detritus. The detritus is an important food source for some tilapia species in the natural ecosystems (Bowen 1982).

Since the aquatic life interacts closely with microbes, a suitable base exists in these ecosystems for development of biofilms.

Biofilms are associated with most aquatic animal surfaces. The mucosubstances form a major component of the biofilms and are associated with many fish tissues including the skin (Pickering and Macey 1977, Zaccone 1983). These mucosubstances may aid the locomotion of fish through the aquatic environment by lubricating of the skin (Rosen and Cornford 1971). The mucosubstances are active in host protection by providing sites for various immune and non-immune reactants such as antibodies or lysozymes (Fletcher and White 1973, Fletcher and Grant 1969). In both the skin and gills, the mucosubstances may be involved in trapping of metals (Handy et al 1989). The mucosubstances, which exist at the interface of the fish gills and the enveloping aquatic environment, play an important role in osmoregulation (Handy et al 1989) by facilitating ammonia and CO_2 excretion (Wright et al 1989).

The intestinal mucous layer in many animals provides an ecological niche (Hoskins 1984) suitable for the development of biofilms (Costerton et al 1987). In this niche the microorganisms interact closely with one another, and with the mucous layer (Hoskins 1984).

An important function of the mucus is protection of the underlying delicate mucosa from mechanical and chemical damage from the digestive secretions (Neutra 1984). The microbes in the tilapia's intestinal environment may play a role in the nutrition of the host (Bowen 1982). Microbial activity on organic matter in the tilapia intestines may provide a mechanism for degradation of complex substances to forms that are

available to the host. However, the access of the exogenous microbes to the intestinal environment may be inhibited by the low pH in the stomach. Moriaty (1973) explained the safe passage of ingested material through the stomach of *O. niloticus*. One mechanism exposes the ingesta which includes microbes, to the low pH environment in the stomach, while a second route makes a direct transition from the oesophagus to the pyloric junction without exposure to low pH. This mechanism provides a conduit to microbes to bypass the harsh gastric environment, to the intestines where they may play a role in the digestive process. Once in the intestines, the microbe's viability is challenged by the presence of proteolytic enzymes (Bowen 1982). Organization of microorganisms into biofilms may enhance bacterial protection and existence in the hostile intestinal environment.

The same mechanism that allows safe passage of the microbes through the stomach, may also permit pathogenic bacteria access to the intestines. Among pathogens of significance is *Aeromonas hydrophila* which is ubiquitous, and is considered autochthonous inhabiting most aquatic environments (Allen et al 1983, Austin and Austin 1987, Hazen et al 1978). Hsu et al (1981, 1985) suggested that the ability of *A. hydrophila* to utilize a diverse range of carbohydrate and proteolytic substrates aids its survival in aquatic and other environments. *A. hydrophila* possesses various factors that enhance colonization (Janda et al 1991) including mucotactic responses (Hazen et al 1982). *A. hydrophila* is associated with diseases in many species (Kokka et al 1991, Llobrera and Gacutan 1987, Khardori and Faistein 1988) and causes septicaemia in tilapia (Lightner et al 1988, Roberts and Sommerville 1982). The role of the large

mixed population of microorganisms in the tilapia intestines has not been fully investigated, neither has the full profile of the functions of the mucus.

1.3 *Mucus component in the intestinal biofilms.*

The mucous layer in the gastrointestinal tract provides a site for concentration of HCO_3^- maintaining a diffusion barrier trap for the H^+ concentration, reflected in the differing pH above and below the mucous layer in the luminal environment of the stomach and the upper intestines (Williams and Turnberg 1980, 1981). The mucous layer also provides a highly selective interface between the lumen and epithelial surface (Mantle and Allen 1989), that allows access for nutrients but prevents toxins and some organisms from reaching the mucosal surface (Forstner et al 1984).

Gastrointestinal mucus in mammals consists of a complex hydrated matrix (Bollard et al 1986, Allen 1984). The major components of the matrix are mucins, which are large molecular weight glycoproteins (Forstner et al 1984), and lipids (Slomiany and Slomiany 1984).

The polymeric mucins of mammals range in molecular weight from $2x10^5$ to $15x10^6$ with an average of $2x10^6$ (Allen 1981, 1984; Forstner et al 1984). The mucins contain 60-80% carbohydrate which exists as hexosamine sugars covalently bound to varying amounts of protein and lipid (Bancroft and Cook 1984). These hexosamine sugars are N-acetylgalactosamine, N-acetylglucosamine, galactose, fucose and sialic acids (N-acetyl/N-glycollyl neuraminic acid) (Neutra and Forstner 1986, Mantle and Allen 1989). These monosaccharides form the basis of oligosaccharide that in turn make up the

chains that are linked non covalently to make the mucous gel (Neutra 1984). The permutations of the different monosaccharide building blocks, presence and location of acid radicals result in the variation in mucus composition in different regions of gastrointestinal tracts and in different species (Reifel and Travil 1979, Mantle and Allen 1989, Grau et al 1992). Lipids constitute the other major component of the mucins (Slomiany and Slomiany 1984) and influence the viscosity and elasticity which in turn influences permeability (Kojima et al 1981). In the intestines, the major lipids are the neutral lipids and phospholipids (Neutra 1984).

The constituent composition of mucins imparts a differential reaction on the mucus from various dyes. Consequently, histochemistry is a useful tool in categorization of mucopolysaccharides (Harris 1973). Mucopolysaccharides have been categorized into acidic mucins which have sulphated or carboxylated acid radicals and neutral mucins that lack these radicals (Bancroft and Cook 1984). The mucopolysaccharides are negatively charged due to their sialic acid and ester sulphate residues, and a net excess of negatively charged amino acids (Bancroft and Cook 1984). The cation trapping property of biofilms is attributable to this net charge (Costerton et al 1987). Mucus histochemistry is important in identification of mucins in different species and areas of the intestines. The physiological significance of the histochemical variation of mucus in the intestinal tract is not well understood, though Grau et al (1982) suggested the presence of neutral mucopolysaccharides could be related to carbohydrate digestion.

In vertebrates mucus is synthesized in and secreted onto the luminal surface by the goblet cells (Fawcett 1986). The mucus proteins are synthesized in the cisternae of the

rough endoplasmic reticulum and then sulphated and glycosylated in the Golgi cisternae (Gauthier and Landis 1972). They are then packaged into granules which migrate to the luminal surface for secretion (Mantle and Allen 1989).

1.4 Rationale and Objectives

The declining stocks from wild fisheries and a higher demand for protein diets from the growing human population challenges aquaculture to meet the shortfall (Chamberlain 1993). In Africa and other tropical countries, the most evident effort is promotion of small-scale community based, integrated subsistence aquaculture systems. These systems have the major advantage of incorporating aquaculture into preexisting agricultural activities thereby enhancing energy recycling.

Tilapia are a major species used in aquaculture, mainly due to efficient use of feeds from diverse sources, fast growth, resistance to disease and easy management. Thus, tilapia are able to consume various low value foods and convert them to high quality protein (Lovell 1989). Tilapia are also very hardy fish that are tolerate many diseases. The development of tilapia culture on an intensive level may result in increased disease prevalence. Roberts and Sommerville (1982) suggested that the currently low prevalence of infectious disease in tilapia may be attributed to low intensification of their culture and\or perhaps inadequate study.

The main objective of the project is to demonstrate the presence of biofilms in the intestines of the Nile tilapia *(Oreochromis niloticus)*. This required the development of techniques that allowed direct examination of microorganisms in their natural

ecosystem. First, the morphology of the intestines of Nile tilapia (*O. niloticus*) was studied to provide an understanding of the structure that biofilm would be associated with. Work then focused on demonstration of a biofilm in tilapia. The project was concluded by investigation of the interaction of the biofilm with *A. hydrophila*, a microbe pathogenic to tilapia and present both in the fresh water and intestinal environment.

1.6 Summary of the Project's objectives.

1. Investigation of the morphology, histology and mucus histochemistry of the intestines of Nile tilapia (*Oreochromis niloticus*).
2. Establishment of a protocol for preservation, and demonstration of biofilm in Nile tilapia (*O. niloticus*) intestines using polyclonal antimucus serum.
3. Investigation of the relationship between *A. hydrophila* and the intestinal biofilm of the Nile tilapia (*O. niloticus*) using monoclonal antibodies.

2. Morphology and histology of Nile tilapia (*Oreochromis niloticus*) intestines.

2.1 Introduction

2.1.1 Digestive systems

The digestive system provides the means for acquiring food organisms needed for energy. To achieve that purpose, this system provides the mechanisms for the ingestion, digestion, and absorption of the food and the formation and egestion of the waste faecal substances (Walker 1987, Wheater et al 1987). The digestive systems range from the simplest form associated with the protozoans, marked by the absence of a gut, and characterized by the movement of molecules across the body wall, to the organisms that have a permanent gut which has evolved regional specialization (Webster and Webster 1974). Specializations include the mouth, pharynx, oesophagus, stomach and the intestines.

The evolution of the digestive system is influenced by various factors including the animal's diet, metabolic rate and size (Walker 1977). The factors lead to specialized morphology reflected in the complexity of gut structure and function (Morton 1979). Consequently, lines of division corresponding to nutrition and feeding habits occur showing an evolutionary convergence, that crosses different phylogenetic classifications (Webster and Webster 1974). These divisions include herbivores, omnivores, carnivores. The herbivores and omnivores have digestive systems fully adapted to bulky and more easily obtained plant diet, with a large indigestible component. Meanwhile the carnivores live on more concentrated and economic diet ingested at

longer intervals. The surface area to volume ratio interrelates closely with the metabolic rate, with higher ratios associated with larger nutrient requirements. The higher the metabolic rate the faster the food is utilised and the sooner replenishment is required.

2.1.2 Comparative intestinal morphology

In most vertebrates the intestines are the primary sites for digestion, absorption of food and formation of feces (Kapoor et al 1975, Anderson 1991). In the intestines, digestion of carbohydrates, proteins and lipids occurs by reduction to simpler units that are absorbed into the body tissues (Stinson and Calhoun 1993).

The intestine is a tubular organ whose length varies between animals, commences at the pyloric junction of the stomach and extends to the anal opening (Patt and Patt 1969). The intestinal wall is structured in concentric layers, with the tunica mucosa forming the innermost layer. The lamina epithelialis mucosa consists of a simple columnar epithelium with an underlying lamina propria consisting of a loose fibrous connective tissue which contains blood and lymphatic vessels. Characteristic of the mammalian mucosa and submucosa are circumferential plications, referred to as plicae circulares or Valves of Kerckring (Fawcett 1986).

The tunica submucosa forms the second layer of the intestinal wall and lies below the mucosa (Stinson and Calhoun 1993). Like the lamina propria, the submucosal layer is a very vascularized, fibrous connective tissue layer. Peripheral to the submucosa lies the tunica muscularis made of two smooth muscle layers. The inner of these layers is

the circular muscle layer, covered by an outer longitudinal layer. Though these layers have slight independent control, they are regulated by the autonomic nervous system. Most muscle cells are innervated by postganglionic sympathetic fibres, while preganglionic parasympathetic fibres terminate in the myenteric and submucosal ganglia lying between the muscle layers and between the submucosa and muscularis respectively (Webster and Webster 1979). The tunica muscularis is covered by the tunica serosa.

The four-layered structure of the intestines is common to all vertebrates, though differences occur between different animal groups on the exact specifications (Walker 1987). In mammals, the small and large intestines are distinctly demarcated (Morton 1979). The small intestine in turn is differentiated into the duodenum, jejunum, ileum. The duodenum which is most proximal is distinguished by presence of mucus and proteolytic enzyme secreting Brunner's glands located in the submucosa (Fawcett 1986). The jejunum and the ileum have no Brunner's glands, though the latter have more goblet cells and smaller villi than the former. The caecum is found at the junction between the small and the large intestine, and is associated large amounts of lymphatic tissue and goblet cells. The large intestine has a larger diameter but has no villi, though long tubular glands that secrete mucus are observed (Fawcett 1986).

Amphibian and reptilian herbivorous species, like their mammallian counterparts have long intestines (Walker 1987). The intestines are differentiated between the large and small intestines. In birds, the intestinal surface has deep crypts of Lieberkuhn reaching into the deep lamina propria at the base of which are large and branched tubular glands

which give the intestines a folded appearance (Patt and Patt 1969). The intestines are lined with numerous villi that have a lamina propria core. A pair of caeca which in herbivore birds may contain symbiotic bacteria, is present between the small and normally short large intestine.

The anatomy and histology of the intestinal tract of fish have been the subject of numerous studies (Dawes 1929, Plaice; Al-Hussaini 1947, Burke 1971; Yatusake and Wales 1983, Salmonids; Grizzle and Rogers 1976, channel catfish; Elbal and Agulliero 1986, *Sparus auratus*; Grau et al 1992, amberjack). These studies indicate variation between species that limit extrapolation of the observations from one species to another (Patt and Patt 1969). The intestinal wall in the fish as in other vertebrates has four layers (Walker 1987). However, unlike the higher vertebrates intestines, no regional distinction exists in most fish (Reifel and Travill 1979), although in some species, the presence of an ileal caecal valve distinguishes the intestine from a rectum (Ezeasor and Stokoe 1981A, Sis et al 1979, Morrison 1967).

In herbivorous teleosts, a common adaptation of increasing the intestinal surface area is an increase in length (Steffens 1989, Patt and Patt 1969). Long intestines which are associated with digestion of carbohydrate rich foods, are efficiently accommodated in the constrained abdominal cavity space by numerous and convoluting loops (Patt and Patt 1969). The arrangement of these loops is modified by the presence of large numbers of ova in mature females (Hale 1965).

Fish have diverse intestinal configurations, evolved by living in different habitats and utilizing a variety of feeds (Ellis et al 1989, Osman and Caceci 1991, Ferguson 1989).

The histology of the gastrointestinal tract of fish is generally less elaborate than that of the mammalian system due to the lower metabolic rate of the fish (Patt and Patt 1969, Ferguson 1989). However, to carry out the major function of absorption, the intestines are morphologically adapted to provide an adequate surface area (Steffens 1989). Fish intestines have numerous mucosal folds that amplify the surface area that is available for digestion and absorption, and aids the mixing of food and digestive juices (Patt and Patt 1969, Mayhew 1984). There are four basic types of folds in the fish. In cyclostomes, the typhosole which is a simple fold runs the length of the intestine, while the spiral valve in elasmobranchs, holosteans, chondrosteans and dipnoi is formed by the mucosa and submucosa is found along most of the intestines. This structure spirals the food as it passes through the intestines enhancing digestion and absorption.

Mucosal folds allow for the distension of the mucosal layer and are probably stretched following feeding (Grau et al 1992). The mucosal folds in fish are dissimilar to the plicae circulares in mammals. Osman and Caceci (1991) suggested the mucosal folds are a common strategy for efficiency since they are found in herbivores, omnivores and carnivores. The villi which are structural features common to mammals as important surface area enhancing strategy, are found only in few fishes (Sis et al 1979, Ezeasor and Stokoe 1981A, 1981B). Unlike mammalian villi, the mucosal foldings of fish lack central lacteals (Ham 1974) and crypts of Lieberkuhn (Kapoor et al 1975).

Pyloric caeca are present in most teleosts (Buddington and Diamond 1987, Steffens 1989). The caeca are blind diverticula associated with an extensive surface area, and are located distally to the pyloric sphincter and vary in number between fish (Ezeasor

and Stokoe 1981B, Elbal and Agulleiro 1986). Changes in surface area of the intestines are influenced by many factors including feeding habits (Kapoor et al 1975), temperature and energy budget (Lee and Cossins 1988), starvation (Gas and Noailliac-Depeyre 1976), heavy metals (Crespo et al 1986) and osmolarity (Nonnote and Nonnote 1986).

The intestinal epithelium is simple columnar in most vertebrates (Patt and Patt 1969). This contrasts to the pseudo-stratified epithelium that was observed in the eel (Thurmond 1979). Vertebrate enterocytes are closely packed and have a mildly basophilic cytoplasm, probably attributable to abundant RER (Gauthier and Landis 1972). The enterocytes have numerous microvilli on the apical border. The presence of these microvilli is typical of absorptive cells (Yamamoto 1966, Bishop and Odense 1966, Krementz and Chapman 1975). The epithelium in some fish has been shown to be ciliated (Ishida 1935, Magid 1975, Burkhardt-Holm and Holmgren 1992, Iwai 1967A), perhaps associated with evolutionarily older fish (Fänge and Grove 1979).

Distributed among the enterocytes in vertebrates are numerous goblet cells. The function of the goblet cells is to produce mucosubstances, whose biochemistry varies along the intestinal tract because of differences in the constituent building blocks. This variation makes histochemistry a useful tool in the investigation of regional differences and specialization (Gaffney 1992). Numerous histochemical studies on mucus reveal there are two general categories (Reifel and Travill 1979). The acid mucins have acid radicals that are either sulphated or carboxylated and are referred to as sulphomucins

and sialomucins respectively. The identification of acid mucins is based on the ester-sulphated groups of the sulphated mucins that are alcianophilic at low pH, and carboxyl groups of the sialic acids in the sialomucins which are alcianophilic at pH greater than 1.5 (Harris et al 1973, Bancroft and Cook 1984).

Other cells identified in the extracellular spaces in the epithelial layer include lymphoid cells, mainly lymphocytes, which are significant in gut associated immunity (Garderen 1991, Jalkanen 1990). They have been identified in *Mulloides auriflamma* (Al-Hussaini 1946), *Poecillia reticulata* and *Gastrosteus aculeatus* (Hale 1965).

The basal plasma membrane of the enterocytes relates to the basal lamina which separates it from the lamina propria. The basal lamina provides structural support to the enterocytes and may be closely involved in their morphogenesis and differentiation (Weiser et al 1990). In most fish, the lamina propria cannot be structurally differentiated from the submucosa, and Hale's (1965) reference to it as a subepithelial areolar (or connective) tissue layer seems appropriate. Some fish species, especially carnivorous fish, have various morphological features associated with the submucosa (Burnstock 1959, Krementz and Chapman 1979). These include the stratum compactum and stratum granulosum which are distinct connective tissue layers, with the latter infiltrated by granular cells (Bergeron and Woodward 1982, Anderson and Mitchum 1974, Ezeasor and Stokoe 1981A). The circulatory system in the lamina propria drains into larger vessels in the submucosa. The blood vessels transport amino acids and monosaccharides, while the lymph vessels deal with the fatty acids and triglycerides. The engorgement of the vasculature following feeding, may extend the mucosal folds

(Stinson and Calhoun 1993). In the submucosal regions, clusters of eosinophilic granular cells (EGCs) are commonly observed in the salmonids (Ezeasor and Stokoe 1980A). Submucosal glands, commonly associated with mammalian intestines are observed in a few fish like sablefish (Bell et al 1986).

In fish, the musculature is made up of a double smooth muscle layer with an inner circular and outer longitudinal components (Sis et al 1979). However, Grau et al (1992) demonstrated a three-layered intestinal musculature in amberjack (*Seriola demerili*) in the lamina muscularis, involving a middle circular component sandwiched between inner and outer longitudinal strata. Curry (1969) observed skeletal muscle in the intestine of carp which is unlike other fish and vertebrates. The serosa forms the outer layer of the intestines and has a thin layer of loose areolar connective tissue, blood and lymph vessels and nerve fibres. These are wrapped externally with a simple squamous mesothelium.

2.1.3 Ultrastructure

The ultrastructure of intestinal tissue is closely similar among vertebrates (Webster and Webster 1974). The plasma membrane on the apical surface of the microvilli is covered by a glycocalyx. The glycocalyx is produced by the cells on which it is attached (Krementz and Chapman 1975). The glycocalyx is believed to be a major site for biochemical activity and participates in synthetic functions of the enterocytes (Egberts et al 1984). The core of microvilli is composed of filaments running down its length and extending to the terminal web region in the apical cytoplasm. These

filaments are muscle-protein (actin) based and give structural support and motility to the microvilli (Burgess 1984, Matsudaira and Burgess 1979).

The apical cytoplasm region of the enterocytes of the Nile tilapia (also called fibrillar exclusion zone), is composed of a complex filament network which stabilizes the cell from osmotic and mechanical shock (Trier 1968). The terminal web plays a role in cytoplasmic transport which is evidenced by the presence of microtubules and membrane-bound vesicles and vacuoles (Gauthier and Landis 1972, Yamamoto 1966, Hale 1965, Iwai 1967B). Yamamoto (1966) indicated the presence of the vacuoles related to presence of food, suggesting they could be fat droplets. The membrane of these vesicles closely resembles the apical plasma membrane suggesting a pinocytotic role (Gauthier and Landis 1972, Burkhardt-Holm and Holmgren 1992). Hull and Stehelin (1979) suggested the terminal web could be sub-divided according to the level corresponding to the junction complexes into the apical, adherence and the basal region. In fish and other vertebrates (Gauthier and Landis 1972, Noaillac-Depeyre and Gas 1978, Fawcett 1986), the terminal web is distinct by absence of cellular organelles.

The cytoplasm region below the terminal web in the Nile tilapia cytoplasm is rich in cellular organelles, mainly the Golgi apparatus, endoplasmic reticulum (ER) and less abundant mitochondria (Gauthier and Landis 1972). This is a major site for cellular metabolism including triacylglycerol metabolism in the ER (Ezeasor and Stokoe 1981A, Bauermeister et al 1979) and their emulsification into chylomicrons in the Golgi apparatus (Iwai and Tanaka 1968, Iwai 1968A, Sire et al 1981).

Adjacent enterocytes relate very intimately to one another due to the presence of

specialized junction complexes. The zonula occludens or the tight junction is closest to the free border of the epithelium and is a region of complete fusion of lateral membranes of adjacent enterocytes. The other junctions include zonula adherens or the intermediate junction and macula adherens, or desmosome. Both are located respectively below the zonula occludens and participate in fastening of adjacent enterocytes. Sire et al (1981) reported at least three rows of desmosomes in rainbow trout. Also occurring between the enterocytes, are the gap junctions characterized by an extremely thin space between apposed regions of the cell membranes (Cormack 1984).

2.1.4 Rationale and Objectives

A comprehensive morphological description of the Nile tilapia (*O. niloticus*) intestine is not currently available. Osman and Caceci (1991) investigated the stomach and Orachunwong et al (1988) focused on the enterocytes of the anterior intestine. Kayanja et al (1975) studied the fine structure of the intestine of *Tilapia grahami*, a high pH, salinity and temperature tolerant species from the alkaline Lake Magadi in Kenya, while Pasha (1964) studied the morphology of the intestine of *Tilapia mossambica*. Knowledge of normal anatomy, histology and ultrastructure provides a base for assessment of disease, environmental toxicity (Crespo et al 1986) and physiological alterations (Lee and Cossins 1988).

The demonstration of the various mucosubstances present in the different regions of the gut can be used to detect changes in the mucins in disease in mammals (Mantle and

Allen 1989). Changes in the proportion of mucins are associated with intestinal pathology, marked by elevated sialomucins in colonic carcinoma (Filipe 1976) and ulcerative colitis (Ehsanullah et al 1982). Elevated sialomucins and neutral mucins are associated with some parasitic infections (Miller and Nawa 1979, Lee and Ogilvie 1981).

The epithelial surface contributes to the development of intestinal biofilms by providing an adherent surface and a major structural component from the secretion of the goblet cells. Therefore, an investigation of morphology of the intestines provides a basis for the study of biofilms.

2.1.5 Summary of objectives

General objective

Investigation of morphologic structure of the intestine of the Nile tilapia (*O. niloticus*).

Specific objectives

I. Investigation of the morphology, histology and ultrastructure of the Nile tilapia intestines.

II. Investigation of the mucus histochemistry of the intestine of Nile tilapia.

2.2 Materials and Methods

2.2.1 Fish maintenance

These studies were carried out on fifteen normal adult Tilapia (*O. niloticus*) obtained from the stocks held at the Atlantic Veterinary College Fish Health Unit's fish holding facility, originally obtained from Dr. Richard Boyle, Department of Biology, Dalhousie University. The fish were maintained in a flow-through system, in 1 metre diameter circular tanks. Temperature was maintained at 27.5 +/- 1^0 centigrade (average +/- SD) to avoid any temperature induced anatomical changes (Lee and Cossins 1988). The fish were fed a 5 mm pellet diet (Moore-Clark Co. Canada Inc.). Water was supplied from a dedicated well source, degassed to prevent supersaturation and maintained at a flow rate between 5-10 litres/minute. Air was delivered to each tank by a rotary vane blower. Daylengths were maintained at 12 hr light and 12 hr dark.

2.2.2 Light microscopy

Ten fish were used in the study and were not fed for 48 hours prior to sampling. They were anaesthetized using Tricaine Methanesulphonate (MS 222 Sigma product Code 18323) at 185mg/L (Ross & Geddes 1979). A state of suitable anaesthesia was achieved when the fish lost muscle tone and equilibrium (Ross & Geddes 1979), floated motionlessly in a dorso-lateral position and did not respond to any stimulation. Immediately after the immobilization, body weight and length were determined and the spinal cord was severed.

The right abdominal wall was dissected and reflected to expose the viscera. Freshly prepared Davidson's fixative (Appendix I) was injected into the intestinal lumen every 2-3 cm with a 23-Gauge needle in the area designated for sectioning, to ensure rapid access of the fixative to the tissue. The intestines were gently removed after severing the intestine at the pylorus and anus. The intestine was divided into 6 parts; 2 anterior, 2 mid and 2 posterior. Sections about 1.5 cm long were obtained from the proximal aspects of each division and immediately placed in labelled cassettes, gently submerged in prechilled fixative and maintained in it at 4°C for 24-48 hours.

Sections were maintained for 1 hour in serial baths of 70%, 70%, 95%, 95%, 95%, 100%, 100% ethanol for dehydration. The sections were then treated with xylene (BDH Inc. Toronto, Ontario) in 2 baths for an hour each, to clear the alcohol. The sections were then embedded, maintained in a longitudinal orientation (Brinkman Integrated Embedder. John's Scientific Inc.) and 6μm thick sections made. The sections were stained with Haematoxylin and Eosin, Alcian Blue, Periodic-Acid Schiff (Bancroft and Cook 1984).

2.2.3 Electron microscopy

2.2.3.1 Section preparation

Five fish maintained and prepared as described in sections 2.2.1 and 2.2.2 respectively, were dissected on the right abdominal wall to expose the viscera. The intestines were divided in 6 divisions and sections obtained from the proximal aspects, trimmed into about 2mm pieces and immediately transferred to labelled vials containing 5 ml of

freshly prepared 2.5% glutaraldehyde containing 0.15% ruthenium red, buffered with sodium cacodylate at pH 7.3 (0.1M). The freshly prepared fixative was maintained at 4°C before use. Sections were fixed at 4°C for 24 hours and then washed in 0.05% ruthenium red-cacodylate buffer for 10 minutes at room temperature (21-25°C).

The sections were then transferred to 2% osmium tetroxide (OsO_4) in cacodylate buffer (pH 7.3) and maintained for 2 hours. Sections were then washed five times for 10 minutes each in fresh 0.05% ruthenium red cacodylate buffer. After the third washing one group of sections was processed for TEM while the other for SEM.

2.2.3.2 Transmission electron microscopy

The sections were washed six times with fresh distilled water and dehydrated by passage through serial ethanol concentrations of 20%, 30%, 50%, 70%, 90% and 100% (twice) for 30 minutes each. Pre-infiltration then followed in two ten-minute serial treatments with propylene oxide. To gradually introduce the resin into the tissues, the sections were placed in propylene oxide mixed (50:50) with the resin and left overnight in a desiccator. The tissues were then placed in fresh resin (Appendix II) and again left overnight. The samples were placed in labelled moulds, then gently flooded with fresh resin and incubated at 60°C for 18 hours to polymerize. Thin and thick sections were prepared with Ultracut E (Reichert-Jung Leica) ultramicrotome using glass knives as described by Bozzola and Russell (1992).

The thin sections were then supported on 200 mesh copper grids and stained using a saturated solution of uranyl acetate in 50% ethanol for thirty minutes, and .22μm

millipore filtered lead citrate in a CO_2 free environment for two minutes. The sections were examined with a Hitachi 7000 electron microscope.

2.2.3.3 Scanning electron microscopy (SEM)

The sections for SEM were washed in distilled water and suspended in 1% thiocarbohydrozide (TCH) for 30 minutes. The sections were washed in distilled water before fixation in 1% OsO_4 for 2 hours followed by 6 washes in distilled water. Subsequently, the sections were floated in 1% TCH for 30 minutes and washed in distilled water. Finally, the sections were fixed in 1% OsO_4 for 2 hours, washed in distilled water and dehydrated by passage through serial changes of ethanol of 30 minutes duration each at the following concentrations; 20%, 30%, 50%, 70%, 90%, 100%, 100%. The sections were then critical point dried in liquid carbon dioxide, sputter coated with gold for 30 seconds and examined using a Hitachi 7000 electron microscope.

2.2.4 Histochemistry of the goblet cells and the mucous layer

Sections previously prepared and processed as indicated in 2.2.2, were stained using periodic-acid Schiff (PAS) and alcian blue-periodic acid schiff (PAS-AB) combination as indicated below. Porcine intestinal tissue obtained from the Atlantic Veterinary College served as controls.

The sections for PAS were deparaffinized by treatment in xylene for 10 minutes then immersed serially for 2 minutes in absolute, 95%, 70% ethanol solutions, and distilled

water. The sections were then treated in 0.5% periodic acid for 5 minutes and rinsed in distilled water. They were then placed in Coleman's Schiff reagent (Bancroft and Cook 1984) for 15 minutes. The sections were washed in running, warm tapwater for 10 minutes. Counterstaining was done in light green (Fisher Scientific Co. NJ USA) for 90 sec. followed by a washing in tap water for 15 minutes. Finally, the sections were dehydrated and cleared by serial treatment through 2 changes of 95% and absolute ethanol and xylene lasting 2 minutes each and mounted using a resinous medium (S/P™ ACCU.MOUNT60™ Baxter Scientific Products. IL USA). Sections were examined using an Olympus BH-2 microscope.

Sections for PAS-AB were deparaffinized and hydrated as described above before staining in alcian blue for 30 minutes. (The alcian blue solution for pH 2.5 was made by making a 1% solution of alcian blue in 3% acetic acid, while that for pH 1.0., a 1% alcian blue solution was made in 0.1 N hydrochloric acid). The pH 2.5 sections were washed in running tap water for 5 minutes, while pH 1.0 sections were blotted using bibulous paper.

All the sections were then placed in 1% Periodic acid for 10 minutes and washed in running tap water for 5 minutes. The sections were then placed to Coleman's schiff's reagent (prepared as described above) for 10 minutes then washed in warm, running tap water for 10 minutes. The sections were then dehydrated, cleared, mounted and examined as described in 2.2.2 above.

The histochemistry was evaluated :

PAS: Red to purple indicated mucin

AB(pH 2.5)-PAS:	Blue indicated acidic carboxylated mucins
	Magenta to red indicated neutral mucins
AB(pH 1.0)-PAS:	Blue indicated sulphated acidic mucins
	Magenta to red indicated neutral mucins
Degree of staining:	+++ intense
	++ moderate
	+ weak
	- no reaction.

2.3 Results

2.3.1 Anatomy, histology and ultrastructure.

In situ observation revealed the intestines of Nile tilapia arranged in numerous loops and spirals. The loops and spirals were more evident in the sexually mature females due to massive accumulations of ova. The length of the intestines was determined to be about four times the body length (Appendix III). The intestines had no distinct structural demarcations such as ileal caecal valve, though a progressive caudal tapering of the intestines was observed grossly.

The intestinal wall along the entire intestine was composed of four layers; tunica mucosa, tunica submucosa, tunica muscularis and tunica serosa (Fig. 1). The tunica mucosa consisted of lamina epithelialis, below which was the lamina muscularis (Fig. 5). The lamina epithelialis or epithelium had numerous gentle folds of varying size and shape, whose core was formed by lamina propria. These folds were prominent in the cranial parts of the gut (Figs. 1,2) and receded progressively caudally (Fig. 3).

The epithelium throughout the intestines was simple columnar with the typically cylindrical enterocytes and numerous goblet cells distributed among them (Fig. 1). Lymphoid cells particularly lymphocytes were found distributed among the enterocytes. No lymphoid cell organization or aggregation as in Peyer's patches was observed. The enterocytes had strongly PAS positive apical borders and round to oval shaped nuclei that were located towards the base of the cells. They had mildly basophilic cytoplasm. The lamina propria was located below the epithelium and above the submucosa,

forming the core of the folds and composed mainly of loose connective tissue with abundant vasculature and lymph vessels (Fig. 3). Lymphoid cells, mainly lymphocytes were found scattered in the lamina propria. The lamina propria was continuous with the submucosa with no demarcation (Figs.1,3,4). In the submucosa, connective tissue elements became less abundant while the blood and lymph vessels were larger. Eosinophilic granular cells were common in the submucosa (Fig. 6).

Subjacent to the submucosa was the tunica muscularis which was made up of two layers, an inner lamina circularis or circular layer and an outer lamina longitudinalis or longitudinal layer (Fig. 4). Interposed between the two muscle layers was the myenteric plexus (Fig. 4). The serosa formed the outer layer of the intestines and was a thin layer of loose areolar connective tissue, blood and lymph vessels and nerve fibres covered externally with a simple squamous mesothelium (Fig. 4).

Adjacent enterocytes adhered closely to each other, with little intercellular space. Microvilli were evident on the apical border (Fig. 7) and were covered by a layer of electron dense material (Fig. 8). The microvilli were composed of an electron dense core of tightly packed microfilaments that run parallel to the long axis of the cell extending to the terminal web region where they contributed to the complex interdigitating meshwork of filaments (Fig. 9). This meshwork of filaments extended across terminal web and terminated at the lateral plasma membrane. The terminal web occupied the apical cytoplasm and was distinct by being devoid of major cellular organelles. Microtubules and vesicles were recognised (Fig. 9). The apical plasma membrane oftentimes made invaginations into the terminal web cytoplasm to form pit-

like depressions.

The region below the terminal web was rich in cellular organelles (Fig. 9) including Golgi apparatus, mitochondria and endoplasmic reticulum (ER). The Golgi apparatus appeared as an aggregation of tubules, vesicles and stacks of cisternae with or without smooth or granular electron-dense, surfaces. These membranes were occasionally connected to the endoplasmic reticulum. The ER was smooth or had granular-like structures, the ribosomes. The mitochondria were rod shaped and characterized by cristae and a matrix with dense granules (Fig. 9). The nucleus was located subjacent to this organelle rich region and had an elliptical shape with a distinct envelope enclosing electron-dense granular chromatin masses (Fig. 7). Like the region above the nucleus, the region below was rich in cellular organelles.

The lateral plasma membrane was marked by specialized junction complexes that defined a close relationship between adjacent enterocytes (Fig. 9). Closest to the free border of the epithelium, was the zonula occludens which was very electron-dense. Below the zonula occludens was the second type of the junction, the zonula adherens. The third type of junction within the complex was the desmosome which was located below the zonula adherens.

Below the enterocytes was the basal lamina which formed the border between the epithelium and the lamina propria. It was composed of randomly oriented filaments closely associated with the connective tissue fibrils in the lamina propria. Mucous cells were distributed widely among the enterocytes along the intestines. They possessed basally located nuclei with cytoplasmic staining that reacted positively with periodic

acid Schiff stain (Fig.1). The goblet cells were characterized by the presence of numerous closely packaged granules that bulged in the apical cytoplasm with a narrow central region (Fig.10). These granules contained a homogeneous material that was bound by a membrane. The supranuclear cytoplasm contained various organelles including Golgi apparatus, rough endoplasmic reticulum, free ribosomes, mitochondria and lysosomes-like structures. They had lateral and basal membranes that closely related to the adjacent enterocytes by tight junctions and the basal lamina respectively. The goblet cells had microvilli that were structurally similar to the enterocytes though less numerous and regular in shape, and a poorly developed terminal web. The goblet cells had openings that appeared as pits on the luminal surface on observation under scanning electron microscope.

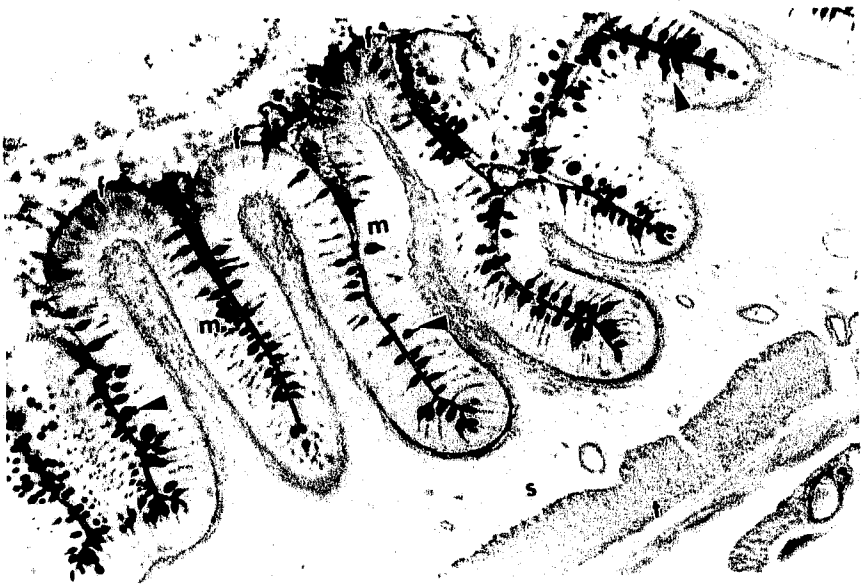


Fig.1 Photomicrograph of section from cranial intestines distal to the pylorus stained with PAS showing the general structure of the intestinal wall. Note the mucosal folding (f), tunica mucosa (m), tunica submucosa (s) and tunica muscularis (t). Numerous goblet cells among the enterocytes *(arrows)*. Mag. x240.

Fig.2 SEM micrograph of the intestines showing the convoluting mucosal folding (*bars*). Mag. x130

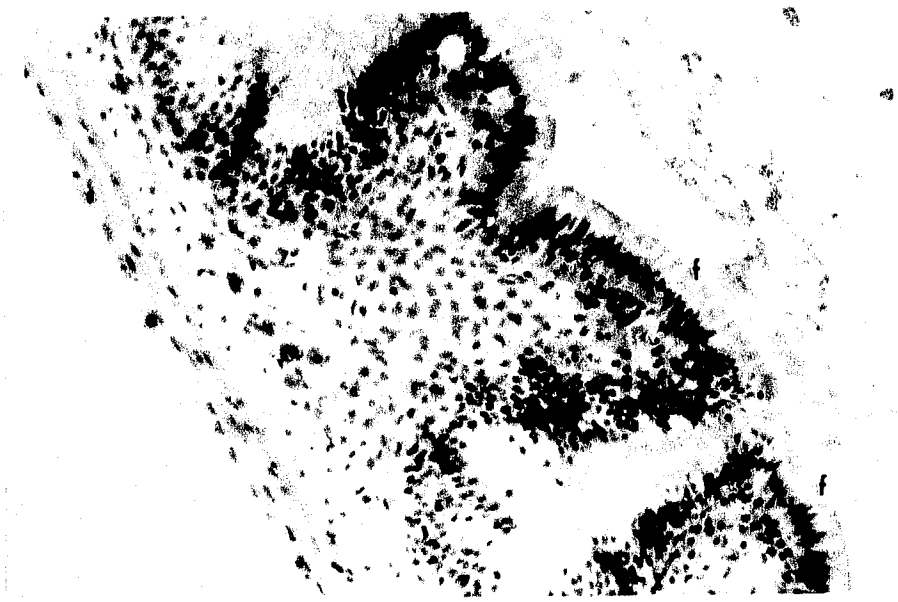


Fig.3 Photomicrograph of a section from the caudal intestines stained with Haematoxylin and Eosin. Note the shorter mucosal foldings (f). Mag. x240

Fig.4 Photomicrograph of a higher magnification of a section from the cranial intestines stained with PAS, showing the absence of any distinction between the lamina propria (P) and the tunica submucosa (sm). The lymphatics (l), blood vessels (b) are observed. The lamina circularis (c) and the lamina longitudinalis (lo) of the tunica muscularis are below the submucosa. The serosa (s) forms the outer border of the intestinal wall. Note and nerve ganglion in the myenteric plexus *(arrowhead)*. Mag. x640

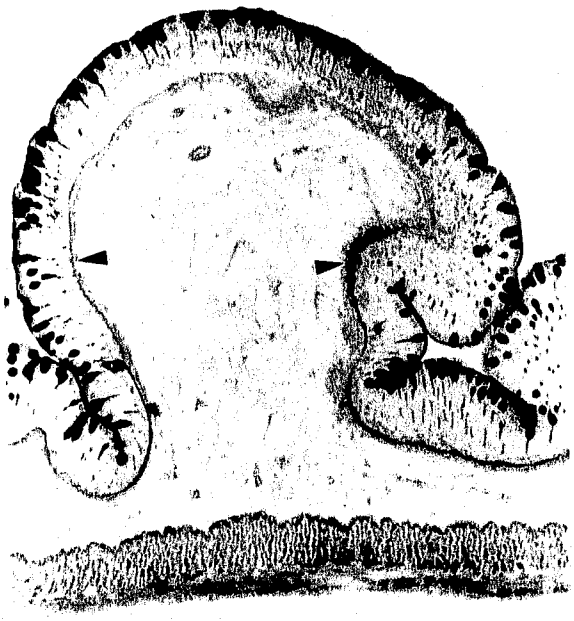


Fig.5 Photomicrograph of a section from the caudal parts of the intestines stained with PAS. Note the basement membrane (arrowheads). Mag. x240

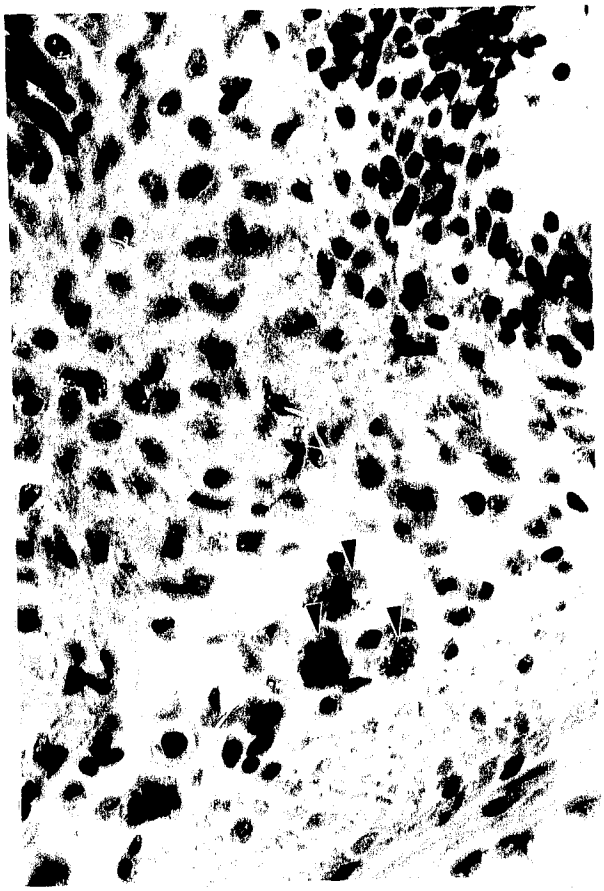


Fig.6 Photomicrograph of a section from the caudal intestines stained with Haematoxylin and Eosin. Note Eosinophilic granular cells (*arrowheads)* in the submucosa. Mag. x750

Fig.7 TEM micrograph showing enterocytes from the cranial aspects of the intestine. The microvilli (mv) form the brush border. Note the terminal web (Tb) region which has very few organelles. The region between the terminal web and the nucleus (n) is rich with cellular organelles. Mag. x3400.

Fig.8 TEM micrograph from the cranial intestine showing the microvilli (mv) and the glycocalyx (g) associated at the apical surface. Note the microvilli actin microfilaments *(arrowheads)*, running parallel to the microvilli. Mag. x30000

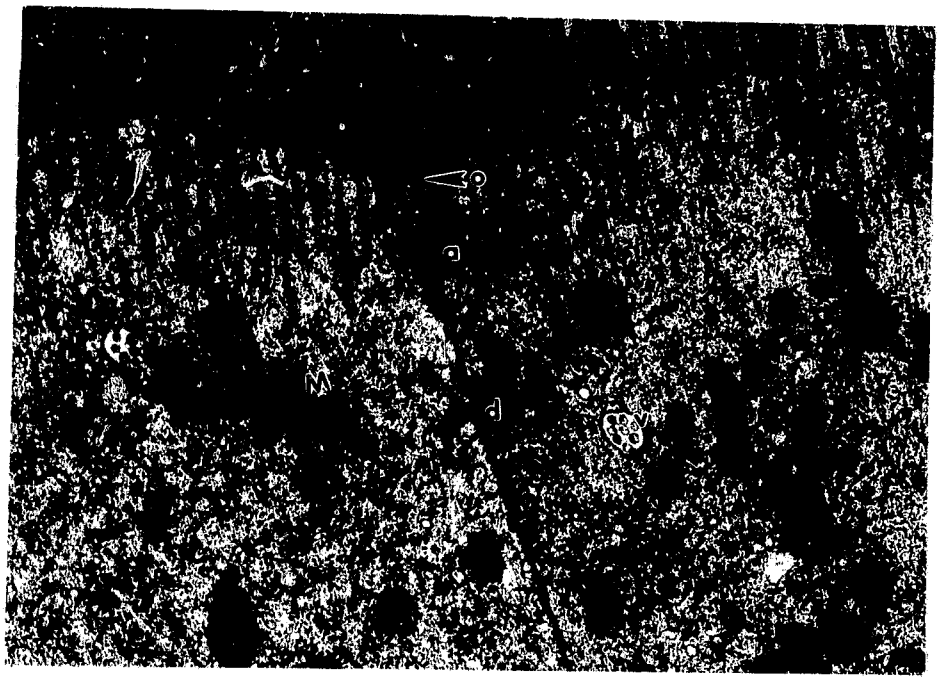


Fig.9 TEM micrograph of two adjacent enterocytes showing cell junctions; zonula occludens (arrowhead o), zonula adherens (a) and desmosome or macula adherens (d). Note the microvillar filaments streaking into the terminal web, cellular organelles including multivesicular bodies (v), mitochondria (m). Mag. x23000.

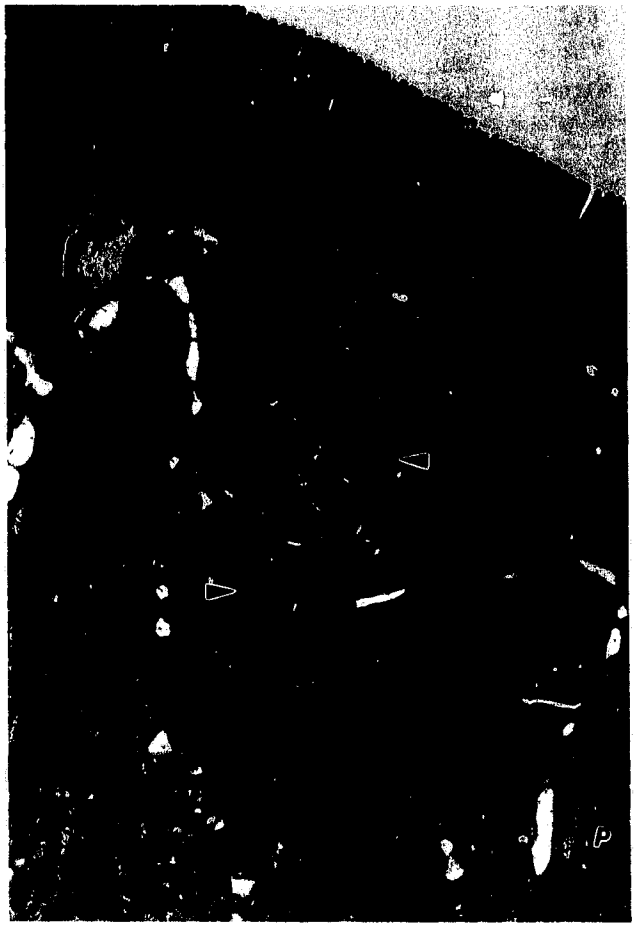


Fig.10 TEM micrograph of enterocytes adjoining a goblet cell. Note the closely packed granules *(arrowheads)*. Mag. x9750.

2.3.2 Histochemistry

The histochemistry reactions revealed the presence of both neutral and acidic mucosubstances in the intestines of Nile tilapia. The neutral mucosubstances were the most prevalent mucopolysaccharides in the goblet cells and in the mucous layer along the intestines (Figs. 11-13). Of the acidic mucosubstances, only sulphated mucin goblet cells were identified in the middle and caudal aspects of the intestines (Figs. 15,16). No carboxylated mucosubstance containing cells were observed. A summary of the histochemical reactions of the mucins are shown in Table I.

The mucus histochemistry of the mucous layer is shown in Table II. Neutral mucosubstances were dominant in the cranial (Fig. 14) sections of the intestines but diminished progressively caudally where traces were observed. The converse was observed with sulphated mucosubstances, whose abundance increased towards the caudal (Figs. 14,15,16). Traces of carboxylated mucosubstances were observed in the cranial aspects of the intestines (Fig. 12).

Table I. Mucus histochemistry of the goblet cells of the intestines of Nile Tilapia (*O. niloticus*).

Sectionof gut[1]	PAS[2]	Carboxylated Mucins AB(pH 2.5[3])	Sulphated Mucins AB(pH1.0[4])	Neutral Mucins
1	+++[5]	-	-	+++
2	+++	-	-	+++
3	+++	-	++	++
4	+++	-	++	++
5	+++	-	+++	-
6	+++	-	+++	+

[1]Section of Gut (1-6): sections representing consecutive division of gut progressing distally (see 2.2.2).

[2]PAS: Periodic acid Schiff.
[3]AB pH 2.5 Alcian blue with pH set at 2.5
[4]AB pH 1.0 Alcian blue with pH set at 1.0
[5]Degree of staining:
+++ intense
++ moderate
+ weak
- no reaction.

Table II. Histochemistry of the mucous layer on the luminal surface of Nile Tilapia (*O. niloticus*).

Section of gut[1]	PAS[2]	Carboxylated AB (pH 2.5[3])	Sulphated Mucins AB(pH1.0[4])	Neutral Mucins
1	+++[5]	++	++	+++
2	+++	++	++	+++
3	+++	+	++	++
4	+++	+	++	++
5	+++	+	+++	+
6	+++	+	+++	+

[1]Section of Gut (1-6): sections representing consecutive division of gut progressing distally (see 2.2.2).

[2]PAS: Periodic acid Schiff.
[3]AB pH 2.5 Alcian blue with pH set at 2.5
[4]AB pH 1.0 Alcian blue with pH set at 1.0
[5]Degree of staining:
+++ intense
++ moderate
+ weak
- no reaction.

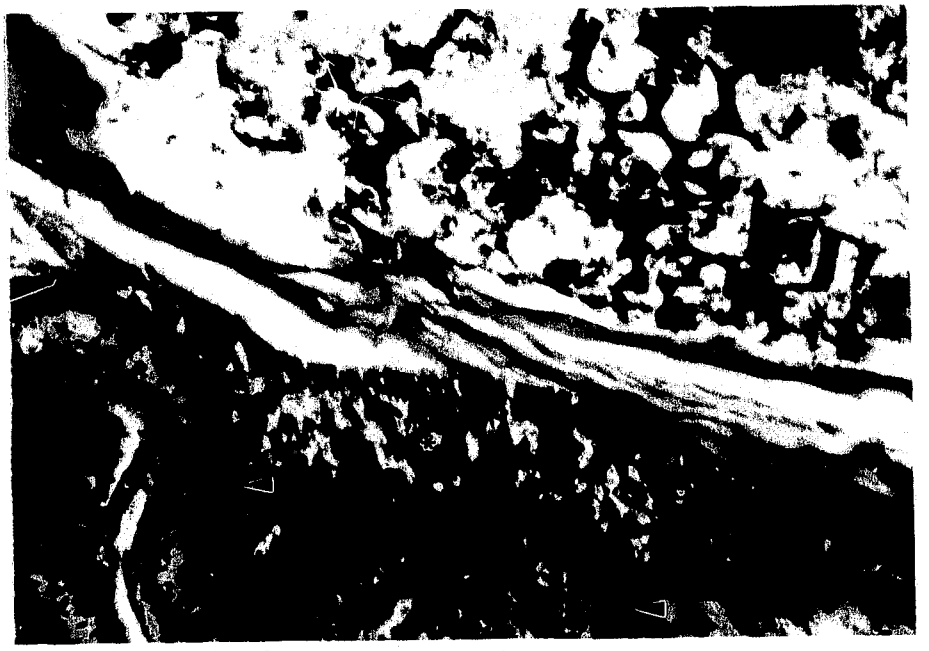


Fig.11 Photomicrograph of section from cranial aspects of the intestines stained with PAS and Alcian Blue (pH 2.5) showing presence of neutral mucosubstances in the goblet cells *(arrowheads)* and in the mucous layer (*). Mag. x320

Fig.12 Photomicrograph of section from middle aspects of the intestines stained with PAS and Alcian Blue (pH 2.5) showing presence of neutral mucosubstances in the goblet cells *(arrowheads)* and in mucous layer (*). Mag. x640

Fig.13 Photomicrograph of section from caudal aspects of the intestines stained with PAS and Alcian Blue (pH 2.5) showing neutral mucosubstances containing goblet cells (*arrowheads*) and in mucous layer (*). Traces of carboxylated mucosubstances are observed in the lumen (c). Mag. 640.

Fig.14 ı ɔtomicrograph of section from cranial aspects of the intestines stained with PAS and Alcian Blue (pH 1.0) showing presence of neutral mucosubstances in the goblet cells *(arrows)* and in the lumen. Note the sulphated mucosubstances in the mucous layer (*). Mag. x640.

Fig.15 Photomicrograph of section from middle aspects of the intestines stained with PAS and Alcian Blue (pH 1.0) showing presence of neutral mucosubstances in the goblet cells and in mucous layer (*arrows*). Note sulphated mucosubstances in the mucous layer (*). Mag. x640

Fig.16 Photomicrograph of section from caudal aspects of the intestines stained with PAS and Alcian Blue (pH 1.0) showing presence of sulphated mucosubstances in the goblet cells and in the mucous layer (*arrows*). Mag. x640

2.4 Discussion

The observations made in the study indicate the intestines of the Nile tilapia (*O. niloticus*) have adaptations consistent with a herbivorous diet. To provide an optimal surface area, the Nile tilapia have lengthy intestines organised into mucosa folds. The presence of a large surface area conforms to observations in other herbivorous fish and vertebrates, and is associated with digestion of carbohydrate rich foods (Steffens 1989). The mucosal folds formed by the bulging of the epithelium over the lamina propria core are a major adaptation for enhancing the surface area of Nile tilapia intestines available for digestion and absorption, and aids the mixing of food and digestive juices. They allow for the distension of the mucosal layer and are probably stretched following feeding. Any surface area lost due to lack of villi in the Nile tilapia is probably compensated for by the folds and the length of the intestines (Al-Hussaini 1947, Yasutake and Wales 1983). These findings are similar to those found in the intestines of most fish (Patt and Patt 1969).

The intestine of Nile tilapia had no regional differentiation, which is similar to observations made in *Tilapia mossambicus* (Pasha 1964), and *T. grahami* (Kayanja et al 1975). Though measurements were not made, a gradual cranial-to-caudal decline, and reduction of the mucosal foldings length and frequency was noted at gross level. A similar trend is noted in most fish (Burnstock 1959, Bishop and Odense 1966, Burke 1971). The intestine of the tilapia has no distinction such as the ileal caecal valve found in the salmonids (Ezeasor and Stokoe 1981), hence a morphologically distinct

rectum was not identifiable.

The tilapia intestinal wall has four layers, similar to that of the stomach of *O. niloticus* (Osman and Caceci 1991) and similar to the intestine of *T. mossambicus* (Pasha 1964). The simple columnar epithelium in tilapia is typical of the intestinal epithelium of vertebrates (Patt and Patt 1969). Being absorptive, the epithelial cells have numerous microvilli (Yamamoto 1966, Bishop and Odense 1966, Krementz and Chapman 1975). In the Nile tilapia intestines, the lamina propria cannot be structurally differentiated from the submucosa, though the density of connective tissue elements is higher towards the epithelia and the size of the blood and lymph vessels increases towards the tunica muscularis. The lack of any delineation between the lamina propria and the submucosa in the Nile tilapia intestines, differs markedly from other fish species especially carnivores that have various morphological features including the stratum compactum and stratum granulosum which also contribute to the overall tensile strength of the intestines (Burnstock 1959). Absence of these layers in Nile tilapia, may contribute to delicateness of the tilapia gut. The blood vessels transport amino acids and monosaccharides, while the lymph vessels deal with the fatty acids and triglycerides. The mucosal folds may be extended by the engorgement of the vasculature following feeding similar to the villi (Stinson and Calhoun 1993). In the submucosal regions, clusters of eosinophilic granular cells (EGCs) were commonly observed. These cells have also been observed in the salmonids (Ezeasor and Stokoe 1980A). Submucosal glands were not observed in tilapia, unlike sablefish (Bell et al 1986) and mammalian intestines (Fawcett 1986).

Numerous goblet cells are distributed among the enterocytes of Nile tilapia. The morphological appearance of the apical membrane of the goblet cells has few microvilli, and may appear incomplete depending on the physiological state (Mantle and Allen 1989). The mobility of these microvilli may serve a minor role in dispersal of mucus. Goblet cells produce mucus that is distributed on the mucosal surface of the gastrointestinal tract forming a hydrated and viscous layer. The main function of the layer is to protect the mucosal cells from potentially injurious contents of the intestinal lumen (Allen 1984).

Neutral mucopolysaccharide was the predominant goblet-cell secretion observed in the cranial intestines, which Grau et al (1992) speculated that this could be related to carbohydrate absorption. However, both carboxylated and sulphated mucus in the goblet cells were observed in the middle and caudal aspects of the intestines. While the mucous layer was predominantly composed of neutral mucopolysaccharide, sulphated types were identified particularly in the middle and caudal aspects of the intestines. The location of sulphated forms did not always coincide with presence of sulphated mucosubstance-containing goblet cells. This may be attributed to chemical changes of the mucous precipitated by luminal environment. The plasma membrane on the apical surface of the microvilli is covered by a glycocalyx. The glycocalyx is produced by the cells on which it is attached (Krementz and Chapman 1975). The glycocalyx is believed to be a major site for biochemical activity and participates in synthetic functions of the enterocytes (Egberts et al 1984).

The core of the microvillus is made of filaments extending to the terminal web region

in the apical cytoplasm, which according to Matsudaira and Burgess (1979) give structural support and some motility to the microvilli. The terminal web is composed of a complex filament network which stabilizes the cell from osmotic and mechanical shock (Trier 1968). The presence of microtubules, membrane-bound vesicles and vacuoles suggests the apical zone play a role in cytoplasmic transport. Yamamoto (1966) indicated the presence of the vacuoles related to presence of food, suggesting they could be fat droplets. Hull and Stehelin (1979) suggested the terminal web could be subdivided according to the level corresponding to the junction complexes into the apical, adherence and the basal region. Like in other fish and vertebrates (Gauthier and Landis 1972, Noaillac-Depeyre and Gas 1978, Fawcett 1986), the terminal web in Nile tilapia is distinguished by absence of cellular organelles.

The region below the terminal web is a major metabolic site characterized by presence of cellular organelles, including Golgi apparatus, mitochondria and endoplasmic reticulum (ER). Mitochondria are less abundant, similar to observations made in other fish (Gauthier and Landis 1972).

The relationship between adjacent enterocytes is very close due to the presence of specialized junctional complexes. The zonula occludens is closest to the free border of the epithelium and ensures that no seepage of undigested food, microorganisms and other luminal contents takes place between the two adjacent membranes. Any communication between the intestinal lumen and the extracellular space can only happen through the microvillar border and apical cytoplasm. The other junctions include zonula adherens and desmosome. Both are located respectively below the

zonula occludens and participate in fastening of adjacent enterocytes.

In conclusion the intestines of the Nile tilapia reflect adaptation for a herbivorous diet and are similar to those of other herbivorous vertebrates. The intestines are lengthy, and have extensive foldings of the mucosa. In order to fit in the abdominal cavity, the intestines are arranged in complex convolutions. The Nile tilapia intestines have no morphological distinction along the length of the intestines though progressive caudal tapering was observed. The intestinal wall has similar stratification like other teleost fish and vertebrates. As with most vertebrates, Nile tilapia have columnar-type epithelium with an apical microvillar brush border and goblet cells distributed among the enterocytes. The major mucosubstances in the cranial intestines are neutral mucosubstances and sulphated acidic mucosubstances in the caudal intestines. The mucus layer followed a similar pattern. The Nile tilapia intestines have no feature delineating the lamina propria from the submucosa, and have a bilayered muscularis composed of an outer longitudinal and inner circular laminae.

3. Demonstration of biofilm in Nile tilapia (*Oreochromis niloticus*) intestines using polyclonal antimucus serum.

3.1 Introduction

3.1.1 General characteristics of a biofilm

Biofilms are dynamic organic polymer matrices (Costerton et al 1987), immobilized at a substratum surface (Marshall 1992) within which mixed natural populations of microorganisms are located (Costerton et al 1987). They are ubiquitous on both biologic and abiologic surfaces immersed in an aqueous environment (Marshall 1992) and distributed in various ecosystems. Some of the studied ecosystems include the bovine rumen (Atkin 1976, Cheng et al 1981, Kudo et al 1987, Patterson et al 1975), oil industry aquatic systems (Costerton 1984, Mittelman and Geesey 1985), human female urethra (Marrie et al 1983), bile ducts (Sung et al 1992), and medical prostheses (Marrie and Costerton 1982, 1983. Marrie et al 1983, Nickel et al 1985). In situ examination of these ecosystems demonstrates that formation of biofilms is the predominant form of microbial growth and provides microorganisms with a protective strategy against various insults. On biological surfaces, the matrix is composed of bacterial exopolysaccarides and components like tissue glycocalyx (Costerton et al 1978, 1981).

Growth in a biofilm confers on the microorganisms shelter from various antibacterial adversaries including antibodies, phagocytic cells, antibiotics and bacteriophage (Costerton et al 1987). Due to the binding nature of the biofilm, nutrient trapping

occurs providing a substrate for microbial metabolism. The metabolic products from these reactions can be utilized by other microorganisms, resulting in microbial consortia communities (Costerton 1988). This organization promotes efficient energy utilization by encouraging microbial complementaries and synergism (Costerton et al 1987).

3.1.2 Formation of Biofilm

Microorganisms show a tendency to adhere to solid surfaces (Marshall 1976) which exert numerous influences on the microbe's behaviour (van Loosdretcht et al 1990). This behaviour is attributed to the ability of microbes to rapidly adapt to various environmental challenges, referred to as phenotypic plasticity (Brown and Williams 1985). The formation of biofilms involves four main processes: transport of the microorganism to the surface, adhesion, attachment and colonization.

Transport of microorganisms to surfaces is achieved by diffusion, convection or active transport. The diffusion mode of microorganism transport is a slow (Marshall 1976) and random process akin to Brownian motion and differs from convection which depends on fluid-flow dynamics (Characklis 1981). Active transport involves the movement of the microorganism by a motility mechanism like flagella or by chemodynamism (Allweiss et al 1977). Once the microorganisms are in close proximity to the surface, the next stage of colonization is adhesion, a physicochemical process that is either reversible or non reversible (van Loosdrecht et al 1989). Reversible adhesion represents a weak localization of the microorganism to the surface. This can be easily removed by small shear forces (Costerton et al 1987). Irreversible

adhesion involves a stronger relationship to the surface and the microorganism can only be removed by very strong shear forces (van Loosdrecht et al 1989).

In animal tissues including the intestines, microorganisms bound to the mucus may maintain a weak adhesion to the surfaces (Arp 1989). Following initial adhesion, organisms attach to the surface creating a stable relationship with the surface mediated by interaction of specialized cell surface structures on the microorganism and the surface. These structures which include fibrils and exopolymers ensure a very strong linkage (Arp 1989). The final step in the formation of biofilm involves the colonization of the surface (Marshall 1992). The microorganisms which are now strongly bound to the surface undergo division. Bacteria within a colony are attached to each another and bound within the exopolysaccharide matrix to finally form a continuous layer (Costerton et al 1981).

3.1.3 Studying Biofilms

Numerous methods of studying biofilms have been reviewed (Ladd and Costerton 1990, Blenkinsopp and Costerton 1991). The use of both scanning and transmission electron microscopy has been useful in establishing the structure and distribution of biofilms in various ecosystems (Cheng et al 1981, Mittelman and Geesey 1985). Scanning electron microscopy is an excellent tool for in-situ study of biofilms due to its three-dimension capability, allowing observation of extensive surfaces (Bozzola and Russell 1992). Transmission electron microscopy allows cross sectional examination of the biofilm and is useful in observing various structural features in its depth, and at higher resolution

than LM and SEM.

While electron microscopy is useful in demonstrating the biofilm, routine tissue processing can alter or destroy the biofilm. Dehydration and washing processes drastically condense, distort or denude the highly hydrated structures during processing (Costerton et al 1981).

The biofilms can be stabilized against condensation during processing by multiple-site anchorage (Costerton et al 1981). Workers have used different methods including specific antibodies against the mucous layer (Rozee et al 1982, Bollard et al 1986, Cornish et al 1987, Chan et al 1983, Morck et al 1987, Caputy and Costerton 1984), lectins (Costerton et al 1986), non-aqueous fixative (Sims et al 1991), and microwaves (Turner et al 1990). Various forms of very low temperature stabilization including rapid freezing (Carr et al 1983), low temperature SEM (Read and Jeffree 1991), freeze substitution (Ichikawa et al 1987) or combinations of acrolein vapour fixation, freeze-drying and osmium vapour (Speare and Mirsalimi 1992) have been used.

The objective of this study was to preserve and demonstrate biofilms on the luminal surface of the intestines of Nile tilapia (*O. niloticus*) by electron microscopy using rabbit antimucus antibody to stabilize the biofilm.

3.1.4 Summary of objectives

General objective

Establish a protocol for preservation and demonstration of biofilms in the intestines of the Nile tilapia (*O. niloticus*).

Specific objectives

1. Production of polyclonal antimucus antibodies.
2. Use of the polyclonal antimucus antibodies to stabilize the biofilms on the luminal surface of the intestines of Nile Tilapia (*O. niloticus*).

3.2 Material and Methods

3.2.1 Use of mucus antiserum to stabilize biofilms.

3.2.1.1 Preparation of antisera.

Five adult fish were anaesthetized as described in section 2.2.1.2.1 and dissected to exteriorize the intestines. The intestines were then incised longitudinally to expose the luminal surface of the intestines and washed gently with Dubelco's phosphate buffered saline (dPBS) at pH 7.4. The mucus was peeled off the luminal surface using the blunt edge of a surgical blade and suspended in dPBS. The pooled mucus was then homogenized by vigorous vortex. The homogenate was centrifuged at 1200g for 20 minutes to separate the large particulate matter. The supernatant fluid was passed through an .45 μ filter and dispensed in 1ml aliquots. Protein concentration was determined by refractometry, set at the absorbency level of 280nm. The filtrate was stored at -20°C prior to immunization.

3.2.1.2 Animal maintenance

Two young adult New Zealand Rabbits were used in the study. The animals were maintained in the Atlantic Veterinary College Animal Research Facility according to the guidelines provided by the Canadian Council on Animal Care. They were housed in spacious wire-mesh cages coupled to an air flushing system. The cages were placed in a well ventilated and lighted room. The rabbits were fed *ad libitum* on commercial rabbit pellets and continuously supplied with fresh clean water.

3.2.1.3 Immunization

One half of a millilitre of undiluted filtrate with a protein concentration of 770μg/ml, was thoroughly emulsified with 0.5ml of incomplete Freund's adjuvant. The rabbits were bled to provide unimmunized control serum and then 1ml of emulsion was administered subcutaneously to the rabbits at several sites in the dorsal aspects of the neck region.

A booster injection (0.5ml) was given after four weeks. The rabbits were bled seven days after the booster injection and blood was collected in non heparin vacutainers. Serum was collected and aliquoted into 1 ml amounts and stored at -20°C. Antibody activity was confirmed by gel diffusion test (Hudson and Hay 1989).

3.2.1.4 Stabilizing the biofilm

3.2.1.4.1 Fish management for stabilizing biofilms

The study was carried out on 10 healthy and normal adults Tilapia (*O. niloticus*) obtained from stocks held at the Atlantic Veterinary College Fish Health Unit's Aquatic Animal Facility. The fish for the study were randomly assigned to either a recirculating or flow-through fish holding system.

The recirculating system fish were maintained in 1-metre diameter circular tanks. Effluent water was drained into a collecting reservoir that allowed sedimentation of the large particulate matter. Sediment- free water was then pumped through a sand filter. The sand filter pressure was monitored frequently and the filter was backflushed when necessary. The water was then directed to the three-way valve titanium-plate heat

exchanger under computer control. Temperature was maintained at 27.5 +/- 1°C (average +/- SD) and was under constant electronic monitoring. The warm water was held in a holding tank before being supplied to the tanks of fish. Water flow was maintained at approximately 6 Litres/minute. Water was constantly being pumped simultaneously from the reservoir tank to a biofilter. The fish were fed a balanced diet in a 5 mm pellet diet. Air was delivered to each tank by a rotary vane blower. Daylengths were maintained at 12 hr light and 12hr dark.

The flow-through holding facility fish were maintained as described in 2.2.1.1

3.2.1.4.2 Dissection

Following euthanasia, the right abdominal wall was dissected and reflected to expose the viscera. Subsequently, the intestines were gently disentangled. Two groups of sections were made from the cranial, mid and caudal intestines of each fish. One group of sections was treated with antimucus antibodies, while the other served as control. The test sections were gently submerged in a vial containing 4 mls of freshly prepared solution of 5% glutaraldehyde containing 0.15% ruthenium red and 1ml of the undiluted antimucous sera. The antimucus sera in the fixative solution used for the control sections was replaced with pre-immunisation sera. Tissues were incubated at room temperature (21-25°C) for 1 hr and maintained at 4°C overnight. The tissues were then processed for scanning electron microscopy as described in 2.2.1.3.

3.3 Results

The use of fixative containing antimucus antibodies allowed the preservation of various forms of amorphous material and numerous microbial morphotypes constituting the biofilm on the microvillar surface of the intestine of Nile tilapia. (These are demonstrated in Figs. 17-30 and described below). The tissues treated with fixative lacking antimucus antibodies, had only remnants of the biofilms on the microvillar surface (Fig. 31).

The biofilms existed in several forms, which were reflected by the mucopolysaccharide present in the specific area. The most common form of the biofilm was a continuous blanket entirely obliterating the microvillar surface as demonstrated in Fig. 17. The surface of the layer was not smooth and appeared corrugated with debris strewn on the surface apparently from its erosion.

A second form of the biofilm was a sheet-like structure made of interdigitating strand-like components. This form of the biofilm appeared to peel from the microvillar surface where the layer was discontinuous (Fig. 18). The strand like components of the layer securely held microbial morphotypes within the matrix.

Different from these "bio-forms" was the mat-like biofilm, with a smooth surface (Fig. 19). This form of the biofilm had numerous discontinuous areas that revealed numerous microbial morphotypes embedded beneath the surface. In another form of the biofilm (Fig. 20) numerous microbial morphotypes are evident bulging beneath a biofilm matrix, that bound them to the microvillar surface.

A fluffy form of the biofilm was also recognized, with morphotypes identifiable within its depths (Figs. 21, 22). The mucopolysaccharide matrix was evidently scarce in some forms, revealing the microvillar surface on which large numbers of different bacterial forms were identified (Figs. 23, 24). Some of the bacterial forms had material condensed on them, which may have been the microbial glycocalyx (Fig. 24).

The presence of large numbers of microbial morphotypes was evident along the entire length of the intestines including the cocci (Fig. 23) and bacilli (Fig. 21) in different arrangements. Larger structures suspected to be protozoa were also observed (Fig.24). Different morphotypes including both the cocci and bacilli, either occurring singly, in chains or in clusters, were distributed heterogeneously on the luminal surface (Figs. 23,24,25). They were observed either within the mucous layer and permeating all levels of the biofilm (Fig. 26) or where the layer was obliterated, they appeared closely interacting and adherent to the mucosal surface (Fig. 25). Most of the microbial morphotypes had material adhering to them which could represent condensed glycocalyx (Fig. 24). The mucous layer was defined by structures of different shapes and sizes and electron densities embedded in a granular matrix (Figs. 27, 28). The layer varied in thickness and was located above a thin layer of denser material that may have been associated with the microvillar surface constituting the tissue glycocalyx (Figs. 28, 29, 30).

The biofilms on the luminal surface from different regions of the intestines of Nile tilapia (*O. niloticus*) showed a uniform pattern of microorganism morphotypes, nature of the matrix and amount of the mucosal surface that was covered. The biofilms on the

luminal surface of the intestines of Nile tilapia (*O. niloticus*) maintained in recirculation systems could not be distinguished from those of the flow-through system (Fig. 32).

Fig.17 SEM micrograph of a section from the cranial intestines, showing the biofilm covering and obliterating the mucosal surface of the intestines. Note the corrugations on the biofilm layer *(arrowheads)*. Mag. x8800.

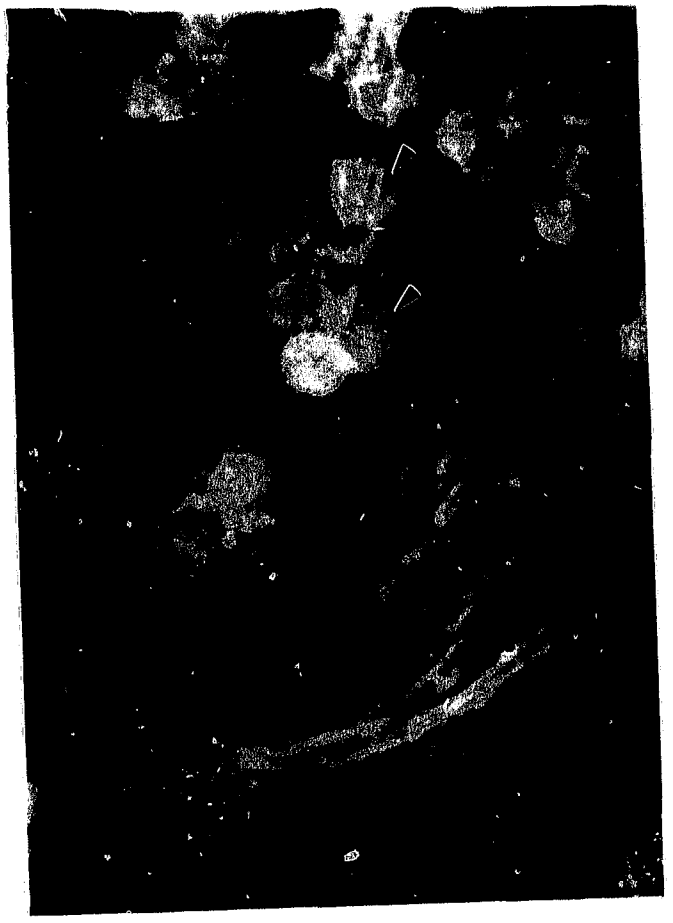


Fig.18 SEM micrograph of a section from the mid intestine, showing an intact sheet-like biofilm peeled from the luminal surface but still intact. Note the spherical structure representing microbes *(arrowheads)* and the strand-like meshwork of the matrix. Mag. x5400.

Fig.19 SEM micrograph of a section from the caudal intestines, showing the flat mat-like surface of the biofilm covering the mucosal surface. Discontinuous areas reveal bacilli microorganisms (*arrows-heads*). Mag. x2000

Fig.20 SEM micrograph of the edge of a section from the cranial intestines, showing the highly condensed matrix binding the underlying cocci and bacilli-microbial morphotypes (*arrowheads*) on the microvillar surface. Mag. x9000

Fig.21 SEM micrograph of section from the mid intestine, showing the fluffy nature of the biofilm. Note the microorganisms within the depths of the biofilm (*arrows-head*). Mag. x2600.

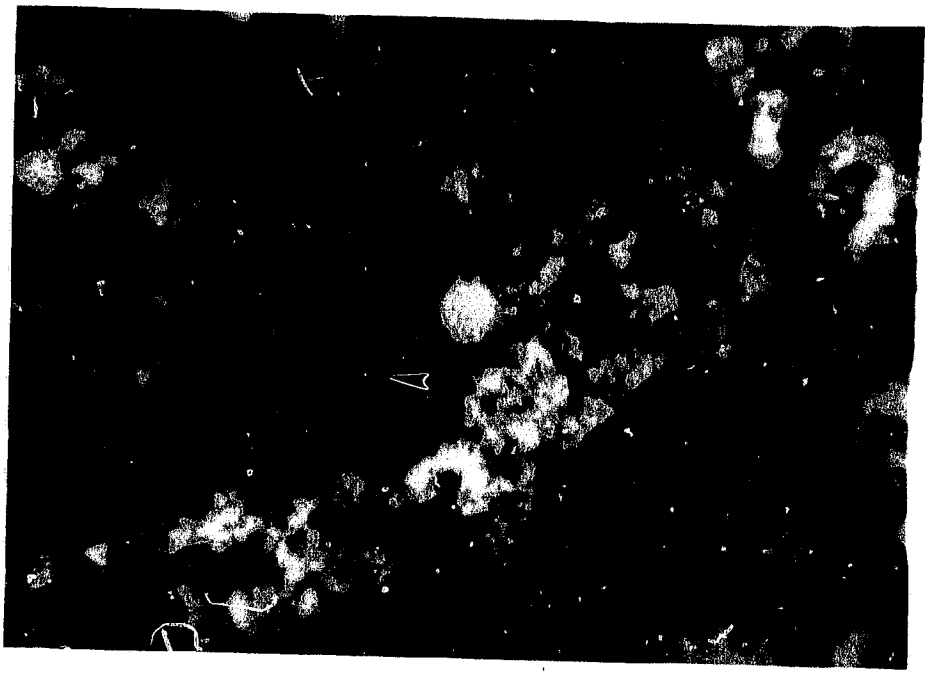


Fig.22 SEM micrograph of section from the caudal intestine showing the fluffy nature of the biofilm. Note the microorganisms within the depths of the biofilm (*arrows-head*). Mag. x2600.

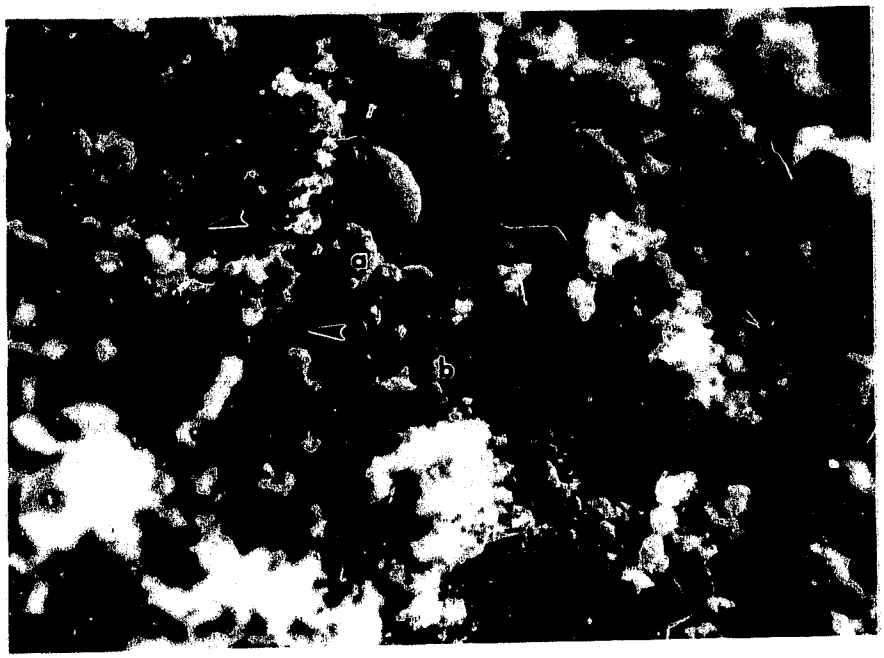


Fig.23 SEM micrograph of a section from cranial intestines, showing area with little mucous layer but numerous and different microorganisms in close spatial association. Note the large (a) and smaller (b) cocci, the latter in long and winding chains. The mucosal surface is visible in the exposed areas *arrows-heads*. Mag. x4200.

Fig.24 SEM of a higher magnification of Fig. 23. Note material on the bacterial surface *arrows-heads*, which may represent condensed bacterial glycocalyx and the presence of a protozoan-like morphotype. Mag. x16300.

Fig.25 SEM micrograph of a section from the mid intestines, showing microorganisms-mucosal surface interaction *arrow-heads*. Note the clustering (c) of the microorganisms and proximal arrangement. Mag. x9000

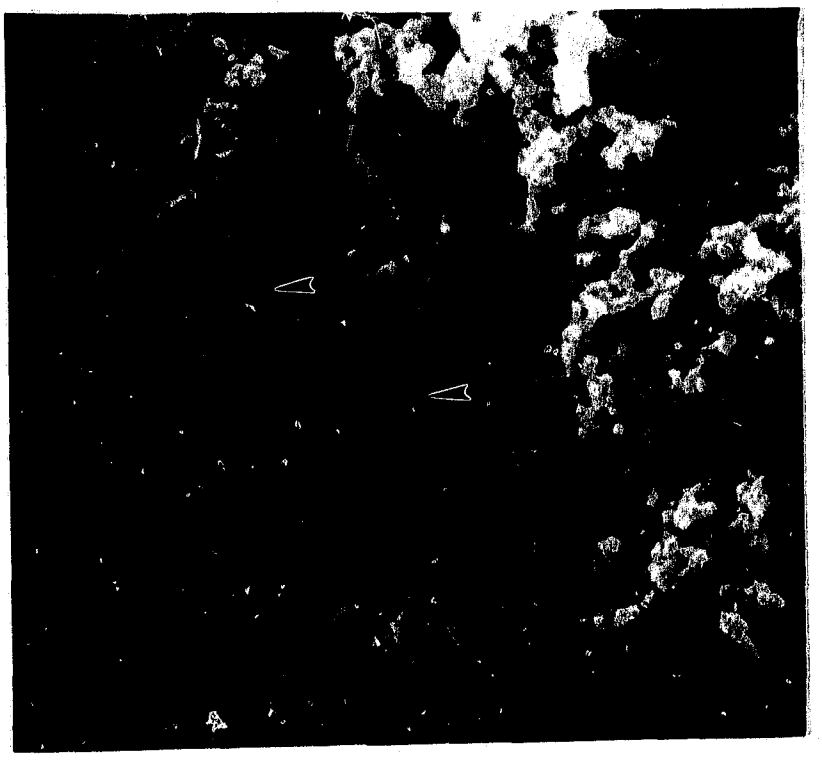


Fig.26 SEM micrograph from the caudal intestines, showing bacilli *arrowheads* microorganisms within the mucous layer at different depths. Mag. x7000

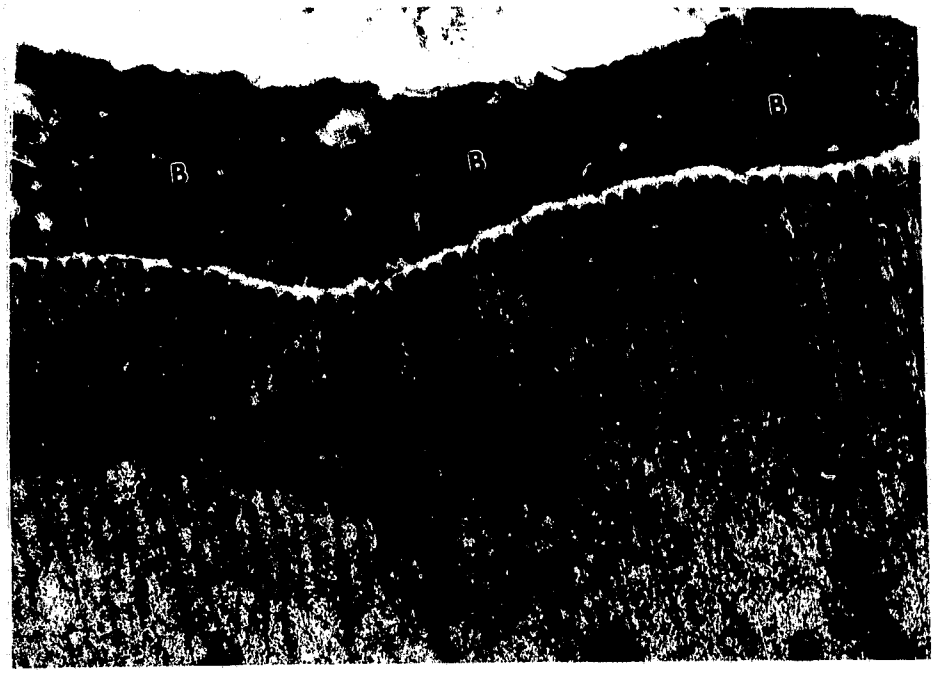


Fig.27 TEM of a section from cranial intestines, showing an electron dense biofilm (B). Mag. x17000.

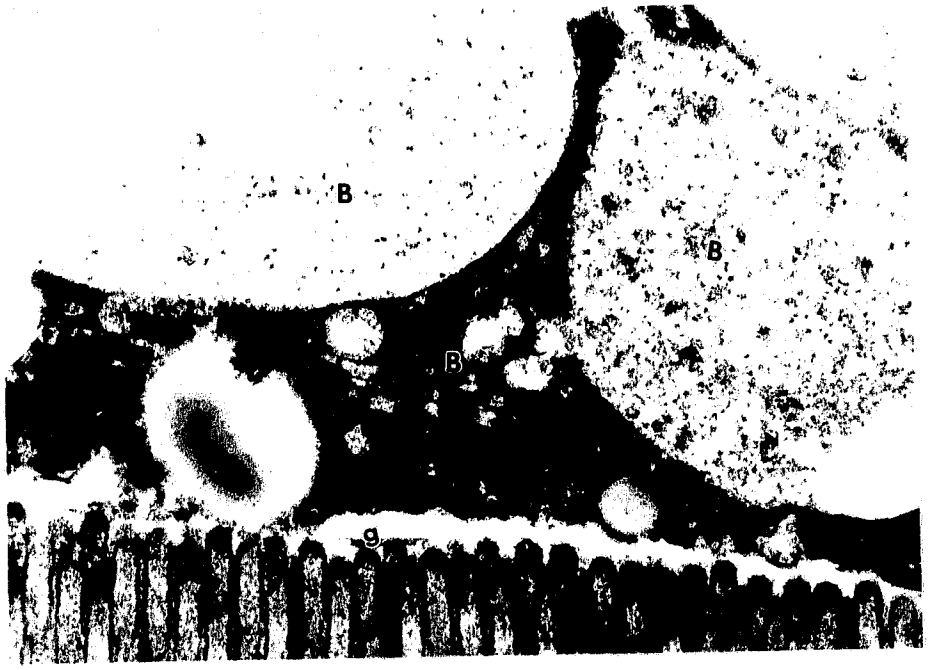


Fig.28 TEM micrograph of a section from the mid intestine, showing a granular-like consistency of the biofilm (B). Note the material (g) on the surface of the microvilli may represent tissue glycocalyx. Mag. x26000

Fig.29 TEM of a section from the caudal intestines, showing opening of the goblet cell (o) to the intestinal lumen. The mucus provides matrix for binding structures within the biofilms (B). The tissue glycocalyx (g) is distinct from the biofilm above the microvillar surface (mv). Mag. x29250.

Fig.31 SEM of luminal processed without antimucus stabilisation sera. Remnants of the biofilm are seen strewn on the luminal surface. Note the mucus pits of the goblet cell opening to the intestinal lumen *arrowheads.* Mag. x2900

Fig.30 TEM micrograph showing structural diversity of the biofilm (B) matrix. The tissue glycocalyx (g) is present on the microvilli (mv). Mag. x29000.

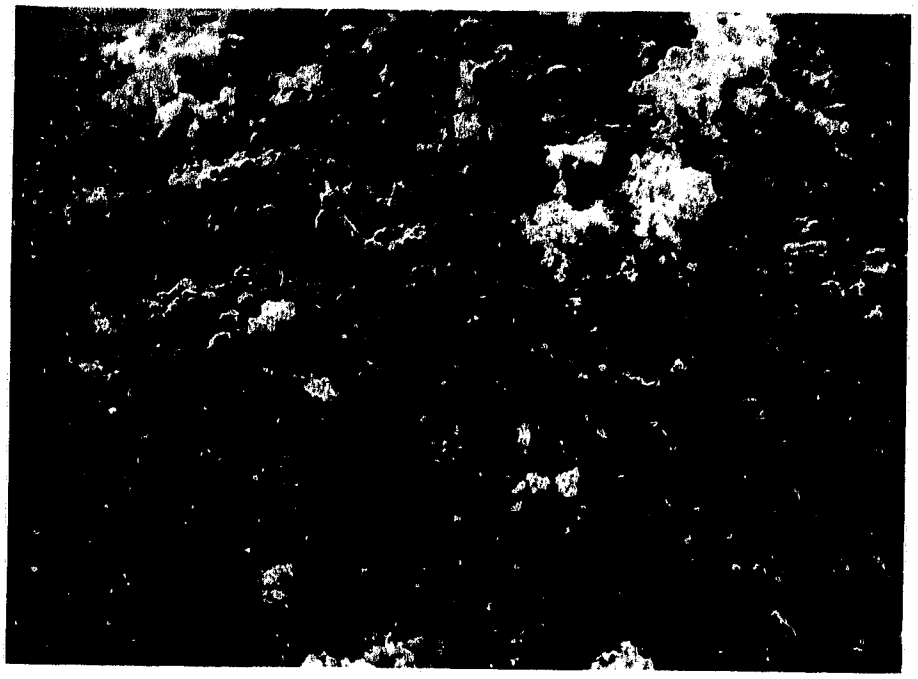


Fig.32 SEM of section from cranial intestines of fish held in flow through holding facility. Mag. x3000

3.4 Discussion

This study demonstrates the presence of a biofilm on the microvillar surface of the intestine of Nile tilapia. The contrast between the sections processed with antimucus antibodies incorporated in the fixative and those without suggests the antisera is effective in stabilisation and preservation of the biofilm.

The biofilm extends from the cranial to the caudal aspects of the intestines. Mucosubstances constitute the structural component of the biofilm forming the organic matrix within which the microbes are lodged.

The biofilm in intestines of Nile tilapia exists in several forms ranging from a continuous blanket layer that masks both the microvillar surface and the microbes in the biofilms, to a biofilm with scanty mucopolysaccharide and numerous visible microbial morphotypes. The presence of these numerous "bio-forms" indicate the biofilms of the Nile tilapia are heterogeneous. The intestine is a very dynamic organ associated with the passage of food through the tract and secretion of copious amounts of a variety of mucosubstances. These forms may reflect the dynamic nature of the biofilms, similar to the descriptions by Costerton (1987) on other biologic and abiologic surfaces. The different forms of the biofilm may also be a reflection of the various mucopolysaccharides found in different regions of the Nile tilapia intestines. The study demonstrated different microbial morphotypes, reflecting the diversity of the microbial ecology of the gut. Microorganisms in the intestines of the Nile tilapia can exist suspended in the biofilm or attached to microvillar surface. The appearance of different

morphotypes in close proximity may suggest the presence of microbial consortia. The development of consortia is characteristic of biofilms (Costerton et al 1987) associated with a close relationship between the microbes. These relationships often reflect a hierarchical microbial arrangement based on nutrition (Costerton et al 1987). Consortia's development in the intestines of the Nile tilapia maybe associated with the microbial component of digestion (Moriaty 1982), mainly involved in the breakdown of the largely vegetative material that the Nile tilapia ingest.

The biofilm demonstrated in intestines of tilapia maintained in flow-through and in recirculating systems had similar patterns in morphological diversity, abundance of microorganisms, and extent of the microvillar surface covered.

The establishment of the biofilms in the intestines of Nile tilapia provides an opportunity for further studies on the association of bacterial population in the Nile tilapia intestines and digestion. In the bovine rumen, about twenty-three taxonomically distinct species have been identified (Cheng and Costerton 1983), including species that produce large amounts of urease thereby playing a physiological role in ruminal component of urea metabolism. In fish, microbes contribute to the digestive process of herbivorous fish (Rimmer and Wiebe 1987).

Costerton et al (1985) suggested the presence of a bacterial biofilm indicates the various bacterial species involved are well adapted to the niche they have established. The implication of the biofilm is that the tissue surface occlusion provides a barrier by the competitive autochthonous population. Consequently, a potential pathogen must gain

dominance in the complex biofilm ecosystem, before penetrating to the mucosa. The significance of this scenario has been demonstrated in the new born calf where the development of a dynamic microbial population was associated with the end of the susceptibility to enterotoxigenic *Escherichia coli* (Moon 1974). Further investigation in the association of biofilms with pathogenic microbes in the Nile tilapia intestines may provide an understanding of bacterial pathogenesis.

4. Use of monoclonal antibodies to identify *Aeromonas hydrophila* in the intestinal biofilm of Nile tilapia (*Oreochromis niloticus*).

4.1 Introduction

4.1.1 Taxonomy

A. hydrophila is a pathogenic bacterium that affects a wide range of both ectothermic and endothermic animals. The genus *Aeromonas* was proposed by Kluyrev and van Niel in 1936 (quoted in Khardori and Fainstein 1988) and is currently classified in the family *Vibrionaceae* along with the genera *Plesiomonas, Vibrio* and *Photobacterium* (Popoff 1984, Schubert 1984). It is divided into two groups, motile and non-motile (Austin and Austin 1987). The non-motile group consists of *Aeromonas salmonicida* while the motile aeromonads encompass the rest of the species including *A. hydrophila.* The taxonomy of *A. hydrophila* remains incomplete due to the phenotypic, serologic and genotypic heterogenicity of the genus (MacLaines et al 1979, LeBlanc et al 1981, Allen et al 1983), and is confounded by a lack of strain specific, and virulence associated markers (Kokka et al 1991). The taxonomy of the genus has been the subject of a long debate, the evolution of which is described by Popoff and Lallier (1984). Use of molecular techniques (Popoff 1981, Kuijper et al 1989A, Kuijper et al 1989B), and the epidemiological linkage of *A. hydrophila* to human gastroenteritis (George et al 1985), has renewed the efforts at classification of the species and identification of the virulence factors (Janda 1991). Popoff et al (1981) found there were seven DNA-DNA hybridization groups (HGs) within the four phenotypes

identified biochemically. Additional groups have been identified bringing the total number to twelve (Fanning et al 1985). Carnahan et al (1991B) have since identified an additional genotype with unique antimicrobial susceptibility. Classification using hybridization techniques is far from complete as the HGs have not been comprehensively matched to the biochemical phenotypes (Arduino et al 1988, Farmer et al 1986). Consequently, MacDonnell and Colwell (1985) proposed the creation of a new family, *Aeromonadaceae* and the transfer of the genus *Plesiomonas* to another family. Meanwhile, various workers have proposed designating names for some of the *Aeromonas hydrophila* strains including, *A. schubertii* (Hickman-Brenner et al 1988), *A. veronii* (Hickman-Brenner et al 1987), *A. jandaei* (Carnahan et al 1991A) and *A. trota* (Carnahan et al 1991B). Schubert and Hegazi (1988) proposed *A. eucrenophila* for a strain resembling *A. caviae*. Evaluation of the monosaccharide profile of the cell wall lipopolysaccharide (Shaw and Hodder 1978), and the use of multilocus enzyme analysis (Altwegg et al 1991) may lead to better strain assignment (Janda 1991).

4.1.2 Morphology and general characteristics

A. hydrophila is a Gram negative rod that appears straight or curved with rounded ends, measuring 0.8-1.0μm by 1.0-3.5μm. It is motile with a single polar flagellum (Austin and Austin 1987) though a lateral flagellum has been observed (Khardori and Fainstein 1988). *A. hydrophila* is a facultative anaerobe, asporogenous and produces oxidase (Hsu et al 1985), with guanine plus cytosine DNA ratios ranging from 58-61.6 moles % (McInnes et al 1979). It is not susceptible to the vibriostat 0/129 (2,4-diamino-6,7-

diisopropylpteridine) (Austin and Austin 1987). Gas liquid chromatography studies on the oligosaccharide core of the cell wall lipopolysaccharides show a monosaccharide profile that is distinct from *A. caviae* and *A. sobria* (Shaw and Hodder 1978).

4.1.3 Isolation and identification

A. hydrophila grows well in routine media like Trypticase Soy Agar (TSA) and nutrient agar where small, white rounded opaque colonies appear after 24-48 hours at temperatures between 25-37° C. The appropriate selective/differential media include Rimler-Shotts media (Shotts and Rimler 1973), Peptone Beef extract Glycogen agar (McCoy and Pilcher 1974) and Pri-xylose-ampicillin agar (Rogol et al 1979). It grows in Eosin Methylene blue agar, Salmonella- Shigella media and Triple-Sugar Iron (Kaper et al 1979, Khardori and Fainstein 1988). The normal temperature range for growth is between 25-37° C with optimum around 30^0 C. However, growth above 35° C inhibits the growth of *A. salmonicida.* This can be used to differentiate these two bacteria (Sanders and Fryer 1988). *A. hydrophila* has an optimum pH range of between 5.5-9.0 and tolerates high turbidity.

A. hydrophila ferments carbohydrates, which has been useful characterizing biochemical phenotypes (Hsu et al 1985, Wakabayashi et al 1981, Kokka et al 1991). It produces catalase, arginine dehydrogenase and oxidase but no lysine or ornithine dehydrogenase (Boulanger et al 1977). These biochemical procedures remain the most feasible method of identifying the species at phenotypic level (Janda 1991). In serology, tube and macroagglutination, indirect fluorescence antibody test (IFAT) and slide agglutination

has been useful, the latter especially in field conditions, for tentative identification (Eurell et al 1978).

4.1.4 Pathogenicity, virulence and colonization

There is controversy as to whether *A. hydrophila* is a primary or secondary pathogen. Bhat et al (1974) reported *A. hydrophila* was involved in acute diarrhoea in humans as a primary pathogen. However, evidence for involvement as a secondary pathogen was given by Groberg et al (1978) who injected fish with *A. hydrophila* and found a stress-induced relationship between infectivity and temperature. Pollutants have been shown to increase the susceptibility of channel catfish to *A. hydrophila* (Hanson and Grizzle 1985) with epizootics linked to stress factors (Nieto et al 1985), while in humans, a fatal septicaemia was associated with a debilitating leukaemia (Davis et al 1978) and exposure of wounds to polluted water led to infection (Joseph et al 1979, Wolff et al 1980).

The virulence of the genus *Aeromonas* is polygenic and involves complex interactions with the host (Janda 1991). The virulence factors of *A. hydrophila* fall under 3 categories; structural features, extracellular features and cell-associated factors. The structural features include pili (Ho et al 1990, Honma and Nakasone, 1990) demonstrable by electron microscopy (Carrello et al 1988), whose expression is temperature related. It is involved in host colonization (Janda 1991).

The presence of an S layer in some strains of *A. hydrophila* isolated from fish has been established (Dooley and Trust 1988). Though the role of this layer has not been

determined, studies on the surface array protein (SAP) which compose the S layer have established substantial similarities to that of *A. salmonicida* (Dooley et al 1988). However, Kokka et al (1990) suggests their roles may be different. The outer membrane proteins (OMP) and the flagellum have been analyzed (Aoki and Holland 1985, Kuijper et al 1989A, Homna and Nakasone, 1990) yet association with virulence has not been determined.

The extracellular factors include haemolysins, proteases, amylase and chitinase. *A. hydrophila* has both α and ß- haemolysins (Brenden and Janda 1987, Janda et al 1984). The haemolysins are part of a larger group of cytolysins, which result in cell destruction due to disruption of the cell membrane integrity (Burke et al 1982, Stelma et al 1986). Studies on the ß-haemolysin have indicated it is an enterotoxigenic cytolysin (Asao et al 1984, Asao et al 1986). An enterotoxin distinguishable from the haemolysin has been demonstrated (Ljungh and Kronevi 1982). Five proteases associated with the genus *Aeromonas* have been identified (Nieto and Ellis 1986) and two have been linked to *A. hydrophila* (Leung and Stevenson 1988) virulence in catfish (Thune et al 1982). The amylase and chitinase have been identified and show similarities to those isolated from other microorganisms (Gobius and Pemberton 1988). A low molecular weight siderophore with high affinity for various forms of iron has also been identified (Barghouthi et al 1989).

The cell associated factors are determinants that are affiliated with the cell surface and cytosol which unlike the extracellular factors are not elaborated externally (Janda 1991). Those identified with *A. hydrophila* include invasins which are invasion factors

(Lawson et al 1985), serum resistance factor (Mittal et al 1980) and adhesins- adherent factor (Levett and Daniel 1981). The adhesins recognize the D-mannose and L-fucose side chains of eucaryotic cell-surface polymers (Trust et al 1980) and may be responsible for the chemotaxis to freshwater fish mucus (Hazen et al 1982), which may enhance the colonization of host cell glycocalyx and the biofilm. Their association with disease has not been conclusively determined. Most *A. hydrophila* strains possess plasmids, with a 20-58% frequency of carriage and high variation (Chang and Bolton 1987, Toranzo et al 1983) and are associated with drug resistance (Toranzo et al 1983, Hedges et al 1985).

4.1.5 Pathology

A. hydrophila has been implicated with disease in many ectothermic and endothermic animal species (Khardori and Fainstein 1988). In snakes and lizards it causes cutaneous haemorrhages and ulcerations with haemoptysis and terminal convulsions (Carlton and Hunt 1978, McGrath et al 1977), septicaemia and stomatitis associated with pneumonia. *A. hydrophila* has been causally linked to red leg in frogs (Shotts et al 1972) and has been associated with abortion in cows (Wohlegemuth et al 1972), sepsis in dogs (Pierce et al 1973), pneumonia and dermatitis in dolphins (Cusick and Bullock 1973) and diarrhoea in pigs (Dobrescu 1978). In humans, it is associated with numerous types of infection; enteritis (Chatterjee and Neogy 1972), meningitis (Quadri et al 1976), endocarditis (Ben-Chetrit et al 1983), and dermatitis (Smith 1980).

The high density of aeromonads in aquatic environments has been implicated in fish

disease (Esch and Hazen 1978) leading to losses in sport and commercial fisheries (Miller and Chapman 1976). Fish are susceptible to *A. hydrophila* which is associated with disease in rainbow trout (Mittal et al 1980), catfish (DeFigueiredo and Plumb 1977) and red sore disease in bass (Hazen et al 1978). *A. hydrophila* is the major cause of haemorrhagic septicaemia in fresh water fish (Frerichs 1989) causing gill haemorrhages and skin lesions including cutaneous ulceration, scale protrusions, and fin rot.

Disease can be acute, chronic or latent (Cipriano et al 1984, Grizzle and Kiryu 1993). The acute disease syndrome is normally accompanied by rapid and often fatal septicaemia with few gross signs. Occasionally clinical signs include exophthalmia, skin erythema, abdominal distension due to ascitic fluid, anaemia and fluid accumulation in scale pockets. The kidney and liver are usually swollen and friable (Huizinga et al 1979). The liver may be pale or green.

The chronic syndrome characteristically involves the skin, with focal haemorrhage and inflammation, erosion and/or ulceration commonly seen as fin rot and scale loss. The vent is protruded and haemorrhagic. The intestines are devoid of food and contain copious amounts of mucus. The histopathology of the spleen reveals intense aggregation of macrophages. In the latent disease, the fish appear healthy with no external lesions though internal lesions including hepatic necrosis may be observed at necropsy.

4.1.6 Objectives

The demonstration of biofilms on the epithelial surface of the intestines of Nile tilapia, shows presence of a heterogenous population of bacterial and protozoan morphotypes embedded in a matrix composed of mucus and bacterial exopolysaccharide (Chapter 3). The objective of the study was to investigate the relationship between *A. hydrophila* and the biofilm of the intestines of the Nile tilapia using an immunofluorescent antibody test (Kawamura 1977). The biofilm of the Nile tilapia intestines was challenged with *A. hydrophila* and the latter monitored over time. The monitoring was done to establish if the *A. hydrophila* associates with the biofilm and subsequently determine its clearance from the biofilm. This information would consequently help determine whether *A. hydrophila* establishes autochthonity in the biofilm. Prior to this study, the *A. hydrophila* endogenous to the fish holding facilities were investigated and reacted to a monoclonal antibody (IgG) generated against the ß haemolysin (Kozaki et al 1988) obtained from Dr. Shunji Kozaki at Department of Veterinary Studies, University of Osaka Prefecture, Osaka, Japan. The reaction to the monoclonal antibody was used to identify and select the strain to be used in the challenge study. The study was carried out in two types of containment facilities viz., the flow through and the recirculating systems. The two systems represented the prevalent systems the tilapia are contained in. The flow through system represented the intensive systems, while the recirculating system simulated the ponds where most of the tilapia are produced.

4.1.7 Summary of objectives

General objective

Investigation of the relationship of *A. hydrophila* and the biofilms in the intestines of Nile tilapia (*O. niloticus*).

Specific objectives

1. Determine the *A. hydrophila* profile of the tilapia containment system.
2. Establish presence of *A. hydrophila* in the biofilm of Nile tilapia.
3. Determine the relationship between *A. hydrophila* numbers, location in the intestines and clearance time in the biofilm of Nile tilapia.

4.2 Materials and Methods

4.2.1 Bacteriological evaluation of the tilapia containment system

4.2.1.1 Water sampling

Bacteriological evaluation was done to determine whether *A. hydrophila* was present in the system, and to determine if any isolates reacted to the B7 monoclonal antibodies (see 4.2.2.1).

The sites of sampling were:

i) Recirculating system (Appendix IV): The fish intestinal tract and the water column.

ii) Flow through system (Appendix V): Water entry point, water column, water exit, and the fish intestines.

Water was collected from all points using 10 ml sterile tubes. To ensure that water sampled for microbes represented both planktonic and sessile phases, the tanks were scraped on the side and mixing done by the aeration and fish activity. During this time the water supply through the flow through tanks was halted.

4.2.1.2 Total bacterial count in Nile tilapia intestines

Three fish were obtained from each of the flow through, and recirculating system. They were euthanized and dissected aseptically. The intestines were divided into six consecutive regions. Sampling was done about 1 cm distal to the cranial aspect of each region. A slit of about five millimetres long was made at the site of bacteriological

sampling. A sterile swab was then inserted and gently agitated. The swab was then rinsed by stirring in 10 mls of dPBS. One ml of this solution was transferred to 9 ml of sterile dPBS and vortexed to disperse the bacteria. Subsequently, 1 ml of the diluted samples was placed into sterile petri-dishes, with each dilution plated in triplicate. Fifteen ml of liquid trypticase soy agar (TSA) were added to the petri dishes and gently agitated on a level surface to ensure the inoculum was evenly distributed in the medium. The plates were incubated at 35°C for 24 hrs and the number of colonies on the TSA plates enumerated using a Quebec dark field colony counter. (The appropriate aerobic incubation temperature was determined before the study by comparing similar trial samples at 22°, 27° and 35° C. Finding no variation between the different temperatures, 35°C was selected.).

4.2.1.3 *A. hydrophila* isolation and identification

From the original dilution, 0.5 ml samples were inoculated on Rimler-Shotts plates and incubated at 35°C. Colonies from the plates were tested for: Gram reaction, cytochrome oxidase reaction, sulphide indole motility, glucose fermentation, 0/129 susceptibility, arginine dehydrogenase, Voges Proskauer, salicin hydrolysis, esculin hydrolysis, blood agar haemolysis. The cytochrome oxidase reaction was carried out on colonies reinoculated in tripticase soy agar. *A. hydrophila* strain # 35 was obtained from the Atlantic Veterinary College Fish Health Unit stocks to serve as a control.

4.2.2 Introduction of *A. hydrophila* to the Nile tilapia's intestinal environment.

4.2.2.1 Purification of B7 monoclonal antibodies.

Monoclonal antibodies (IgG) generated against the ß haemolysin (Kozaki et al 1988) were obtained from Dr. Shunji Kozaki at Department of Veterinary Studies, University of Osaka Prefecture, Osaka, Japan. These monoclonal antibodies designated B7, were shipped in mouse ascitic fluid.

The antibodies were purified using the Protein G affinity chromatography technique, using a HiTrap™ (Pharmacia LKB Biotechnology) affinity column. The column contained agarose beads cross-linked to recombinant Protein G (Sepharose^R, Pharmacia LKB) gel by N-hydroxysuccinimide activation. The gel supplied was preserved in 20% ethanol.

The mouse ascitic fluid was dialysed overnight at 4°C against 20mM Na-Phosphate pH 7.0 to adjust the pH and ionic strength. The gel was washed with 20 ml of the buffer to remove the ethanol preservative. Subsequently, 10 mls of the buffer were passed through the column to equilibrate the gel to the buffer. The dialysed mouse ascitic fluid was applied to the column. Twenty ml of the buffer were then flushed through the column to remove any unbound protein. The column was washed with 15 ml of 0.1 M glycine-HCl at pH 2.7 to elute the monoclonal antibodies which were collected in 5 ml tubes containing 110 µl of Tris-HCl buffer pH 9.0 per tube to bring the final pH to 7. Protein concentration was determined by absorbance standardized at 280nm.

4.2.2.2 Preparation of the *A. hydrophila* bath.

Introduction of *A. hydrophila* to the intestinal environment of Nile tilapia (*O. niloticus*) was achieved by exposing the fish to high concentrations of the bacteria (Beveridge et al 1989) selected using the B7 monoclonal antibodies. Strain # 35 was selected since the endogenous *Aeromonas* did not react with the monoclonal antibodies. *A. hydrophila* strain #35 was inoculated in brain heart infusion (BHI) media at 35°C for 24 hr. Broth cultures were vortexed to disperse the microbes and avoid clusters, and then transferred to the 20 litre experimental tank. The suspension was dispersed by agitation using a sterile applicator and aeration. Twelve fish, six from the flow through and six from the recirculating systems respectively were then introduced into the container for 15 minutes and subsequently transferred to new tanks maintained as in the original stocks. Two fish, one from each of the flow through and the recirculating systems, were then sacrificed at 12, 24, 48, 72, 96, 120 hrs post exposure. A similar number of control fish were transferred to new tanks and similarly maintained as in the original stocks.

4.2.2.3 Demonstration of *A. hydrophila* by Indirect Fluorescent Antibody Test (IFAT).

4.2.2.3.1 Tissue preparation

The test fish and the controls were euthanized, dissected and the intestines gently exteriorized as described in section 2.2.1.2.2. Immediately, the gut was divided into 6 consecutive regions. The intestinal regions were then opened longitudinally and gently washed using with sterile DPBS prechilled at 4°C. Using the blunt edge of a surgical

blade the mucous layer was gently peeled off and pooled in 0.5ml of sterile PBS. Samples were homogenized by vortexing and placed on treated microscope slides. These slides were allowed to air dry at room temperature.

4.2.2.3.2 Indirect Fluorescent Antibody Test

The smears were fixed in acetone for 10 minutes at -20°C then rinsed with PBS solution. Subsequently, the smears were incubated with 20% normal goat serum in PBS containing 0.5% Bovine Serum Albumin (BSA) for 20 minutes at room temperature. The slides were then divided into 3 groups. Slides in the test group were incubated and treated with 100 µl of B7 monoclonal antibodies diluted at 1:100 at 37°C for 10 minutes in a moist chamber. Slides from the second and third groups were treated with an unrelated monoclonal antibody and PBS respectively replacing the B7 monoclonal antibodies and acting as controls.

The slides were then washed in DPBS for 30 minutes and incubated with a fluorescein-isothiocyanate (FITC) conjugated goat antimouse IgG-Fc fragment (Cedar Lane Laboratories Ltd. Hornby, Ontario) diluted at 1:50 and containing a drop of Evan's Blue stain for 30 minutes at room temperature in a light-proof moist chamber. They were then rinsed and washed in dPBS for 20 minutes at room temperature. The slides were mounted with FA mounting media and examined using an epifluorescence microscope.

The number of fluorescent microbes was determined and scored on a numerical reaction index ranging from 0-5. The index was designed as follows:

0 No fluorescence was observed.

1 Fluorescence observed and number of identifiable fluorescent microbes under 10.

2 Fluorescence observed and number of microbes between 10-50

3 Fluorescence observed and number of microbes between 51-100

4 Fluorescence observed and number of microbes between 101-200

5 Fluorescence observed and number of microbes >200 and corresponding to the positive control made from a pure culture.

4.2.2.3.3 Controls

Controls were set up as follows:

a. Fish not transferred in the *A. hydrophila* suspension served as control for the suspension.

b. A pure *A. hydrophila* culture suspension served as a positive control.

c. An unrelated monoclonal antibody and dPBS served as negative controls for the antibody.

4.2.2.3.4 Statistical analysis

The data was examined using a three-way factorial analysis of variance with system, region and time as factors (Minitab Computer Software. Minitab Inc.). The analysis was carried out to determine the relationship between the distribution of the *A. hydrophila* and the factors. The 3-way analysis factorial of variance was adopted for

its sensitivity, hence the ability to assign treatment effect. The hypotheses analyzed included:

i. The fish holding system had no effect on the numbers of *A. hydrophila* enumerated.

ii. The region of the intestines had no effect on the numbers of *A. hydrophila* enumerated.

iii. Time following exposure had no effect on the numbers of *A. hydrophila* enumerated.

iv. The effect of system was not dependant on the region of the intestines.

v. The effect of system was not dependant on the time.

vi. The effect of time was not dependant on the region of the intestines.

4.3 Results

4.3.1 *A. hydrophila* isolates

Seventeen isolates of *A. hydrophila* were identified, eight associated with fish intestines (Table III) while the rest were obtained from the water (Table IV). Isolates were determined to be *A. hydrophila* by growth as small round yellow colonies on Rimler-Shotts medium after 24 hours at 35°C, Gram negative reaction and rod-shaped appearance on microscopic examination. Further confirmation was made by resistance to vibriostatic 0/129, a positive cytochrome oxidase reaction, acid production when grown in glucose and hydrolysis of esculin. While all the isolates from the intestines produced acid with salicin, only four out of the nine isolated from the water did. Three out of the eight isolates from the intestines and six out of the nine from the water developed round and shiny, whitish colonies in 24 hours following incubation at 35°C, and were associated with haemolysis. When grown on sulphide indole and motility medium, all the isolates had a black deposit indicating production of hydrogen sulphide. Addition of Kovac's reagent revealed the production of indole. All the isolates produced arginine dehydrolase. None of the isolates reacted with the B7 monoclonal antibodies.

The intestines and the water from the recirculating system had significantly higher viable microbial counts than the corresponding samples from the flow-through system (Tables V,VI). There was no significant difference between the different regions of the intestines ($p > 0.05$). In the flow-through system, the samples obtained from the exit had significantly higher viable counts ($p < 0.05$) than did either the entry point or the

water column. The microbial count at the water entry point of the recirculating system was twenty-one times higher than the flow through system. Similarly the water column from the recirculating system had more organisms than it did in the flow through system. In the flow through system, there was no significant difference between the water column and the exit point sample ($p > 0.05$). The sedimentation tank column, biofilter exit and the replenishing tanks had progressively fewer viable microbes.

4.3.2 *A. hydrophila* detection with IFAT

The presence of *A. hydrophila* in the intestines was transient and not detected in any region 120 hrs following exposure to the microbe bath (Table VII, VIII). Following exposure, organisms could be detected only in the cranial two-thirds of the intestines in both the recirculating and flow-through systems (Fig. 35). However, there were significantly more microbes (p 0.05) in the fish in the recirculating system (Table V, Fig. 36).

Twelve hours after exposure, detection could be made in the cranial two-thirds of the intestines in both systems. There was a decline in the numbers detected cranial-caudally in the recirculating system, however, no significant difference was observed in the flow-through system ($p > 0.05$). After 24 hours (Table VII), there was marked reduction in the numbers of *A. hydrophila* detected in the intestines of the fish from the flow through system. A similar trend was noted in the recirculating system fish but it was less marked. There was difference between regions in the flow-through system ($p< 0.05$).

Forty-eight hours after exposure, only a few bacteria could be detected in the cranial intestines of the fish maintained in flow through system (Table VIII). In the recirculating system, detection was made in the cranial two-thirds of the intestines.

No A. hydrophila was detected in fish after 72 hours (Table VIII) in the flow through system. After 96 hours the only detection made was in the caudal aspects of the cranial intestines of fish from the recirculating system. No detection was made in both systems after 120 hours.

The fish holding system, region of the intestines and time following exposure had a significant effect on the distribution of *A. hydrophila* (Figs. 35, 36., Table IX). The number of microbes was higher in the cranial region of the intestines, and progressively declined towards the caudal intestines (Fig 35). Highly significant interactions were detected between the holding system and the region of the intestines, and between the latter and time following exposure to *A. hydrophila* (Table IX).

Fig.33 Photomicrograph of intestinal biofilm stained with IFAT following 24 hours after expose of tilapia held in a flow-through aquatic system, to *A. hydrophila.* Note the rod shaped streaks representing the microbe. Mag. x1600.

Fig.34 Photomicrograph of intestinal biofilm stained with IFAT following 24 hours after expose of tilapia held in recirculating fish holding system, to *A. hydrophila.* Note the rod shaped streaks representing the microbe. Mag. x1600

Table III. Reaction profile of *A. hydrophila* isolates from the fish intestines.

0 Test	Positive reaction	No. of +ve/ total No. of strains.	% of +ve strains.
RS	Yellow colonies	8/8	100
Gram reaction	negative rods	8/8	100
C-O	Purple colour	8/8	100
SIM	motility	8/8	100
SIM	H_2S Present	8/8	100
Glucose	Fermentative	8/8	100
0/129	Insensitive	8/8	100
Arginine	positive	8/8	100
VP	positive	8/8	100
Salicin	hydrolysis	8/8	100
Esculin	no hydrolysis	8/8	100
Blood agar	haemolysis	3/8	37.5

+ve Positive reactors.
RS Rimler-Scotts medium.
C-O Cytochrome Oxidase reaction.
SIM Sulphide Indole Motility medium.
0\129 Vibriostatic agent (2, 4-diamino -6, 7-diisopropylpteridine).
VP Voges Prokauer reaction.

Table IV. Reaction profile of the *A. hydrophila* isolates from the water.

Test	Positive reaction	No. of +ve/ total No. of strains.	% of +ve[1] strains.
RS	Yellow colonies	9/9	100
Gram reaction	negative rods	9/9	100
C-O	Purple colour	9/9	100
SIM	motility	9/9	100
SIM	H_2S present	9/9	100
Glucose	Fermentative	9/9	100
0/129	Insensitive	9/9	100
Arginine	positive	9/9	100
VP	positive	9/9	100
Salicin	hydrolysis	4/9	44.44
Esculin	no hydrolysis	9/9	100
Blood agar	Haemolysin	3/8	37.5

+ve Positive reactors.
RS Rimler-Scotts medium.
C-O Cytochrome Oxidase reaction.
SIM Sulphide Indole Motility medium.
0\129 Vibriostatic agent (2, 4-diamino -6, 7-diisopropylpteridine).
VP Voges Prokauer reaction.

Table V. Number of microbes isolated from the intestines of Nile Tilapia (*O. niloticus)* in the flow-through and recirculating systems.

System sampled	Region of the Intestine	Number of colonies ($x10^4$)
FT	1	2.47
FT	2	1.94
FT	3	2.06
FT	4	1.22
RC	1	>3.00
RC	2	>3.00
RC	3	2.94
RC	4	>3.00

RC Recirculating fish holding facility.
FT Flow-through fish holding facility.

T test analysis of the two fish holding facilities: T= -4.08. This value is significant at the 0.05 level.
(While 6 regions of the intestines were sampled for the viable microbial count, results from regions 5 and 6 were unreliable due to contamination associated with the narrow diameter of the gut.)

Table VI. Number of viable microbes (x100) per ml of the holding water in both flow-through and reciculating systems*.

System.	Sampling site	No. of colonies (x100)/ml
FT	Fish tank entry	1
FT	Fish tank water column	9.5
FT	Fish tank exit	17
RC	Fish tank entry	21
RC	Water column	>300
RC	Fish tank exit	>300
RC	Sedimentation Tank column	163
RC	Biofilter	52
RC	Replenishing Tank	2

RC Recirculating fish holding facility.
FT Flow-through fish holding facility.

Analysis of variance of the fish holding facilities: F=5.89 (DF=11). This value is significant ($P < 0.05$).

* See appendix 4 for diagramatic representation of the recirculating system.

Table VII. Indirect fluorescent antibody (IFAT) test and the corresponding Reaction Indices of the smears after 0, 12, 24 hours following exposure to *A. hydrophila.**

Sys.	Gut region	IFAT Test			Reaction Index		
		0	12	24	0	12	24
RC	1	+	+	+	4	4	3
RC	2	+	+	+	2	3	2
RC	3	-	+	+	0	3	2
RC	4	-	+	+	0	2	1
RC	5	-	-	-	0	0	0
RC	6	-	-	-	0	0	0
FT	1	+	+	+	3	3	3
FT	2	+	+	+	2	3	2
FT	3	-	+	+	0	2	2
FT	4	-	+	+	0	3	1
FT	5	-	-	-	0	0	0
FT	6	-	-	-	0	0	0

Sys Fish holding facility.
RC Recirculating, FT Flow-through fish holding facility.
\+ Positive IFAT test, - Negative IFAT test.
0 No fluorescence was observed.
1 Fluorescence observed and number of identifiable fluorescent microbes under 10.
2 Fluorescence observed and number of microbes between 10-50
3 Fluorescence observed and number of microbes between 51-100
4 Fluorescence observed and number of microbes between 101-200
5 Fluorescence observed and number of microbes >200 and corresponding to the positive control made from a pure culture.
n Number of fish exposed to *A. hydrophila* = 18
* Refer table IX for statistics analysis result.

Table VIII. Indirect fluorescent antibody (IFAT) test the corresponding Reaction Indices of the smears after 48, 72, 96 hours following exposure to *A. hydrophila*.*

Sys.	Gut region	IFAT Test			Reaction Index		
		48	72	96	48	72	96
RC	1	+	+	-	1	1	0
RC	2	+	+	+	3	1	1
RC	3	+	+	-	1	1	0
RC	4	+	-	-	1	0	0
RC	5	-	-	-	0	0	0
RC	6	-	-	-	0	0	0
FT	1	-	-	-	0	0	0
FT	2	+	-	-	1	0	0
FT	3	-	-	-	0	0	0
FT	4	-	-	-	0	0	0
FT	5	-	-	-	0	0	0
FT	6	-	-	-	0	0	0

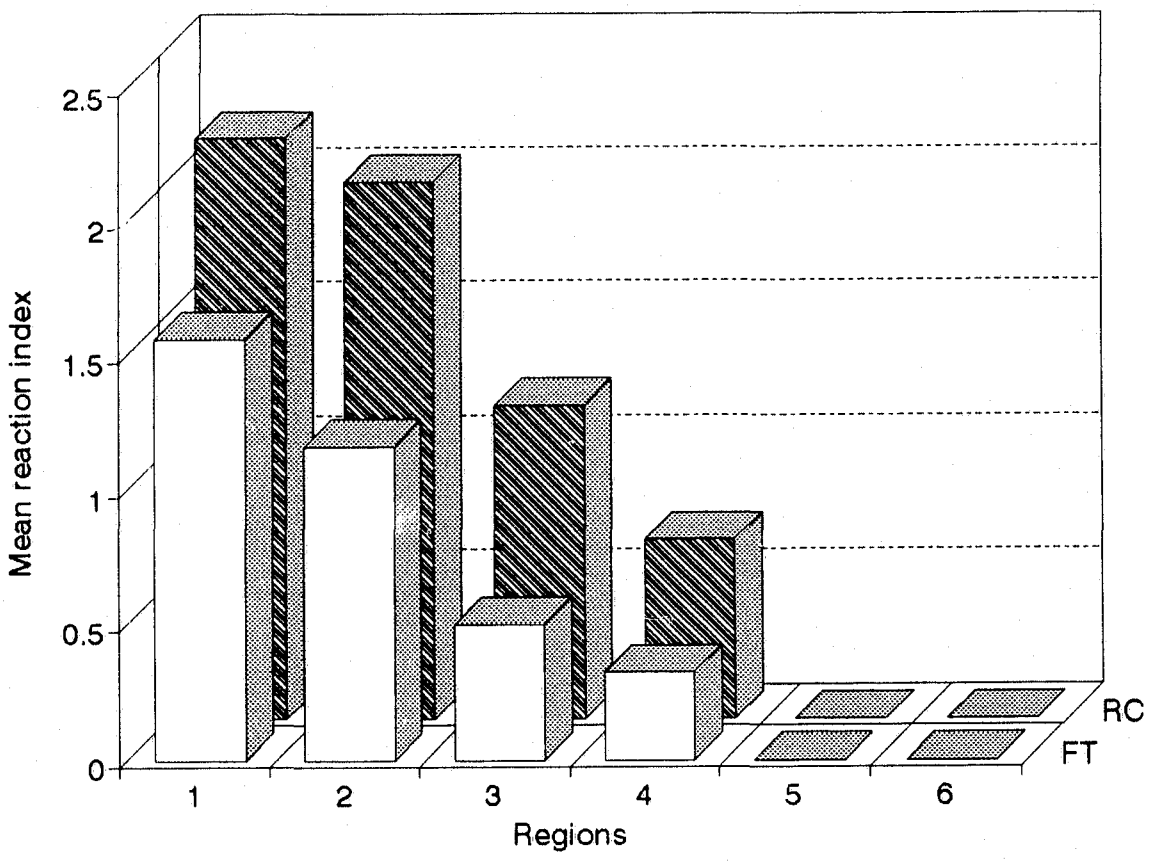
Sys Fish holding facility.
RC Recirculating, FT Flow-through fish holding facility.
\+ Positive IFAT test, - Negative IFAT test.
0 No fluorescence was observed.
1 Fluorescence observed and number of identifiable fluorescent microbes under 10.
2 Fluorescence observed and number of microbes between 10-50
3 Fluorescence observed and number of microbes between 51-100
4 Fluorescence observed and number of microbes between 101-200
5 Fluorescence observed and number of microbes >200 and corresponding to the positive control made from a pure culture.
n Number of fish exposed to *A. hydrophila* = 18
* Refer table IX for statistics analysis result.

Table IX. Analysis of variance of the reaction indices.

Source	DF	F-value	P-value
System	1	30.77	0.000
region	5	55.87	0.000
time	5	44.33	0.000
sysreg	5	5.10	0.002
systim	5	2.79	0.039
regtim	25	9.54	0.000
Error	25		
Total	71		

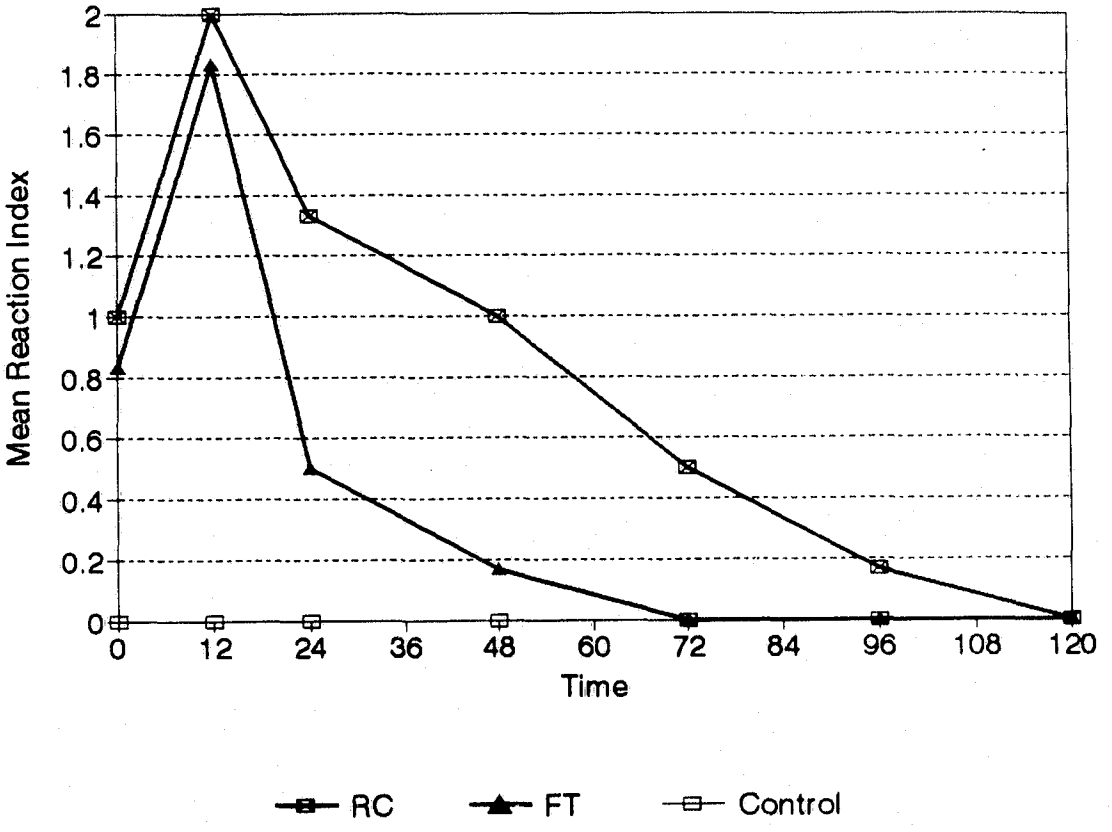
DF	Degrees of freedom
Source	Origin of the variance
System	Fish holding Facility.
Region	Regions along the intestine.
Time	Time period after exposure to *A. hydrophila.*
Sysreg	System x Region interaction.
Systim	System x Time interaction.
Regtim	Region x Time interaction.

Fig. 35 Immunofluorescence reaction indices associated with the various regions of the intestines.



RC Recirculating system
FT Flow through system

Fig. 36 Immunofluorescence reaction indices plotted against time following exposure to *A. hydrophila*.



RC Recirculating system
FT Flow through system

4.4 Discussion

A. hydrophila was isolated from both the recirculating and flow through systems which confirms earlier studies that showed it was frequently associated with freshwater environments (Hazen et al 1978, Allen et al 1983). *A. hydrophila* was also isolated from the intestines of the Nile tilapia corresponding to findings in other freshwater fish (Trust and Sparrow 1974, MacMillan and Santucci 1990).

The isolates were identified as *A. hydrophila* by growth on Rimler-Shotts medium (Shotts and Rimler 1973), and Gram and cytochrome-oxidase reactions. The yellow colonies on the Rimler-Shotts medium, attributed to maltose fermentation, differentiate *A. hydrophila* from other microorganisms growing on the medium (Shotts and Rimler 1973). Both *A. salmonicida* and *Citrobacter* species may produce yellow colonies, but are distinguished by incubation at 35° C and the cytochrome oxidase reaction respectively. The cytochrome oxidase test had to be carried out on *A. hydrophila* reinoculated on TSA to avoid acid insensitivity on Rimler-Shotts medium (Amos 1985). The Rimler-Shotts medium inhibits the growth of *Vibrio* species due to incorporation of novobiocin and sodium deoxycholate. *A. hydrophila* utilizes various types of carbohydrates including mono-, di- and poly-saccharides, mucopolysaccharide and glucosides and sugar alcohols (Hsu et al 1985). The isolates demonstrated an ability to utilise glucose, salicin and esculin, producing acid and gas in the former and acid in the latter, further indicating conformity with established *A. hydrophila* profiles (Austin and Austin 1987, Hsu et al 1985). The two genera *Aeromonas* and *Vibrio* are under the

same family *Vibrionaceae* (Popoff 1984, Schubert 1984) and show phenotypic similarities. The vibriostat 0/129 is a useful differential test because *Aeromonads* are insensitive to it. When challenged, the isolates used in this study were resistant to 0/129. Most of the isolates, had similar phenotypic profiles suggesting they were likely to be the similar. However, a few isolates from the water from the recirculating system demonstrated some differences in utilization of salicin and in blood haemolysis. This phenotypic incongruence is not uncharacteristic of the genus (MacInnes et al 1979, Allen et al 1983).

The number of viable microbes isolated from both the fish intestines and the water differed significantly depending on the fish holding system, with higher counts in the recirculating compared to the flow through system. The low microbial count in the flow through system is attributable to the system design, which allows greater flushing. The continuous addition of fresh water to the system precludes the build up of heavy microbial loads due to the flushing activity. The incoming water had significantly very low microbial count compared to both the water column and at the point of exit in the flow through system. The difference between the exit and the column is attributable to the microbes that are associated with the sediments.

The number of viable microorganisms from the intestines of Nile tilapia held in the recirculating tanks was significantly higher than those isolated from the intestines of the fish maintained in the flow through system ($p< 0.05$). Similarly, the number isolated from the water in the recirculating system was higher than in the flow through system ($p < 0.05$). This suggests that the environmental microbial load may influence the

number of microorganisms in the intestines.

Beveridge et al (1989) demonstrated that tilapia could detect and subsequently deliberately ingest microbes, though they (tilapia) are fresh water fish species and do not drink a lot of water (Bone and Marshall 1982). The detection of *A. hydrophila* following bath exposure, in both the intestines of fish maintained in flow through and recirculating systems confirmed the ingestion took place during exposure since no detection was made in the control fish.

Twelve hours after exposure, distribution of the microbes extended to the mid region of the intestines in both containment systems. Though a higher count was made in the region immediately distal to the stomach in the fish from the recirculating system no significant difference was observed between the two systems. However, twenty-four hours after the bath, there was a significantly higher number of *A. hydrophila* in the recirculating system, than in the flow through system ($p< 0.05$). This suggests a faster clearance rate of *A. hydrophila* in the flow through than in the recirculating system. This trend continued between twenty-four and forty hours after the bath, with very dramatic differences between the two containment systems. By seventy-two hours, *A. hydrophila* could not detected from the intestines in the fish held in flow-through system indicating there was complete flushing of *A. hydrophila* in the intestines.

The clearance of *A. hydrophila* in the intestines took significantly longer in the fish maintained in recirculating, than in the flow through system ($p <0.05$). The environmental microbial flora can affect the quantities and quality of gut microbes (Buras et al 1985) suggesting the different microbial densities in the respective holding

systems influenced the dynamics of the *A. hydrophila* introduced in the intestines. MacMillan and Santucci (1990) noted there was seasonal variation of the intestinal microbes. The seasons have a direct impact on the abundance of microbes in the environment probably through the influence of temperature. This finding suggests *A. hydrophila* species may be transient in the gastrointestinal tract rather than being part of the normal flora of the gut (Trust and Sparrow 1974, Sakata et al 1980, Toranzo et al 1985, Nieto et al 1984). It appears that the strain of *A. hydrophila* that was introduced to the biofilm, does not establish autochthonity in the gut though the presence in the biofilm may be associated with the numbers of microbes in the fishes' aquatic environment.

In conclusion, the fish holding facility influences on the number of viable microbes isolated from it. The number of viable microbes in the flow through system was significantly different from those in the recirculating system. The *A. hydrophila* introduced in the systems was associated with the biofilm in the intestines of the Nile tilapia, and was cleared from the biofilm of the Nile tilapia by 120 hours.

5. General discussion

Studies of the morphology, histology and ultrastructure indicate the structure of the intestinal tract of Nile tilapia reflects the herbivorous nature. The major features of herbivorous adaptation include, the lengthy intestines with a high intestinal length: body length ratio, and, extensive mucosal foldings. These features result in extensive surface area that is appropriate for carbohydrate rich diets compared to the protein rich diets of the carnivores. In aquaculture the cost of protein is much higher than carbohydrates, making tilapia a more economical fish to culture. This increases the appeal of tilapia culture especially in the small subsistence enterprises prevalent in tropical countries. The ability to consume plant materials makes tilapia culture appropriate for integration into established agricultural practice.

Morphologically, the intestine of the Nile tilapia is not very differentiated. However, to carry out the functions of digestion, the intestines should be physiologically differentiated. Variation in mucus histochemistry may reflect such regional differentiation. Notable in the submucosa was an absence of strata compactum and granulosus which may contribute to the intestine's fragility. This fragility of the intestines required very gentle handling of the intestines during specimen preparation. This fragility of the Nile tilapia intestine may be associated with the ability to be packaged in the limited abdominal space.

Observations made in the study indicated the presence of biofilms on microvillar surfaces of the epithelium of the intestines of the Nile tilapia. The presence of the

biofilms is consistent with other biotic or abiotic flowing aqueous ecosystems where microbes are organized in organic polymer matrices (Costerton et al 1987).

The biofilms are composed structurally of the exopolysaccharide matrix, within which the microbes reside. The intestines provide a unique site for biofilm development, due to the large and regular supply of nutrients accessible to the microbes, and large amount of mucus from the numerous goblet cells available for development of the organic matrix. The microbes form a major component of the intestinal biofilms of Nile tilapia. The microbes are a mixed population of microorganisms that are evident as the different morphotypes.

A variety of biofilm forms are recognized in the intestines of the Nile tilapia, defined by the amount of mucopolysaccharide. The various forms were not associated with any specific region of the intestines. The biofilms were demonstrated following stabilization using antimucus antibodies, which were effective in anchoring the biofilm and sustaining it through the processing of the tissues. This technique is suitable since it allows direct observation of the biofilms in the intestines, by overcoming the inherent limitations in conventional electron microscopy protocols attributed to dehydration and washing that lead to biofilm distortion or destruction.

While the technique stabilizes the biofilm, it does not prevent condensation. Since the biofilm is very hydrated, the observations were on the compacted polymer structure. Details on the actual depth of the biofilm and variations between different sites in the intestines and impact of the holding facility could not be fully assessed and can only be estimated. Further investigation using other techniques would therefore be

necessary.

Tilapia are hardy fish that are associated with environments where *A. hydrophila* is ubiquitous. Th *A. hydrophila* strain that was introduced into the system was associated with the biofilm which provides an interactive phase between the lumen and the microvillar surface of the tilapia intestines. However, the presence of *A. hydrophila* in the biofilm is only for a limited period of time. For disease to occur, microbes should establish dominance in the biofilm environment upsetting the stable ecosystem present prior to infection. This suggests the biofilm may be involved in the enteric defence. However, the diminishing presence of *A. hydrophila* in the biofilm suggests the biofilm plays a role larger than simply a physical barrier to the microbes.

Further investigations which may benefit from understanding the presence of biofilms in the Nile tilapia intestines include, the digestive physiology and nutrition, local immunity and relationship of the biofilm to the environmental microbial populations. One of the tenets of use of tilapia in fisheries and aquaculture is their ability to utilise low value unrefined food including material low in the food chain. These are then converted to high quality protein in a process that may involve a microbial role similar to the mammalian herbivores. Further investigations could be directed towards determination of the significance of biofilms and whether an opportunity for manipulation exists.

A major characteristic of biofilm is protection of the microorganisms from various antimicrobial agents by the shelter provided by the polymer matrix. Speculations can be made that in an environment such as the intestines where the local microbial

population interacts with the external environment, the embedding of microorganisms in biofilms may sequester the microbes and their products from the host. This would mean the biofilm may have a role in local intestinal immunity. Of significance in this scenario is the consortia organization of the microorganisms with hierarchical arrangements that allow the utilization of products of one microbial group by another. Following introduction, the presence of *A. hydrophila* was transient in the intestines with a greater transit time observed in the recirculating system. Greater numbers of viable organisms isolated from the recirculating system suggests interaction between the environmental microbial population with those of the intestine. This has significance in aquaculture in bacteriological fouling especially in holding facilities including recirculating holding facilities and ponds.

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7. Appendices

Appendix I Davidson's fixative.

Glycerine	400ml
30-40% Formaldehyde	800mls
95% Ethanol	1200mls
Distilled Water	1200mls

Refrigerate at 4°C.

Prior to fixation, add 1 part acetic acid to 9 parts stock Davidson's.

Appendix II Resin

1. Mix in a plastic beaker.

[a]Araldite	20ml
[b]Epon 812	25mls
[c]DDSA	60mls

2. Mix by hand, then seal to keep dry and stir (magnetic stirrer) for 30 minutes.

3. Add 2.4 ml [d]DMP-30

4. Stir again for 30 minutes.

5. Desiccate for 1 hr and store in freezer.

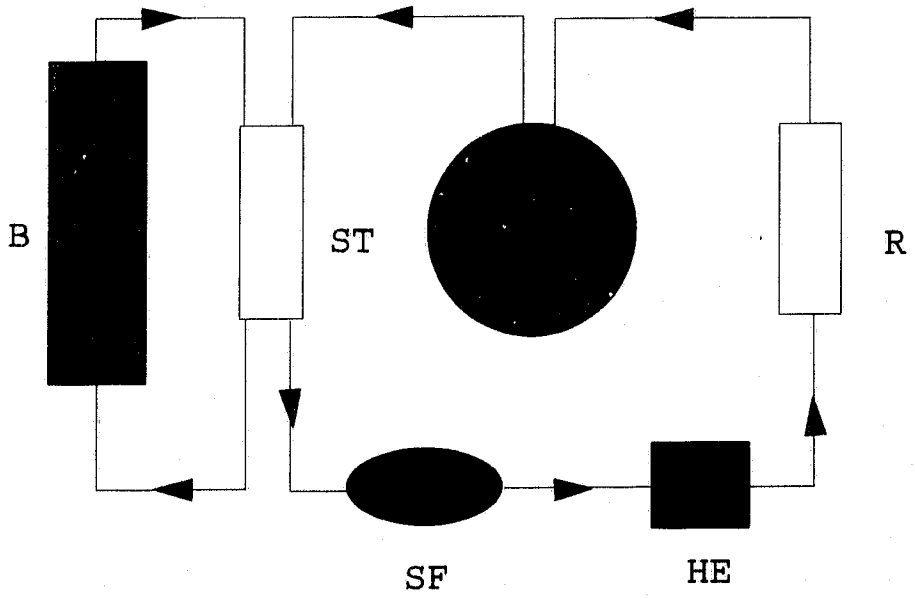
[a] Araldite 502 solution (JB EM services Inc. Que. Canada).
[b] (JB EM services Inc. Que. Canada).
[c] (Dodecyl Succinic Anhydride (JB EM services Inc. Que. Canada).
[d] (2,4,-6tri,-(dimethylaminomethyl) phenol Marivac Ltd. Halifax. canada.

Appendix III. **Intestinal:body length ratios of a random number of fish used in the study.**

Fish #	Body length (cm)	Body wt. (g)	Gut length (cm)
1	16.8	84.4	85
2	24	174.6	95
3	20	155	92
4	19.8	134.5	80
5	20.5	157.7	83
6	19.5	145.1	76
7	20.5	155.2	77.5
8	19	117.4	82
9	21	179.4	88
10	18.7	117.9	90

Gut length:body length ratio 4.45

Appendix IV Diagrammatic representation of the recirculating fish holding system.



B Biofilter
ST Sedimentation tank
FT Fish tank
R Reservoir
SF Sand filter
HR Heat exchanger

Appendix V Diagrammatic representation of the flow-through fish holding system.