IDENTIFICATION AND ANTIBIOGRAM PROFILES OF BACTERIA ASSOCIATED WITH DISEASED *OREOCHROMIS NILOTICUS* IN LAKE KARIBA, ZAMBIA

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A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN HEALTH OF AQUATIC ANIMAL RESOURCES OFSOKOINE UNIVERSITY OF AGRICULTURE.MOROGORO, TANZANIA.

2017

ABSTRACT

The recent intensified aquaculture projects in Lake Kariba have brought about disease outbreaks in cultured *Oreochromis niloticus*. This has brought on an awakening to the scientific knowledge gap surrounding these events. This study aimed at identifying bacteria associated with diseased *O.niloticus* and establishes theirantibacterial resistance patterns. The diseased fish in cages were identified based on behavioural and physical abnormalities including swimming in circles, swimming in lateral or dorsal recumbency, ocular opacity, hyperpigmentation, fin erosions and ulcerations. A total of 25 sick and 4 apparently healthy fish were sampled. Samples from the liver, kidney, spleen, brain, abdomen, blood and ulcers were inoculated on 10% sheep blood agar and nutrient agar. Isolates were then classified by genera using standard culture and biochemical tests. The bacterial isolates were also tested for resistance to commonly used antibacterial compounds in aquaculture using the disc diffusion technique. The eyes, spleen and liver were found to have the highest number of pathologies, in descending order. A total of 15 bacteria genera were identified. *Lactococcus/Streptococcus* genera had the highest prevalence with 46.2% followed by *Aeromonas* at 11.5%. Multiple drug resistance was observed in all isolates, with two isolates each of *Aeromonas* and *Lactococcus/Streptococcus* exhibiting complete resistance to majority antibiotics tested. The results suggest that an increased biomass in diseased cages may be the main risk factor for the disease, with the immune and regulatory organs being first to succumb. The lake environment has been shown to be a mixing vessel of various microorganisms that are already showing multiple antibacterial resistance. Risk factors surrounding the presence of these bacteria genera must be accessedand a more comprehensive,comparative study in antibiotic resistanceon farmed,in contrast to wild species in the Lake Kariba.

DECLARATION

I, Tipezenji Sakala, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor concurrently submitted for a higher degree award in any other institution.

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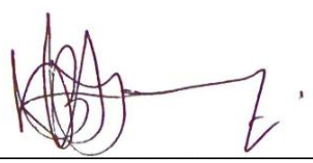
(Supervisor)



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Prof. Bernard M. Hang'ombe Date

(Supervisor)

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ACKNOWLEDGEMENTS

I would like to express my deepest and sincerest gratitude to the NORAD-NORHED-TRAHESA (Training and Research in Aquatic Health for Eastern and Southern Africa) project (No TAN 13/0027) for having given me this opportunity, against all odds. Thank you to the entire administration team and technical staff for facilitating this adventurous and unique two years of study.I would like to appreciate my supervisors Prof. Bernard Hang'ombe, Prof. Robinson Mdegela, Prof. Stephen Mutoloki, Prof. Øystein Evensen and the late, Dr. Huruma Tuntufye for guiding and challenging me enough to the point of producing excellence. I would like to thank the technical and advisory staff at The University of Zambia (UNZA) and Sokoine University of Zambia (SUA) for all the assistance, special appreciation to Mr Evans Mulenga, who so faithfully and diligently assisted me with the heavy workload in the field and laboratory.All thanks goes to my family; My Mother, Mary Chisanga Sakala,my brothers; Tanje David Jnr, Bwalya, Kapya, Grandma, and my wonderful Partner Mr Bwale George Chembe, you have been just so wonderful and supportive. Last but not the least I would like to acknowledge and give praise to the all mighty God, for carrying me through, I am nothing without Him.

DEDICATION

To the late David Sakala, I shall forever love and miss you Dad.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percent
°C	Degrees Celsius
µ	micro
A	Adenine
A/B	Antibiotic
Abs	Abscess
AMC	Amoxiclav
AMX	Amoxicillin
B	Brain
BA	Blood Agar
BC	Before Christ
Bd	Blood
bp	base pairs
C	Cytosine
CIP	Ciprofloxacin
cm	centimetres
COT	Co-trimoxazole
CTX	Cefotaxime
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
E	Erythromycin
ELISA	Enzyme-Linked Immunosorbent Assay
FAO	Food and Agricultural Organisation
FAT	Fluorescent Antibody Technique

g	grams
G	Guanine
hrs	hours
IFAT	Indirect Fluorescent Antibody Techniques
K	Kidney
kg	kilogram
L	Litre
M	mole
m^2	metre squared
m^3	metre cubic
MCA	MacConkey Agar
mg	milligram
ml	millilitres
mm	millimetres
N	Nitrogen
NX	Norfloxacin
OIE	World Organisation for Animal Health
P	Penicillin
PCR	Polymerase chain reaction
pH	Hydrogen potential
RNA	Ribonucleic acid
S	Spleen
SIM	Sulphur Indole Motility
sp(p).	Specie(s)
T	Thymine
T_1	Esculin (Scullion)

T_{10}	Sorbitol
T_{11}	Lactose Monohydrate
T_{12}	Urease
T_{13g}	gas production
T_{13G}	Glucose
T_{13L}	Lactose
T_{13S}	Sucrose
T_{14}	Simmon’s citrate
T_{15I}	Indole
T_{15M}	Motility
T_{15S}	Sulphur
T_{16}	Glucose
T_{2}	Galactose
T_{3}	Raffinose
T_{4}	Salicin
T_{5}	Maltose Monohydrate
T_{6}	Xylose
T_{7}	Mannitol
T_{8}	Trehalose
T_{9}	Inulin
TE	Tetracycline
TL	Total Length
TSA	Tryptone soya agar

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Fish are a crucial nutritional component worldwide, providing energy, protein and a range of essential nutrients. Eating fish is part of the cultural traditions of many peoples, and fish and fishery products are a major source of food and essential nutrients for some populations, more especially the densely populated developing countries where in many cases, there may be no alternative affordable food sources. Fish provide over 2.9 billion people (20%) and 4.5 billion (15%) of animal protein (FAO, 2014). With capture fisheries production in general, levelled off, the growing demand for fish in practice may mainly be met by increased production from aquaculture. People have never consumed so much fish or depended so greatly on the fisheries and aquaculture sector for their nutrition as they do today (FAO, 2016).

Aquaculture has been intensified in many parts of the world including Chile, Brazil, China, India, Norway, Bangladesh, Morocco, Nigeria, Uganda, Ghana and Egypt (FAO, 2014; FAO, 2016). This intensive production has with it, brought the development of disease outbreaks. The host (fish), pathogen and environment triad are generally maintained in a balanced relationship, regulated primarily by the immune system. In the eve of aquaculture related practices and consequences such as high densities, lack of oxygen, water contamination and management practices including transportation and vaccination, causing stress leading to lowered immunity, and ultimate disruption of the balanced triad relationship (Huicab-pech *et al*., 2016).This results in disease outbreaks, primarily bacterial, in which microflora found within the natural aquatic environment, may be implicated (Austin, 2006; Helmy and Atallah, 2015). Antibacterial agents, in practice,

have been used in small sub-therapeutic doses in feed as growth promoters or to prevent disease in fish, though rarely. Most commonly, in the presence of a disease problem, they have been used in the practice of treating an entire population to protect the healthy ones until the sick fish die and infection subsides. This form of oral treatment leads to sub-therapeutic doses which can enable selection for resistance in bacteria (Thorsen, 2014).

Currently, the most common type of species farmed in Zambian aquaculture are mostly from the Cichlid family, namely, *O. andersonii* (64%), *Tilapia rendalli* (20%), *O. niloticus* (5.2%), *O. macrochir* (5%), and to a lesser extent, but in order of significance Carp, crayfish (red claw, maron and yabbies), and catfish <1%). In this regard, commercial Nile tilapia (*O. niloticus*) culture, due to its hardy nature and economical production (Popma and Masser, 1999), has been a growing, profitable source of income on Lake Kariba, Zambia. The use of antibacterials has been reported in an effort to avert disease. With reports of resistant bacteria having recently been investigated and confirmed in Nile tilapia and catfish in aquaculture within Africa (Ekundayo *et al.*, 2014; Tiamiyu *et al.*, 2015), this study aims to lay a foundation for resistance to antibacterials and prevalence ofbacteria associated with sick fish in cages fish in Lake Kariba.

1.2 Problem Statement and Justification

1.2.1 Problem statement

The growing profitable cage culture of Nile Tilapia (*O. niloticus*) on Lake Kariba has been on a steady rise, due to the many advantageous qualities of the species. Recently, however, there has been an outbreak of bacterial disease in cultured *O. niloticus*, with reported cases stemming from the year 2015 to date.This venture is being threatened by economic losses that are a result of disease. On-going anthropogenic activities on the lake Kariba, coupled with the aquaculture projects recently established, may have affected the aquatic environment. This situation may have changed the environmental set up, leading to a

situation where bacteria populations are on the increase as the host *O. niloticus*, gets concentrated in one place. There is therefore a need to look at the bacteria make-up of sick fish cultured commercially to enhance knowledge on this topic. Furthermore there exists no information on public health issues surrounding fish found in this lake.This includes sensitivity to antibacterial agents of the cultured fish, and establishment of lacking baseline information.

1.2.2 Justification

Therefore, bearing this in mind, there is need for novel research to investigate the most prevalent bacterial isolates present as well as their sensitivity towards commonly used antimicrobials in aquaculture or present within the lake environment.Passive surveillance of this nature is essential for the prevention of possible disease outbreak associated with the potential pathogenic bacteria observed especially during critical periods of environmental stress. An understanding of bacteria detected will be of value during the development of strategies for preventing or managing diseases caused by the potential fish pathogens in future. This data base shall be a reference point for creation of an avenue for establishment of preventive and control measures by aquaculturalists and policy makers.

1.2.3 Objectives of the study

1.2.3.1 Overall objective

To investigate the magnitude of bacteria isolates and antibiogram profile of isolates from diseased *O. niloticus*in commercial cage farms on Lake Kariba,Zambia.

1.2.3.2 Specific objectives

i. To establish the prevalence and characteristics of bacterial isolates from diseased *O. niloticus;*

ii. To determine the extent of sensitivity of the bacterial isolates to commonly used antibiotics.

1.2.3.3 Hypotheses

1. A varying composite of bacteria are present in diseased *O. niloticus*
2. The varying array of bacteria may express different levels of resistance to commonly used antibiotic

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1Fisheries and Aquaculture Trends

Global consumption of fish has doubled since 1973. The developing world has been responsible for nearly all of this growth due to rapid population growth, rapid income growth and urbanization trends(Delgado *et al.*,2003). Through diversification and high nutrient value, the significant growth in fish consumption has enhanced people's diets around the world. According to the Food and Agricultural Organisation,fish accounted for about 17% of the global population's intake of animal protein and 6.7% of all protein consumed in 2013 (FAO, 2016). Fish also provided more than 3.1 billion people with almost 20% of their average per capita intake of animal derived protein. In addition to being a rich source of easily digestible, high quality proteins containing all essential amino acids, fish provides essential fats (long chain omega-3 fatty acids), vitamins (D, A and B) and minerals (including calcium, iodine, zinc, iron and selenium), particularly if eaten whole(USAID SPARE Fisheries and Aquacullture Panel, 2001). Small quantities of fish also have a significant positive nutritional impact on plant based diets particularly in least-developed countries. Fish is usually high in unsaturated fats, provides health benefits in protection against cardiovascular diseases, and also aids foetal and infant development of the brain and nervous system. It has many valuable nutritional properties and can also play a major role in correcting unbalanced diets and, through substitution, in countering obesity(FAO,2016).

World inland capture fisheries production has dramatically increased since the mid-2000s, with many water bodies being considered overfished in many parts of the world.

Degradation of many important water bodies of freshwater has been attributed to human pressure and changes in environmental conditions (FAO, 2012).

Aquaculture has evolved in terms of technical innovation and adaptation over the past years, to meet changing requirements. This expansion has been responsible for the impressive growth in the supply of fish for human consumption. Aquaculture has been the engine during growth in total fish production since the 1990s, as global capture production has levelled off (FAO, 2012)(Fig.1 and Fig.2).

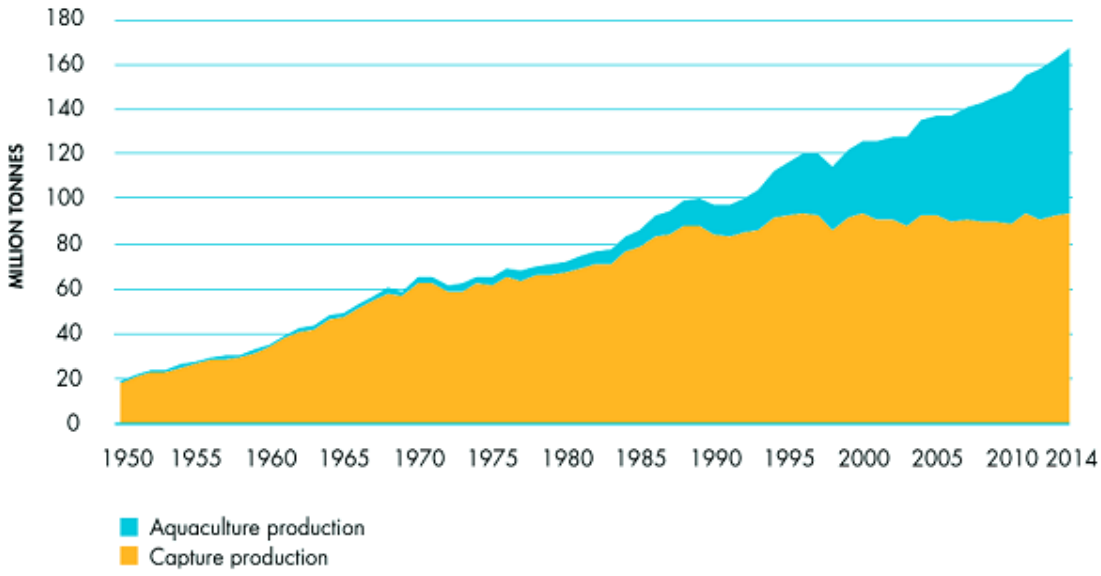


Figure 1:World capture fisheries and aquaculture production (FAO, 2016)

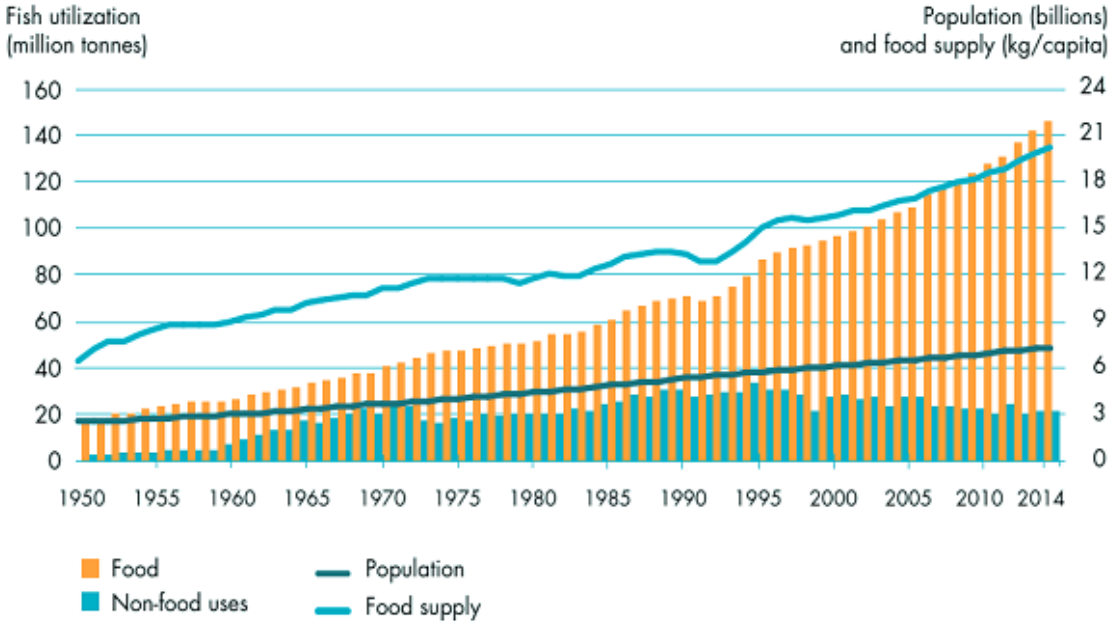


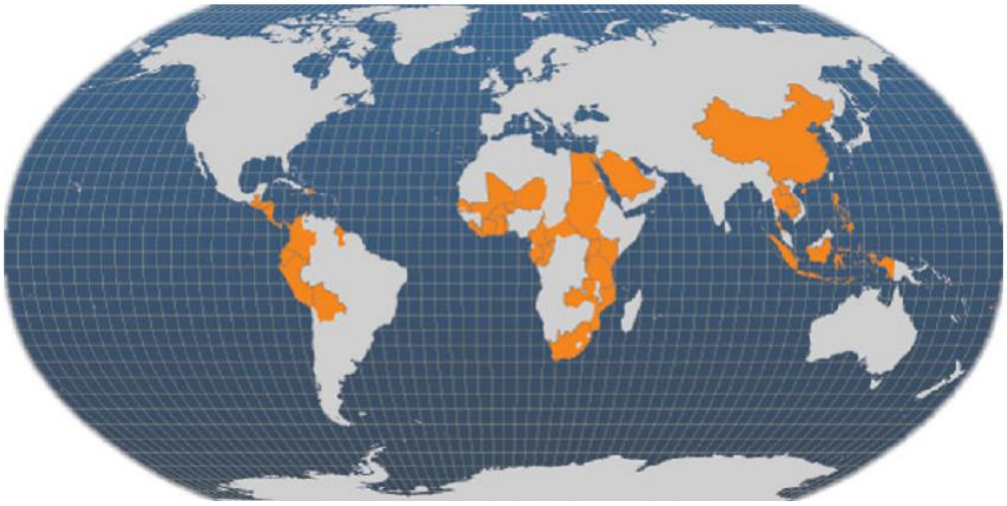
Figure 2: World fish utilization and supply (FAO, 2016)

The major categories of species produced in aquaculture include: freshwater fishes (56.4 percent, 33.7 million tonnes), molluscs (23.6 percent, 14.2 million tonnes), crustaceans (9.6 percent, 5.7 million tonnes), diadromous fishes (6.0 percent, 3.6 million tonnes), marine fishes (3.1 percent, 1.8 million tonnes) and other aquatic animals (1.4 percent, 814 300 tonnes). Aquaculture production exceeds capture production for many of the staple species Production of freshwater fishes has always been dominated by carps (71.9 percent, 24.2 million tonnes, in 2010). Production of tilapia species has a wide distribution, and 72% are raised in Asia (particularly in China and Southeast Asia), 19% in Africa, and 9% in America (FAO, 2012).

In Zambia, all natural water body fish stocks have either been fully fished or over-exploited, as it has been confirmed by decreased catches. Aquaculture development had been encouraged in order to minimise fishing pressure on the natural stocks(Mudenda, 1994). Fish farming in Zambia has attained a high level of diversity, with systems

practiced including: extensive and non-commercial fish farming; semi-intensive; and intensive aquaculture practices(Albert and Simbotwe, 2014; SADC Protocol, 2016).

According to (FAO, 2014),farming of tilapias including *O. niloticus* and other Cichlid species is the most widespread type of aquaculture in the world. Records state that farmed tilapia production is active in 135 countries on all continents. Several species of tilapia are cultured commercially, but *O. niloticus* is the predominant cultured species worldwide, including local producers in Africa, China, Indonesia, Mexico, Honduras, Colombia and Brazil(FAO,2012). Asia accounts for over 70% of world tilapia production with Africa accounting for about 12%, the Americas 15% and others 3%; 73% of this is farmed (Fig. 3).



-Main producercountries worldwide

Figure 3: Main producer countries of *Oreochromis niloticus* in Africa, Asia and South America (FAO Fisheries and Aquaculture Department, 2013)

*Oreochromis niloticus*is a tropical species that prefers to live in shallow water of lower and upper lethal temperatures of 11-12℃ and 42°C, respectively, with the preferred temperature ranging from 31-36°C(Stander,2000). It is a filter feeding, omnivorous grazer

that feeds on phytoplankton, periphyton, aquatic plants, small vertebrates, benthic fauna, detritus and bacterial films associated with detritus.

The culture of Nile tilapia at high densities in floating cages is practiced in large lakes and reservoirs of several countries including China, Indonesia, Mexico, Honduras, Colombia and Brazil (FAO, 2012).Tilapias have many attributes including its basic biology, feeding and veterinary requirements that make it suitable for culturing by farmers at a low cost. These attributes include its general hardiness and high tolerance to adverse environmental conditions and overcrowding.It is able to withstand low oxygen and a wide range of salinity concentrations as well as high disease resistance. According to FAO,(2014)tilapia is also able to survive and grow on a wide range of artificial and natural feeds, converting food efficiently, having fast growth and high yield potential accepted by a wide range of consumers. Furthermore it can be grown in a wide variety of culture systems from simple, low mechanized systems to more complex, intensive systems. It is an inexpensive flavourful, versatile whitefish(Stander, 2000).

2.1.2 Significant requirements of aquaculture

The practice of aquaculture varies widely. The type of aquaculture system a farmer will use depends on geographic location in which land with a gentle slope is desirable and flood-prone areas must be avoided. The soil type should not allow more seepage than 10-20mm per day; clay-type soils have good water-holding properties (Stander, 2000). Resources for feeding and/or improvement of water quality through aeration and partial water exchange (dependant on the intensity of the establishment), are also to be considered. The level of intensification is also dependant on individual financial situationof the farmer (Slonski *et al.*,2005). The difference in the intensity of culture, which can be broadly grouped into three categories; Extensive, which uses large stagnant

ponds that allow only a low stocking density and rely on natural sources of feed (i.e. there is no supplemental feeding), management and skills input are low; Semi-intensive which is similar to extensive culture with a greater degree of intervention. This allows for an increase in the production of livestock when compared to extensive systems. Management and skills input occur at a medium level; Intensive systems are maintained at high stocking density and feeding comes solely from introduced feeds. The culture systems are highly technical and rely on electricity to operate. The space required is relatively small and the system is designed to optimize water use and quality, management and skills input are also high(Rural Fisheries Programme Science, 2010).

The selection of the site, location and climate in particular, must be carefully considered prior to production system. Production of tilapias including *O. niloticus* has a wide distribution in the warmer regions, being raised in Asia (particularly in China and Southeast Asia), Africa, and a small percentage in America. *O. niloticus* thrive inwater temperatures ranging from 24-34°C (Stander, 2000). The top producers in Africa, include Egypt, Nigeria, Uganda, Kenya, Zambia, Ghana, Madagascar, Tunisia, Malawi and South Africa, in descending order (FAO, 2012).

The majority of freshwater fish are raised in ponds with water being channelled from a lake, river, well or other natural source. The water should be of good quality and free of polluting agents such as excrement and chemicals. It is important that there is sufficient water and maintenance of the same water level. Water is either retained in the pond, up until such a point that it is all discharged, or partially replaced to retain a certain percentage of the total water.In cage culture, there is no control of the water quality and the water in which the fishare placed must be suitable for the species cultured. The mesh

of the cage should be small enough to preventescape of fish, yet large enough to allow water and waste to pass through to the outside(Rural Fisheries Programme Science, 2010).

The kind of pond built by a farmer is dependent on local resources, equipment and conditions (Carballo *et al*., 2008). In this case, slope and topography must also be considered. Control against predators including birds, frogs, snakes and lizards must be in place, as these may prey on the small fish. Fences and screens at water inlets and outlets may need to be installed for this reason.

2.1.3 General effects of aquaculture

Aquaculture has many positive effects including food security and conservation of wild fisheries. Besides this, it also poses some serious environmental risks and create sustainability challenge (Gamble, 2012). One of the main environmental impacts of cage aquaculture is that ofnutrient and effluent build-up around and below the cages. In this system, the fish are contained in one place at high densities.Nutrients are discharged as excess feed particles or as fish waste, composed of both solids and in dissolved state (primarily carbon, nitrogen, and phosphorus).This results in a series of chemical and biological responses as they are consumed by bottom feeding animals or decomposed by bacteria. This build-up below and around the cages creates the potential for algal blooms, which deplete the water of oxygen and can create damaging dead zones near aquaculture sites(Price and Stimpert, 2014).

Another environmental concern is the effect of the farmed fish on local wild fisheries. Disease and parasitic(ecotparasites- and endoparasites) outbreaks in fish farms can spread rapidly among farmed fish because of the high densities at which they are kept, and disease may spread to wild fish populations(Aquaculture New Zealand, 2013). In the event

when fish farmers mitigate these outbreaks with antibiotics and other chemicals in fish feed, the drugs may have an effect on the ecosystems around the cages, as well as residual antibacterials winding up on consumers' plates. Land-based aquaculture systems often require huge amounts of water to be pumped into their systems. Pumping the water alone requires electricity, and depending on the design of the aquaculture system, cleaning and filtering the water may also require high energy input. Furthermore, this waste water rich in nitrogen, solids and other waste materials is pumped back into water bodies such as lakes, rivers and streams creating potential for algal blooms(Gamble, 2012).Energy and freshwater are both limited resources, high in demand in the aquaculture industry.

2.2 Bacterial Microflora of Freshwater Fish

Microflora is a community of organisms including bacteria, fungi and algae, that live in a particular habitat or in another living organism(Hale, 2005). Thenumbers and taxonomic composition of the bacterial population microorganisms are influenced by the environment in which the host, in this case fish, thrives. They are often a reflection of the surrounding water. The role of some of these bacteria may include the ability to degrade complex molecules (therefore exercising a potential benefit in nutrition), to produce vitamins and polymers, and to be responsible for the emission of light by the light-emitting organs of deep-sea fish, whilst others may not have beneficial roles (Austin, 2006).

2.2.1 Environmental Microflora

Fish are continuously being exposed to microorganisms present in water, sediment and contaminants such as sewage/faeces. Many bacterial taxa representatives present have at one time or another, been associated with fish diseases. Some constitute primary pathogens, whilst the majorities are categorized as opportunistic pathogens, which colonize and cause disease in hosts already weakened or damaged by pollution, physical

injury, nutritional imbalance or a natural physiological state such as reproduction (Austin and Austin, 2007). These pathogens are often regarded as contaminants or even innocent saprophytes.

A bacterium isolated from the surface of fish has been thought to be a direct reflection of the microflora of the aquatic environment in which it resides. Some bacteria from the surface of freshwater fish have been reported to include *Acinetobacterjohnsonii*, *Aeromonads* (notably *Aeromonas hydrophila*, *A. bestiarum*, *A. caviae*, *A. jandaei*, *A.schubertii* and *A. veronii biovar sobria*).Others include*Alcaligenes piechaudii*, *Enterobacter aerogenes*, *Escherichia coli*, *Flavobacterium*, *Flexibacter spp*., *Micrococcus luteus*, *Moraxella spp*., *Pseudomonas fluorescens*, *psychrobacters*, and *Vibrio fluvialis*(Austin, 2006).

Aquatic animals take a large number of bacteria into their gut and gills from water, sediment and food. The two broad groups of bacteria that will contaminate fish include, the indigenous microflora occurring naturally in the environment, and the other group is the non-indigenous bacteria that include the members of the*Enterobacteriaceae and Enterococcus* families (Austin and Austin, 2007; Marcel and Sabri, 2013).

2.2.2 Normal internal microflora

There is no certainty about the existence of strictly aquatic bacteria, although the most widespread opinion among the various authors is that the majority of the bacteria found in aquatic environments is of soil origin and carried into the water due to rain or accidental introduction of natural or a direct consequence of human activity such as waste defecation(Latha and Mohan, 2013). Generally bacteria isolated from fish vary with the water source and pollution level.According to work done by Marcel and Sabri (2013),

predominant bacteria present in which *Oreochromis* species were being cultured included *Staphylococcus* species, *Klebsiella terrigenia* and *Kocuria varians*. *Aeromonas hydrophila* was found to be part of the normal flora of freshwater fish and thought to be an opportunistic pathogen that manifests itself in times of heavily stressed fish.

2.2.3 Non-indigenousmicroflora

The other group of microflora found on/in the fish or within the aquatic environment are the non-indigenous bacteria. They are termed 'non-indigenous' in that they are known normal flora of the gastrointestinal tract of mammals and humans. They include the members of the family *Enterobacteriaceae* such as *Salmonella* species, *Shigella* species and *Escherichia coli*. Marcel and Sabri(2013) hypothesized that the presence of *Enterococcusavium* in fish may originate from contaminated water through excreta of animals and human waste.Latha and Mohan (2013) further added that in urban and densely populated rural areas, the microbiological quality of fresh water is frequently threatened by contamination with untreated domestic wastewater.

2.3 Mechanism of Disease Causation and Triad

According to Merriam-Webster (2017), a disease refers to an impairment of the normal state of the living animal or plant body or one of its parts. It is an interruption or modification the performance of the vital functions, is typically manifested by distinguishing signs and symptoms, and is a response to environmental factors (as malnutrition, industrial hazards, or climate), to specific infective agents (as bacteria, viruses, protozoa and fungi), to inherent defects of the organism (as genetic anomalies), or to combinations of these factors. Long ago, prior to the year 1450 BC (Before Christ), it was believed that fish did not get sick or diseased.Bibliographic documentation on fish

parasites and diseases has been dated back as far as 330BC, although most developments in fish medicine had begun around the 1970s(Snieszko, 1975).

Disease is usually the outcome of an interaction between the host (fish), the disease-causing situation (pathogen) and external stressor(s) (unsuitable changes in the environment; poor hygiene; stress) (Austin and Austin, 2007). Communicable diseases of fish occur when a susceptible host and virulent pathogen meet a favourable environment that facilitates such an occurence. Infectious disease is dependent on the interaction and cyclical balance among pathogens, hosts and their environment as illustrated in Fig. 4.

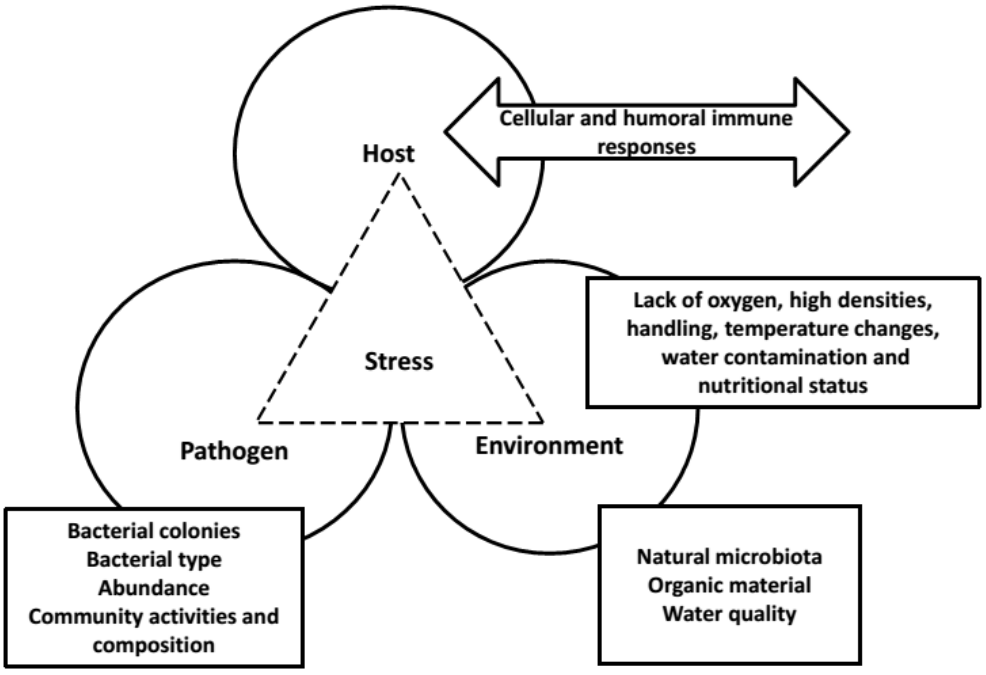


Figure 4: Conceptual diagram of equilibrium among hosts, pathogens and the environment in aquatic systems(Huicab-pech *et al*., 2016)

Host variables that determine the degree of resistance and susceptibility to a disease include its immune defence mechanism system, genetic characteristics; species, age, size and development, nutrition and reproductive needs(Huicab-pech *et al*., 2016). These are

all intertwined within the immune system whose primary function is to defend the host against diseases that impact optimal biological, chemical and physical functions.

The host, pathogen and environment balanced relationship is basically maintained by the pathogen-host systems, with the immune system being the primary regulator of this interaction. In reference to bacterial pathogens, the abundance, type and nature of colonies play a very important role (Fig.4). Roberts (2012) deduced that most causative microorganisms of bacteria disease are essentially natural occurring bacterial flora of fish, originating from the environment in which they swim. These are thought to be the opportunist pathogens which invade the tissues of a fish host rendered susceptible to infection by stress factors or other disease processes. The pathogens otherwise referred to as facultative pathogens survive in the absence of a host and are associated with structures such as capsules, flagella, hair, endospores and cytoplasmic inclusions (Huicab-pech *et al*., 2016). Obligatory pathogens are those thought to be directly related to host infection, and are contained within structures such as cell walls, plasma membranes, cytoplasm, ribosomes and nuclear inclusions.

In fish few bacterial species, however appear to be obligatory parasites. They are unable to multiply to any significant extent outside the host, although they may survive for variable lengths of time in the aquatic environment. Disease caused by these primary pathogens is invariably stress-mediated. Fish that appear clinically healthy yet latently infected, generally do not succumb to infection, provided favourable environmental conditions prevail. They may remain long term carriers of the pathogen, able to infect other fish especially when subjected to stress (Roberts, 2012). In such cases, overt clinical disease may manifest upon major change in the physiology of the fish, due to action of an external stressor, or occasionally associated with internally driven changes such as spawning. A

number of pathogenic microorganisms including *Aeromonas,Pseudomonas, Edwardsiella* and *Streptococcus* have been implicated in bacterial epidemics in (*Oreochromis* species) cultures (Dong *et al*., 2015; Karimi, 2015; Huicab-pech *et al*., 2016).

The pathogenicity and infection of an organism are associated with stress and environmental dynamics including the natural microbiota, organic material and water quality(Fig.4). Vital water quality parameters include pH, temperature, salinity, oxygen, ammonium, nitrates and nitrites (Huicab-pech *et al*., 2016). Ultimately, significant deviations from what is considered normal, results in response otherwise known as stress.
In aquaculture stress in fish is determined by the period of adaptation to changes in their aquatic environment, lack of oxygen, high densities, handling and changes in the temperature and pollutants in the water associated with symptoms pointing to no balance among the host, pathogen and aquatic environment (Huicab-pech *et al*., 2016). Waters with a high organic load, which favour the multiplication of bacteria, rapidly changing temperatures, overcrowding, trauma and transportation are the most commonly encountered environmental stress factors which predispose to clinical disease in fish. Intensive fish culture systems are particularly likely to give rise to these factors(Roberts, 2012).

2.4 Diagnosis of Diseases in Fish

Diagnosis in a disease situation begins with standardized collection otherwise known as sampling of fish specimens. According to international standards (OIE, 2003), a minimum number of ten moribund fish or ten fish exhibiting clinical signs of disease in question must be sampled; these fish must be alive. The transportation of samples to the laboratory,in aseptically sealed refrigerated containers, is optional. However the most preferable and recommended is sampling or collection of organ samples from the fish

immediately they have been selected, on-site at the fish production site then stored. The place and time of sampling must be noted in form of a label attached to the sample.

The collection of fish must encompass a statistically significant number of specimens, but it is obvious that failure to detect certain pathogens from the sample does not guarantee the absence of these agents in the specimen examined or in the stock(OIE 2003). In the case of clinical infection, the sample material to be used for bacteriological tests include the entire viscera or whole alevin (immature fish), organs to be sampled include the kidney and spleen. Isolation and identification also be sourced from other organs including liver, brain, gills, intestine or blood(Darwish *et al*., 2004; Al-harbi *et al*., 2005; Jeffery *et al*., 2010; Amal and Zamri-Saad, 2011; Roberts, 2012; Ahmed, 2013;Dong *et al*.,2016) depending on the suspected potential disease situation and accompanying clinical signs. The organ of choice for isolating systemic bacterial pathogens in fish is the kidney(Noga, 2010). Swabs or samples of skin are appropriate for skin disease(Buller, 2014).

Preliminary detection, isolation and identification are based on clinical signs exhibited in the sick fish,post-mortem observations, histopathology, morphological culture characteristics, Gram staining and biochemical test results. Definitive identification is by molecular techniques and serological assays. The bacterial species including *Aeromonas hydrophila, Streptococcus iniae, Streptococcus agalactiae* and *Lactococcus garvicae*, *Pseudomonas aeroginosa* and *Edwardsiella tarda* have been reportedly isolated from cultured and commonly associated with disease outbreak in *O. niloticus* worldwide (Iregui *et al*., 2004; Belém-costa *et al*., 2006; Austin and Austin, 2007; Baiano and Barnes, 2009; Musa *et al*., 2009; Woo, 2011; Roberts, 2012; Anshary *et al*., 2014; Karimi, 2015; Amutha and Kokila, 2016; Huicab-pech *et al*., 2016).Bacterial species*Streptococcus Iniae*, *Lactococcus garvicae* and *Aeromonas hydrophila* have been isolated and confirmed to

molecular level, and implicated as causative agents of disease outbreak in farmed *O. niloticus* on Lake Kariba, Zambia (Hang'ombe and Ndashe, 2015). These have been as a result of on-going active disease surveillance and diagnosis by Veterinary health professionals within the Commercial farms catchment area.Illustrated in Appendix 1 are identification features of bacteria pathogens associated with*O. niloticus*.

(a) Clinical Signs

Disease detection at the earliest possible opportunity is of extreme importance as prompt treatment may be able to save the fish, and equally important, prevent spread of the disease to other fish(Loh,2003).However, most clinical signs are non-specific. Recognition of signs of illness depends upon regular observation of the fish, so as to be familiar with the normal physical appearance and behaviour(Aly, 2013).

A thorough history is pertinent before fish are examined for behavioural abnormalities in their natural setting. Sick fish often congregate together, separating themselves from their healthier cohorts, and position in the water column (surface, bottom and shoreline) often changes in sickness(Noga,2010). Extremely sick fish may be in dorsal or lateral recumbency as well as exhibit other behavioural signs, including staying near the surface of the water due to hypoxia, scrapping of the body or holding the fins close to the body due to parasitic irritation or perhaps showing behavioural abnormalities from nervous system involvement(Loh, 2003).

Sick fish are often abnormally coloured compared to their healthy cohorts;the melanin pigmentation in fish's skin is under neuroendocrine control and is thus affected by hormones, such as epinephrine(Helfman *et al.*, 2009). Maintenance of a normal pigmentation pattern presumably takes less precedence than homeostasis of more vital

body functions when fish are sick.Blindness can also cause a colour change, which eliminates the normal visual cues that are needed to maintain a normal colour pattern in daylight(Noga, 2010). The chemical signals that control pigmentation are transmitted via the nerves, peripheral nerve damage, such as from vertebral instability, can cause a focal change in pigmentation pattern. Focal colour change can also be caused by local tissue irritation/damage, such as parasite feeding, chronic wounds, or healing wounds, which cause a change in the pigment cell distribution at that site.Haemorrhage usually causes reddening of the body,resulting from systemic bacterial or viral infectionssuch as in *Aeromonas* infection, or skin wounds such as ulcers(Austin,2012).

Other common gross signs of disease include loss of fin tissue, resulting in eroded or irregular fins, most often resulting from poor water quality. This is bearing in mind that acute confinement may quickly lead to iatrogenic skin erosion and ulceration, thus one must be certain that such changes were not caused by the acute stress of capturing and transporting of the fish. Trauma to the eyes or mouth often is present in large fish in aquaria or in any fish exhibiting a strong pressing behaviour against the sides of a tank. Swelling of the abdomen, also known as dropsy, is most commonly caused by an infectious peritonitis (viral, bacterial, or parasitic) but can also be caused by a metabolic disturbance (e.g., renal failure), neoplasia, obesity, or egg retention (“egg bound”)(Noga,2010).

Chronically ill fish are often emaciated as was noted by Tang and Nelson (1998)where many of the smaller fish had wasted bodies, which gave the appearance of a large head.Most of the diseased fish were also noticed to be much smaller than the healthier individuals. Emaciation is evident by loss of dorsal (back) muscle, a concave abdomen, and enophthalmos. Eye lesions, such as exophthalmos are found to be common in several

infectious diseases, including several viral and bacterial infections. Unilateral eye lesions most often indicate possible traumatic cause, especially in large fish. Many nutritional deficiencies are also associated with ocular pathology. Skeletal deformities, especially of the vertebral column, may have many causes, including hereditary factors, defective embryonic development, water temperature that is unsuitable, salinity fluctuation, environmental hypoxia, x-irradiation, ultraviolet radiation, ascorbate deficiency, parasitic infection, electric current, and certain toxins. The gills may also exhibit gross lesions, whose examination is most easily done when taking biopsies(Noga, 2010).

Once fish, swabs or tissues have been collected, they must then be placed into a transport medium for preservation during transportation to the laboratory. Amies transport media may be used for this purpose, as soon as possible after collection preferably on ice at 4°C (Buller,2014).

(b) Post-mortem Observations

It is always desirable to do a complete necropsy examination on selected individuals, provided circumstances are permitting.Approximately four to six individuals showing clinical signs typical of the disease outbreak are a sufficient representative of the population(Noga, 2010).The diagnostic usefulness of the post-mortem examination is highly dependent upon the quality of specimens; recently died fish are often of no diagnostic value.Fish decompose much more rapidly than mammals under similar conditions; this is especially true for small fish, therefore, whenever possible, live fish should be examined. Bacterial invasion of both skin and internal organs occurs rapidly after death, making interpretation of culture results difficult, and additionally, certain parasites die within minutes to hours of the host death.

The skin and gills should be examined first as they deteriorate rapidly. The internal organs; intestines, liver, spleens, gonads, swim bladder, kidney, peritoneal cavity and braincase must be examined systematically with biopsies, swabs and smears collected as per required. Blood may also be collected and smears prepared on a clean microscope slide(Meyers, 2000).

(c) Histopathology

Histology is a useful tool for differentiating many of the diseases affecting internal organs, such as parasites or pathologies due to chronic bacterial infection. Affected tissues containing anomalies such as granulomas may also be histologically processed for a diagnosis(Noga,2010). Histological samples should be fixed in Bouin's solution, Helly's solution or 10% buffered formalin.Ideally 10 moribund fish and 10 that are apparently normal from the same lot must be sampled whole or have individual organs separated. Dead fish are unsuitable for histology. The volume of fixative must be 10 times the volume of tissue.

At the time of processing organs and tissue samples from a single fish should be placed in one cassette, and labelled according to the fish from which it was taken. The tissues are in cassettes, preserved with fixative go through standard histopathology processing being moved into 70% alcohol, dehydrated then rehydrated in the tissue processor. Once the tissues are embedded, cooled and cut, they are fixed on a slide and ready to be viewed under the microscope(Meyers, 2000).

(d) Culture and Isolation of the Agent

Bacterial culture is a term that refers to proliferation of bacteria with a suitable nutrient substance (Kayzer *et al.*, 2005). It is important to use standardized bacteriological culture

methods and perform the work in a precise and logical step-by-step manner, to ensure successful isolation and accurate identification of a suspected pathogen(Buller, 2014).

Specimens collected ought to be inoculated to general purpose media for culture. The medium is then incubated at 25℃ for 2-5days for general culture conditions.Noga (2010) states it is best to culture fish isolates at room temperature (22-25℃), not 37℃ as is routinely done in commercial microbiology laboratories. Some organisms have special growth requirements or may be better detected with the use of a selective and/or enrichment medium. Freshwater specimens are cultured in blood agar and MacConkey agar, Tryptone soya agar (TSA) may also be used, but with the addition of blood to improve isolation of some organisms (Buller, 2014). Nutrient-rich blood agar is a good general-purpose medium for both freshwater and marine bacterial pathogens.

In Saudi Arabia, Al-harbi *et al*. (2005) isolated bacterial strains from the gills and intestine of tilapia, among other sites. He was able to divide the bacterial colonies into different types according to colony characteristics of shape, size, elevation, structure, surface, edge, colour and opacity.Representatives of each colony type were then streaked on additional TSA plates repeatedly until pure cultures were obtained. Seven bacterial species that included*Vibrio parahaemolyticus, V.carchariae, V. alginolyticus, Chryseomonas sp., V. vulnificus, Streptococcus sp.* and *Shewanella putrefaciens* were able to be isolated from different populations.

(e) Biochemical Characteristics

Classification of bacterial pathogens is according to their biochemical characteristics. The evaluation of such characteristics gives insight into the ability of bacteria to alter specific substrates and synthesize various products. Most bacterial cultures are similar in their morphological and microbiological culture characteristics, yet some bacteria are capable

of exhibiting differences, according to metabolic reactions regarding catalase, oxidase, indole, lysine and arginine production, and the fermentation of glucose and lactose, among other sugars and tests. Other verification tests include Voges–Proskaüer, Methyl-red and Gram-stain (Huicab-pech *et al*., 2016).

Pure subculture growths are used for the inoculation into biochemical identification tests.Primary verification include the Gram stain and microscopic examination of smears, catalase, oxidase, presence of haemolysis, motility and growth on MacConkey agar(Buller,2014). The secondary identification is by biochemical characterisation, which may be used to catalogue an organism to species level. These include tube media tests of carbohydrate fermentation of L-arabinose, glucose, inositol, lactose, maltose, mannitol, mannose, salicin, sorbitol, sucrose, trehalose and xylose. The carbohydrates are commonly termed as 'sugars'. Decarboxylases include Arginine dihydrolase (ADH), lysine decarboxylase (LDC), ornithine decarboxylase (ODC), decarboxylase control tube. Other biochemical tests include Aesculin, motility, methyl red Voges–Proskaüer (MRVP), nitrate, oxidative fermentative tubes, *o*-nitrophenyl b-d-galactopyranoside (ONPG), triple sugar iron (TSI) for hydrogen sulphide gas, indole and urea tests(Buller,2014).

The API identification systems have been established for the confirmation of bacteria from medical laboratories, however, their use for the identification also of bacteria from the aquatic environment has been reported in the literature (Swaminathan *et al*., 2004;Roberts, 2012; Buller, 2014). BioMérieux, France, produces a number of these kits for the identification of bacteria including *Streptococcus, Aeromonas, Corynebacteriaspp.*and *Eschericia coli.*

(f) Serological Assays

With the evolution of disease diagnostic procedures in aquaculture, antibody-based (protein-based) immunodiagnosis have been playing a crucial role. These methods have the advantage over other traditional methods in being able to detect sub-clinical/latent/carrier sate of infection and also discriminate the antigenic difference, in a relatively rapid and more specific and sensitive way (Sudhagar *et al.*, 2017). Many immunological methods have already been developed and a wide range of antibodies and a variety of kits are now commercially available to detect fish and shrimp pathogens.

Detection of pathogens Immunological methods such as fluorescent antibody technique (FAT) and indirect fluorescent antibody techniques (IFAT), immunohistochemistry (IHC), enzyme-linked immunosorbent assay (ELISA) and dot blot/Western blot have enabled rapid, specific detection of pathogens without the need to first isolate the pathogen. Monoclonal antibodies (mAbs) provide ideal standardised reagents for such tests and many are now commercially available against a variety of fish pathogens(Adams and Thompson, 2008).The FAT and IFAT are very simple, sensitive methods that can be performed within two hours, with the requirement of specialised equipment (i.e. fluorescent microscope or confocal microscope) and a skilled operator to read results. This method is widely used for the detection of fish pathogens in samples cultured from infected fish, formalin-fixed tissue sections, or on imprints made directly from infected tissue. It is particularly useful for the identification of viruses and bacteria that are difficult to culture.The ELISA can be used in a variety of formats, both for the detection of pathogen and for serology (detection of antibodies to the pathogen). The sandwich ELISA is useful for the detection of pathogens during clinical disease, but is limited in its application to subclinical infection. It does have the advantage of high throughput, automated equipment is available, and it is quantitative. Western blot and dot blot are not

used routinely as diagnostic methods, but their application can be useful in certain situations, e.g. they are used to confirm the presence of viruses such as those in shrimp (e.g.white spot shrimp virus)(Sudhagar *et al.*, 2017).

Serology has been found to offer an alternative, indirect approach to pathogen detection. Though due to insufficient development of serological methodology, the detection of antibodies to pathogens in fish is yet to be established as a routine method for assessing the health status of fish populations (Adams and Thompson, 2008; OIE, 2003).

(g) Molecular techniques

Over the past 15 years or so, molecular techniques have been increasingly employed to diagnose fish diseases. In the past,post-mortem necropsy and histopathology have been the primary methods of diagnosis, however these methods have been shown to lack specificity, sensitivity along with being time-consuming and costly(Sudhagar *et al*., 2017). In addition, many pathogens are difficult to detect when present in low numbers or when clinical signs of disease are not present.

In an effort to overcome challenges in diagnostics, DNA (Deoxyribonucleic acid)-based diagnostic methods have been developed. The use of these DNA-based methods is derived from the premise that each species of pathogen carries unique DNA or RNA (Ribonucleic acid) sequences that differentiate it from other organisms. These techniques offer high sensitivity and specificity, as well as diagnostics kits allowing rapid screening for the presence of pathogen DNA. Variations of the DNA amplification techniques include multiplex polymerase chain reaction (PCR), labelling and detection of nucleic acids, Restriction enzyme digestion, Restriction Fragment Length Polymorphism(RFLP),

Random Amplified Polymorphic DNA (RAPD), *In-situ* Hybridization, DNA microarrays and Loop-Mediated Isothermal Amplification (LAMP) (Sudhagar *et al.,* 2017).

Besides use in specialized laboratories, DNA probes are expected to find increasing use in routine disease monitoring and treatment programs in aquaculture, in field epidemiology and in efforts to prevent the international spread of pathogens (national quarantine and certification programs) (FAO,1999).

2.5 Management and Control of Bacterial Fish Disease

Disease management in farmed fish systems, especially in developing countries, is fairly challenging as production is dependent on natural environmental conditions, in contrast to terrestrial animal production systems where environmental conditions can be very closely controlled (Roberts, 2012).

Majority of disease conditions in aquaculture will be significantly reduced if proper attention is paid to good husbandry and the maintenance of optimum environmental conditions, especially water quality.Effective fish health management programs are focused on keeping stressful conditions to a minimum, prevention of the introduction of pathogens, negligible use of drugs, and use of vaccines when available. It is axiomatic that well-nourished fish reared in highly favourable environmental conditions will be resistant tomost pathogens and in many cases, prompt reduction of the stressful conditions may lead to self-cures without the need to resort to chemotherapy (Meyer *et al*., 1991).

2.5.1 Husbandry and maintenance of optimal conditions

2.5.1.1 Water quality

Disease prevention and control in aquaculture is a function of the nature of a facility and how it is managed, to a large degree. Effective environmental manipulation often results in successful fish culture. Infectious disease occurrence is often closely related to

environmental stress, which is determined by conditions including site selection, quality of water supply, facility design, fish handling,transport systems and the efficiency of waste removal(Aly, 2013).

Good water quality is essential to the health of the fish, which differs between species and life stages of individuals. Unlike mammals, fish are unable to regulate their own body temperature, making temperature the most important water-quality variable. It affects growth rate, feed conversion rate, metabolism and reproductive ability of the fish (Rural Fisheries Programme, 2010). *Oreochromis niloticus* is a species that prefers to live in shallow water, with lower and upper lethal temperatures of 11-12°C and 42°C, respectively. The preferred temperature ranges from 31-36°C (FAO, 2012) whilst optimal temperature is from 24-32°C. Growth rate declines rapidly at temperature below 20°C with little or no growth registered at temperature below 15°C. At these temperatures the fish is also more susceptible to diseases and with mortality often experienced at temperatures of 11°C and below(Stander, 2000).

Water quality is an area of paramount concern and plays a major role in the efficiency of production and the quality of the end product, therefore it is important that the quality of the water is constantly analysed for vital parameters including pH, temperature, salinity, oxygen, ammonium, nitrates and nitrites (Huicab-pech *et al*., 2016).

Fish, like other terrestrial animals use oxygen, however oxygen available to them is that which is dissolved in the water. Dissolved oxygen(DO) level refers to the amount of molecular oxygen within the water, and it is measured in milligrams per litre. Oxygen dissolves directly into the water surface from the atmosphere and in natural conditions; fish can survive in such water. In intensive production systems with higher fish densities,

this amount of DO diffusion may be insufficient to meet the demands of fish, plants and bacteria, and thus needs to be supplemented through management strategies. This included the use of aerators, paddlewheels and air-stones (Rural Fisheries Programme, 2010).

Dissolved oxygen is the water quality parameter that has the most immediate and drastic effect, therefore must be diligently monitored. This may be done so through use of measuring devices and through frequent monitoring of fish behaviour and plant growth.The amount of oxygen in the water is closely linked to temperature, with lower oxygen levels occurring at higher temperatures (Ngugi *et al*., 2007). This is due to the fact that,at higher temperature past that best for the species, fish metabolism increases, creating more waste resulting in faster bacterial growth from utilization of this waste. The bacteria deplete oxygen supply from the water as they thrive. A second reason is that, as water temperature rises, the solubility of oxygen decreases; warm water holds less oxygen than does cold water. The optimum DO level for *Oreochromis* species is a minimum of 5mg/L(Stander, 2000; Ngugi *et al*., 2007), within acceptable units of 4-6 mg/litre.

The pH scale refers to the degree to which water is acid or alkaline, which ranges from 0-14;acid substances have a pH from 0-7 where 7 is neutral (neither acidic nor alkaline), and alkaline is between 7 and 14. A change in a single pH unit represents a large change in water quality, fish generally prefer water that is neither too acidic nor too, alkaline maintained within one unit from neutral (pH 6-8) (Beveridge, 2004). An experiment by El-Sherif and El-Feky (2009) to investigate the effect of different levels of pH on growth performance and some blood parameters of Nile tilapia (*O. niloticus*) fingerlings further proved the sensitivity of pH as a water parameter. A great difference in the growth rates among different pH levels was shown at the end of the experimental period, with a decrease at low pH. It was concluded that the average individual body weights of tilapia

observed in the experimental groups of pH 7 and 8 were found to be the best, with no significant mean weight gains between the two. Also no significant differences among average haemoglobin levels in pH 7, 8 and 9 were observed. The water pH can be measured using simple pH test-strips, chemical test kits or digital probes (Rural Fisheries Programme, 2010).

Nitrogen is required by all life as part of proteins, and enters the aquatic environment through fish feed as crude protein. Some of this protein is used by the fish for growth, with the remainder being released by the fish in the form of ammonia (NH_3) waste. It is released through the gills ,urine andin solid waste (faeces)(Masser,2007). Ammonia is thought to be the next important water-quality factor after dissolved oxygen. Ammonia is toxic to fish. *Oreochromis* species can show symptoms of ammonia poisoning at levels as low as 1.0 mg/litre, with prolonged exposure at or above this level leading to damage to the fishes' central nervous system and gills, resulting in loss of equilibrium, impaired respiration and convulsions. At higher levels of ammonia, effects are immediate and numerous deaths can occur rapidly. Lower levels over a long period result in fish stress and increased incidence of disease, resulting in more fish loss. Ammonia toxicity is actually also dependent on both pH and temperature; higher pH and water temperature make ammonia more toxic. The formation of nitrite (NO_2) is the step between the conversions of ammonia to nitrate by nitrifying bacteria.At sub-lethal levels, nitrite reduces oxygen transport into the fish by reducing the oxygen-carrying capacity of the fish's blood. High levels also result in poor feed conversion, reduced growth, and increased susceptibility to disease(Masser, 2007; Ngugi *et al*., 2007). Recommended levels of nitrite and nitrate according toStander (2000) are,5mg/L and 100mg/L, respectively. Normal nitrite range is <1 mg/litre,and <400mg/ litre of nitrate. These parameters are measured using a water test-kit in mg/L.

Stocking density in aquaculture is also a very important factor in relation to water quality, in that it must be within optimum range in order to maintain water quality suitable for fish health and growth(Table 1). Rahmatullah *et al*. (2010) performed a study to determine the effects of stocking density on the growth and production parameters of *Oreochromis* species in an aquaponic system. With all water quality parameters including temperature, dissolved oxygen, pH, alkalinity, nitrite-nitrogen and nitrate-nitrogen maintained within suitable ranges for culture of these species, indicated an inverse relationship between weight gain and stocking density. Survival rate was also found to be highest in the lowest stocking density. Recommended stocking rate of *Oreochromis* specie fingerlings depends on cage volume, desired harvest size and production level, as well as the length of the culture period(Mcginty and Rakocy, 2015).

Table 1: Standard stocking densities in aquaculture systems

System	**Stocking Density**
Ponds	3 fish/m^2
Floating Cages	$4m^3$ cages -200-300 fish/m^2
	$\geq 100m^3$ cages- 25-50 fish/m^3
Tanks and Raceways	160-185 kg/m^3
Recirculation Systems	60-120 kg/m^3 of rearing tank volume

Source: MSD Animal Health (2006)

2.5.1.2 Husbandry

In order for fish health management to be successful, disease prevention rather than treatment should be imperative. Health management includes good water quality management,nutrition and good sanitation practices. There is no substitute for proper animal husbandry in fish health and management(Francis-Floyd, 1990).

The foundation for good husbandry is in maintaining good records, which should also be available for immediate reference in the event of a fish disease outbreak. Records include dates fish were stocked, size of the fish at stocking, source of fish, feeding rate, growth rate, daily mortality and water quality(Parker, 2012). Daily observation of fish at regular intervals, to get an overview of behaviour and feeding activity is also of good practice as it allows early detection of problems when they occur. This is as illustrated in Appendix 2.

With careful observation fish that are sick can usually be distinguished before they start dying. Sick fish often stop feeding (healthy fish eat aggressively if fed at regularly scheduled times) and may appear lethargic.Behaviouralabnormalities such as hanging listlessly in shallow water, gasping at the surface, or rubbing against sharp objects indicate something may be wrong. Upon suspicion that fish are getting sick, water quality is the first that must be checked to ensure the vital parameters are within optimal range, and rectified if need be. Good records, a description of behavioural and physical signs exhibited by sick fish, results of water quality tests and accompanying daily records provide a complete case history for the diagnostician at work on the case(Francis-Floyd, 1990). Submission of organ samples including the kidney, spleen, brain, eye and liver, to a diagnostic laboratory may also be recommended. Diagnosis may be made before the majority of the population becomes sick or corrective action indicated, early implementation will be most successful whilst the fish are still in fairly good condition.Fish care or husbandry practices in cage culture are quite simplified. Observation of fish behaviour, especially feeding behaviour is essential for good husbandry.

2.5.1.3 Biosecurity practices

Biosecurity in aquaculture consists of practices that minimize the risk of introduction and spread of infectious agents or disease, and the risk that these infections will leave the

faculty and spread to other sites(Yanong and Erlacher-reid, 2012).Good biosecurity minimized the fish's exposure and susceptibility to pathogens and reduces economic losses from mortalities.The major goals of biosecurity are animal, pathogen and people management. The ease with which a specific pathogen can enter a facility and spread is dependent on host factors including species immune status, life stage, physiological state and genetics. Major environmental factors include water quality and husbandry practices. Pathogen characteristics that include biology and lifecycle, potential reservoirs, survival on inanimate objects are also of importance. It is also imperative that workers understand biosecurity principles as well as comply with biosecurity protocols.

A. Animal Management

This critically entails that healthy fish are sourced from a reputable source. The assistance of animal health professionals ought to be utilized to obtainas much information about an animal's source and whether they have undergone any health examinations. Unavailable or unclear information must be compensated through testing shortly after they are received. Lineage of the broodstock must also be taken note of to target more hardy or specific disease resistant strainsas well as avoid inbreeding(Rural Programme, 2010).

Good husbandry to prevent stressful conditions, good water quality/chemistry, nutrition and handling methods go far towards preventing disease. Medical practices including quarantine, routine observations, vaccination and use of immunostimulants, probiotics and diagnosis for disease management are good preventive measures worth employing. One of the most important animal management and biosecurity measures is quarantine, which involves isolation of an individual or population, in which acclimatization,observation and if necessary, treatment for diseases is performed, prior to release on the farm(Lio-Po *et al.*, 2001).The principles of quarantine apply to new fish coming onto the facility, fish moving from one area or system to another within a facility or resident fish that become diseased.

It is for this reason that quarantine facilities must be well designed to ensure efficient physical separation as well as proper handling of discharges. The quarantine period should exceed the length of the longest latent period of suspected pathogens. The major components of quarantine include all-in-all-out stocking isolation or separation, observation and diet adjustment, and sampling and treatment.

B. Pathogen Management

Pathogens vary in their regulatory significance with disease surveillance boards, survivability in reservoirs and hosts, pathogenicity, diagnostics and control, and though they vary in disease causation, ultimately the environmental and host factors will determine whether fish will become sick. Regulatory significance of diseases and pathogens must be taken into consideration. Due to the economic and environmental importance of some internationally listed diseases by the OIE, certain outbreaks may require depopulation, due to potentially serious socio-economic consequences(FAO, 2010).

Reservoirs such as water system components, equipment, floors, walls, even feeds can harbour pathogens. Living reservoirs including aquacultured animals themselves, other animals (such as frogs and birds),plants and live(or frozen) food may present an environment in which these pathogens survive and thrive. It is for this reason that the biology and factors that permit pathogen survival in reservoirs, as well as how easily they can be killed by common disinfectants are very crucial(Yanong and Erlacher-reid, 2012). High animal densities, biofilms and sediments can help concentrate and amplify pathogenic microorganismssuch as *Aeromonas* and *Vibrio* species.Pathogens enter facilities via many routes including aerosol (fine water droplets), on equipment and

vehicles, on people's hands, feet and arms or may be spread by other animals living near the water body (rodents, reptiles, insects, aquatic vertebrates).

Diagnostics coupled with routine tests are a very useful tool. The major drawback to this is with disease conditions that are more difficult to identify in fish that are carriers with hidden pathogens or that are present in very small numbers. The fish are otherwise healthy-looking in this synario.Good sanitation or disinfection or use of drugs may be the key to controlling some infectious diseases, whilst other pathogens including mycobacteria, some parasites, and many viruses cannot be treated effectively or easily.

It is of great importance that a cleaning and disinfection protocol be established(Price and Beck-Stimpert, 2014). This would include manual removal of dirt and organic matter, vigorous cleaning with detergent or soap and water, followed by rinsing, then application of a disinfectant with appropriate contact time, rinsing again or neutralizing, then finally completely drying(preferably in the sun). Good sanitation involves cleanliness including making sure that filtration and aeration systems are well maintained and that excess suspended particulates, uneaten food, dead or dying fish(a major pathogen reservoir), detritus and other organics are removed and disposed of appropriately according to relevant regulations.All equipment, surfaces, vehicles/tyres within the facility must be disinfected; vehicle access may also be limited to specific areas especially if vehicles have been driven to other aquaculture facilities.

A number of disinfection methods that can be used in aquaculture can be broadly divided into two; physical and chemical methods (Lio-Po *et al.*,2001; Yanong and Erlacher-reid, 2012). Physical methods include heat, sunlight and drying(dessication). Chemical disinfection involves the use of chemical disinfectants such as chlorine products (bleach as sodium hypochlorite or calcium hypochlorite), Virkon® Aquatic,quaternary ammonium compounds(QACs), chlorohexidines, alcohols (such as isopropyl alcohol), iodophors,

hydrogen peroxide, phenol derivatives and formaldehyde. The methods used are determined by logistics, residue toxicity and cost best suitable to the scale of an aquaculture facility.

C. People Management

This involves the successful understanding and cooperation of managers, staff and visitors(Aquaculture New Zealand, 2016). It has been demonstrated that a lack of following the established protocols increases the risk of disease. Large enough workforces should be divided with specific personnel being assigned specific work areas based on age, species and perhaps disease status. In a scenario where personnel are not enough, handling should begin from the clean and healthy to the diseased fish, or chronologically, where the younger (most disease susceptible) are handled first,then to the oldest fish.

Some of the measures that may be employed to prevent introduction of diseases includes disinfection stations for people and equipment. These include disinfectant footbaths, handwashing stations or alcohol spray bottles, net disinfection stations, showers and vehicle disinfection stations, to be placed at strategic places(Yanong and Erlacher-reid, 2012).

Critical control points are the production steps or physical locations at which the fish may be exposed to disease pathogens or at which pathogens may enter the aquaculture systems. These include, on the fish themselves, in the water, from the environment, on other organisms and in commercial feeds or live and frozen foods. Therefore it is of utmost importance that the ways these possible pathogens may arise are evaluated and preventive measures put into effect (Volta, 2011).

Bearing all the aforementioned risk factors into consideration, a written biosecurity programme based on risk analysis may be developed. This includes a detailed assessment of the risk, followed byappropriate management strategies and lastly communication of the risk to involved parties(Volta,2011; Aquaculture New Zealand, 2016).Once in place, on-going educational programs on a biosecurity programme should be provided for employees. It is of importance that the biosecurity program, employee compliance, disease risks, andproductivity should undergo periodic review(Yanong and Erlacher-reid, 2012).

2.6 Use of Antibacterials in Aquaculture

2.6.1 Background information

Human consumption has resulted in an increased demand for animal protein, which has been on the rise globally. This has resulted in the association of modern animal production with the regular use of antimicrobials. This is according to an analysis on global trends in antimicrobials used in food animals by Boeckel *et al*. (2015). Growth in aquaculture globally, has been accompanied by disease constraint, with reports from many regions of the world on the increase. Maintenance of animals of specified health status, vaccination, eradication and good hygiene have been used to control bacterial diseases in some situations. Despite this there still remains need for chemotherapy in treating, and in some cases, preventing some bacterial diseases. Appropriate use cures sick animals, speeding up the recovery of others, improves their welfare and reduces the spread of infection to other animals (Aly and Albutti, 2014).

A wide range of chemicals are used in aquaculture including antibacterials, pesticides, hormones, anaesthetics, various pigments, minerals and vitamins. Not all of the aforementioned are antibacterial agents. Antibiotics or antibacterials are drugs of natural

or synthetic origin with the capacity to kill or to inhibit the growth of microorganisms(Sekkin and Kum, 2011).

In aquaculture, the vast majority of antibacterial treatments are administered by cage/pond or to the entire site or unit. Antibacterials are used therapeutically, when an outbreak of disease occurs within the system. The use of antibacterials as growth promoters is often voluntary, although this practice is generally rare in aquaculture. Prophylactic treatments, when employed, are mostly confined to the hatchery, the juvenile or larval stages of an aquatic animal production system. Prophylactic treatments are also thought to be more common in small-scale production units that perhaps cannot afford, or cannot gain access to, the advice of health care professionals(Sekkin and Kum, 2011). Metaphylaxis treatments are initiated post-isolation of a target bacterium or the presence of a particular bacterium is assumed, based on disease signs (Austin, 2012).

Antibacterial chemotherapy has been in use for over 60 years, with peaks being in the late 1980s into the 1990s(Sekkin and Kum, 2011; Aly and Albutti, 2014). The use of antibiotics in Norwegian salmon farming is shown in Fig. 5, and it demonstrates the impact on antibiotic usage upon introduction of vaccines against bacterial infections. Vaccines were introduced in the late 80s after which there was a marked drop in the use of antibiotics, and it has remained low (Norwegian Medicines Agency, 2017).

In Zambia, records up until the year 2015 state there have been no records of antibiotic usage in aquaculture (Personal Communication, 2015). Disease prevention through improved management practices has performed both at small-scale and commercial farming.

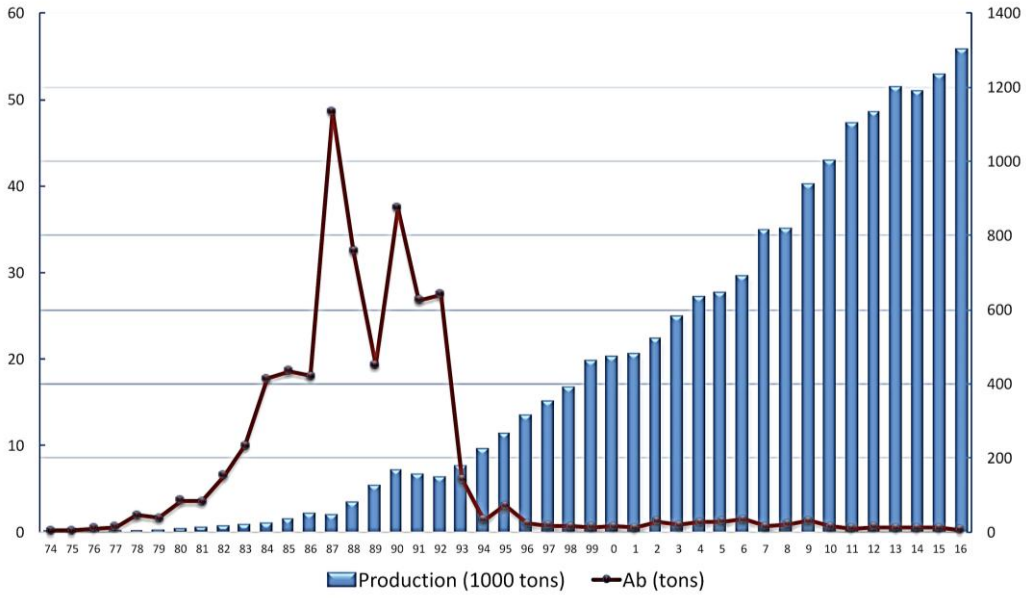


Figure 5: Use of antibiotics (line)and production volume (bars) of Atlantic salmon in Norway between 1980 and 2016 (Norwegian Medicines Agency, 2017)

2.6.2 Commonly used antibacterials

Antibacterials authorized for use in aquaculture include oxytetracycline, florfenicol, sarafloxacin erythromycin and sulphonamides potentiated with trimethoprim or ormethoprim (Serrano, 2005; Kümmerer, 2009). Treatment may be either localized or administered into the diet of the fish. Commercial feeds already containing antibacterial agents are fairly cheap and easy to use. Where commercially medicated feed is not available, medicated feed may be prepared on site. The drugs are suspended in oil such as cod liver oil, soy bean or corn oils, during preparation of the feed to prevent leaching (Aly and Albutti, 2014). Applications include externally, internally via injection; intraperitoneal, subcutaneous or intramuscularly and also by use of bath treatments which could range from a dip bath for a few seconds to a short bath of up to an hour(Aly and Albutti, 2014). The chemical concentrations of the drugs administered are dependent on the growth stage of the fish, systems of management, properties of the drug and route of administration.Antibacterial drugs have different types of chemical structures and act

differently on parts of the target bacterial machinery. Appendix 13 summarizes the mechanisms of action of some antibiotics.

Drug metabolismin fish and mammals is similar, with the primary organ of detoxification of drugs in the fish being the liver. The elimination rate of antibacterial drugs from fish tissues varies greatly with the temperature; elimination half-life increases significantly as the temperature decreases. It is for this reason that temperature dependency of drug pharmacokinetics is an important consideration for drug residues(Sekkin and Kum, 2011).Antibacterial use should always be based upon examination of the clinical case, diagnosis of bacterial infection and selection of a clinically efficacious antibacterial agent. Despite the widespread use of antibacterials in aquaculture facilities, limited data is available on the specific types and amounts to be used. The general considerations in the selection and use of antibacterial drugs are given in Fig. 6.

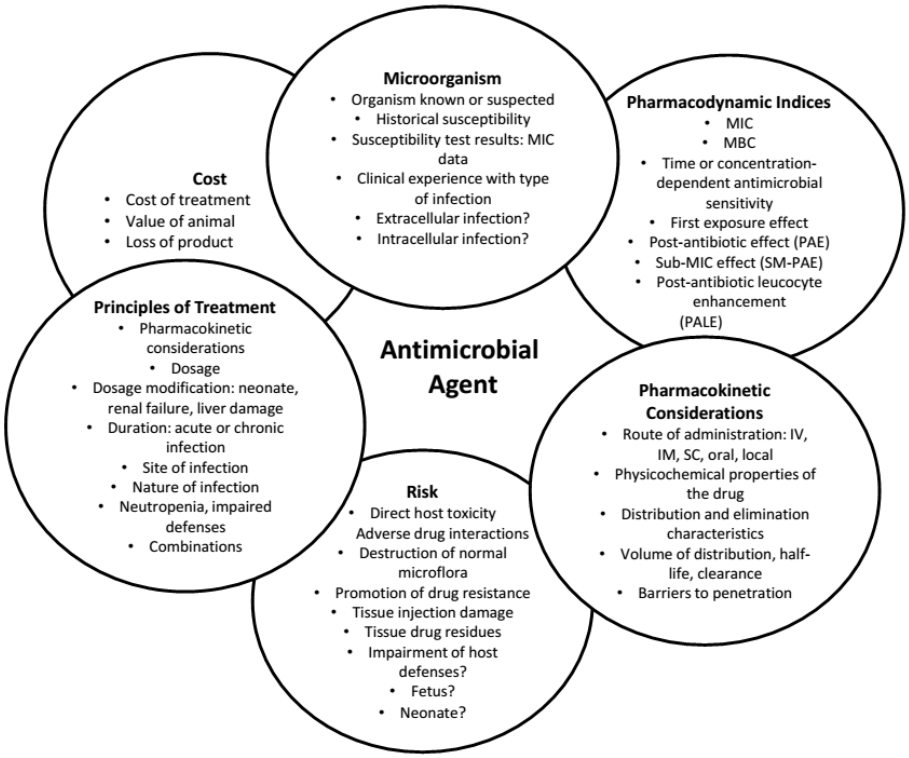


Figure 6: Some considerations in selecting and using antibacterials(Walker and Giguére, 2008)

2.6.3 Antibacterial Resistance

Growing intensification of aquaculture establishments has brought on the indiscriminate use of common antibiotics including ROMET 30® (sulphadimethoxine 25% and Ormetoprim 5%), ROMET TC® and Terramycin® in feed formulations. These events have left room for the development of resistant bacterial strains. Resistance can be described as the relative susceptibility of a microorganism to a particular treatment under known conditions(Sekkin and Kum, 2011). Antibacterial resistance takes two forms; inherent or intrinsic resistance, referring to species that are not normally susceptible to a particular drug due to their inherent characteristics, or, resistance that is acquired through transfer of DNA responsible for the expression of drug resistance through transformation, transduction or conjugation mechanisms.

These processes result in a natural or acquired ability by a pathogen to resist the effect of an antibacterial or chemical, where it was initially susceptible (Huicab-pech *et al.*, 2016).The spread of antibacterial resistance due to exposure to antibacterial agents is well documented in both human and veterinary medicine(Romero *et al*., 2012). The major concerns with treating fish with antibacterials are the potential impact of these compounds on the aquatic environment, both marine and fresh water, and the wider theoretical risks associated with the development of antibacterial resistance by fish pathogens; bacteria in aquatic environments can develop resistance as a result of antibacterialexposure(Kümmerer, 2009; Romero *et al*., 2012).Examples of these bacteria include *A. salmonicida, A.hydrophila, E. tarda, Y. ruckeri, P. damselae* and *V. anguillarum*.

2.7 Vaccination

2.7.1 Background

The term ‘vaccine’ refers to all biological preparations, produced from living organisms, that enhance immunity against a specific disease and either prevent (prophylactic vaccines) or, in some cases, treat disease (therapeutic vaccines)(Jenner,2012). They have been used for many years in humans, terrestrial livestock, and companion animals against a variety of diseases. A vaccine is composed of either the entire disease-causing microorganism, or some of its components, and works by exposing the immune system of an animal to an “antigen”. This then allows time for the immune system to develop a response and lasting “memory”, also to accelerate this response in later infections by the targeted disease-causing organism. Theanalogy used for vaccines is like that of an insurance policy; if effective, can help prevent a future disaster from being a major economic drain. The disclaimer is that vaccines, like insurance, have a premium, or cost. The producer must weigh the cost in materials and labour against the risk and cost of a disease outbreak to determine whether vaccination is warranted.

Ideal properties of a vaccine include one which is safe for the fish, the person(s) vaccinating the fish, and the consumer; protection against a broad strain or pathogen type and gives good protection; provides long-lasting protection, preferably as long as the production cycle;is easily applied; is cost effective; and is readily licensed and registered(Yanong,2014). Of the different types already in use and continuously under development, those most commonly used include bacterins (killed bacteria), live attenuated vaccines, toxoids, or subunit vaccines. They are administered to fish by oral delivery, by immersion, or by injection.These methodseach have different advantages and disadvantages, taking into consideration the nature of the pathogen and its natural route of infection, the life stage of the fish, production techniques and logistical issues. The choice of which one to use will depend upon each unique production situation.

2.7.2 Present Status

Vaccination has played an important role in large-scale commercial fish salmon cultivation farming. In general, empirically developed vaccines based on inactivated bacterial pathogens have proven to be very efficacious, dramatically reducing the use of antibiotics. In addition to salmon and trout, commercial vaccines are available for channel catfish, European seabass and seabream, Japanese amberjack and yellowtail, tilapia (*Oreochromis* species) and Atlantic cod(Sommerset, 2005).The main diseases in these groups of fish controlled by vaccination and their associated infectious agents include furunculosis caused by*A. salmonicida*,vibriosis caused by *V. anguillarum* serotypes 01 and 02, Enteric redmouth (ERM) – *Y. ruckeri*, Infectious pancreatic necrosis caused by IPN virus, Salmon pancreas disease (SPD) caused by SPD virus(NOAH, 2006).

According to Pridgeon and Klesius (2012),vaccines are commercially available for protection against 18 diseases worldwide (Appendix 3).Particularly for *Oreochromis* species these include, Streptococcosis caused by *S. iniae* or *S.phocae* and Edwardsiellosis or putrefactive disease caused by *E. tarda*.There still remain other important diseases in aquaculture, including motile aeromonads septicaemia(*A. hydroplila*), Francisellosis (*Francisella* species), streptococcosis caused by *S. agalactiae*, and other emerging diseases, for which vaccines are still at an experimental level.

A review by Muktar *et al*. (2016) discussed the major limitations in fish vaccine development.These included little understanding of fish immunology, many unlicensed vaccines, a lack of cost effectiveness and stress on fish at administration. It is hoped that next generation vaccines would rely on multiple killed antigens delivered with an adjuvant to enhance vaccine effectiveness.

Pridgeon and Klesius (2012)added on that a majority of the vaccines available are bacterins which can only provide partial protection against certain strains for a limited time frame. Furthermore, delivery by injection(as is the case with most vaccines), is also labour-intensive as well as stressful on the fish. Therefore, user-friendly (immersion or oral delivery) efficacious vaccines that can offer broader protection for a longer duration are urgently needed for the aquaculture industry.It is also of importance that attention must be given to the larval or fry stages, as major disease problems may appear. At these stages, the animal is not large enough to be vaccinated or have even developed functional immune system; the apparent lack of maternal immunity also limits the possibilities to protect these offspring by parental vaccination.

Currently, vaccines are available for some economically important bacterial and only few vaccines for viral diseases and no vaccine developed for fish parasites and fungus(Muktar *et al*., 2016).To achieve progress in fish vaccinology, an increase in the co-operation between basic and applied science (i.e., between the immunologist / microbiologist and the vaccinologist) is required.

At the time of the study, vaccine production against *Streptococcus iniae* was under laboratory trial at the University of Zambia and Norwegian University of Life Sciences. This was in an effort to arrest disease outbreaks that have been causing production losses on Lake Kariba cage *O. niloticus* farms (Personal Communication, University of Zambia, 2015). Field vaccine trials are to commence once laboratory trials are complete.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study area for this research was a commercial cage farm within the Siavonga town, in the Southern Province of Zambia. The site is located at latitude 16°S 28.318', longitude 28°E 38.52', on the Lake Kariba, within Strata IV (Appendix 5).Lake Kariba is the world's largest man-made lake and reservoir by volume, covering an area of 5,580 square kilometres. It lies 1300 kilometres upstream from the Indian Ocean, along the border between Zambia and Zimbabwe.

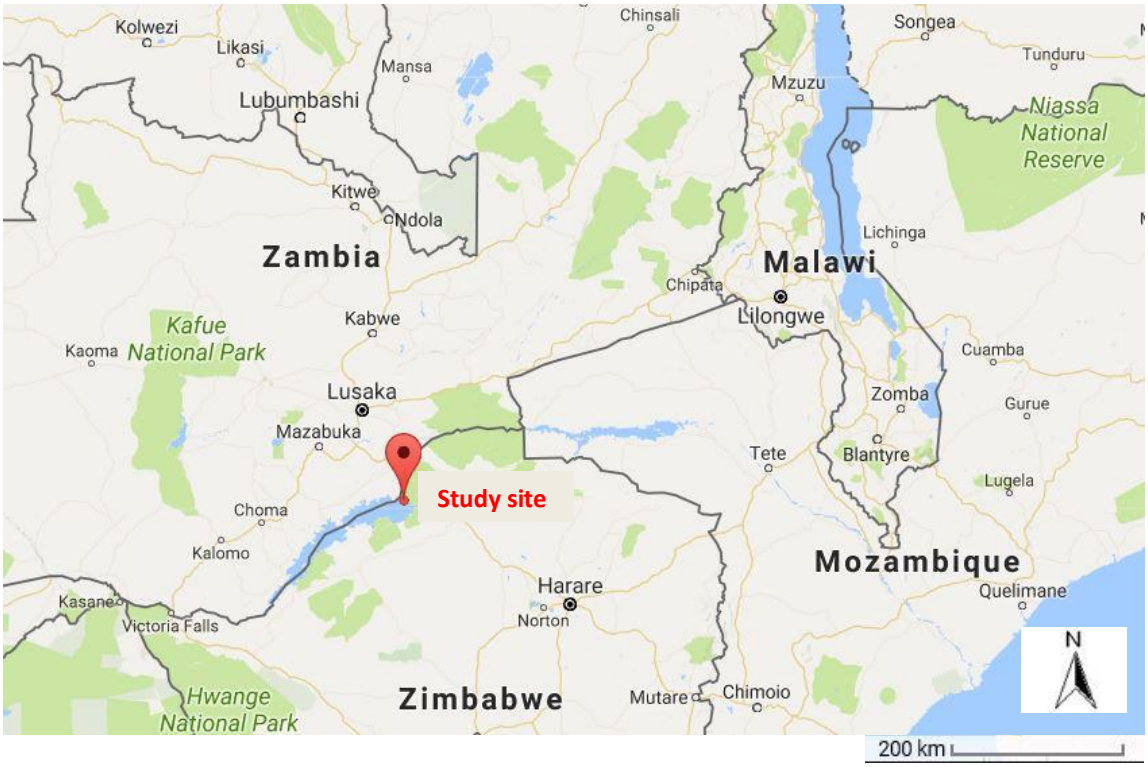


Figure 7: Study Area- Commercial cage farm on Lake Kariba, Zambia

Lake Kariba is home to a vast expanse of freshwater fish species including Tiger fish (*Hydrocynus vittatus*), Catfish (*Clarias gariepinus*), Barbel (*Barbus barbus*), Labeo (*Labeo rohita*), Jack (*Caranx lugubris*), Vundu(*Heterobranchus longifilis*) and Bream/Tilapia(*O. niloticus*); introduced and reared under cage aquaculture facilities(Mudenda, 1994; Fish Site, 2006).

3.2 Study Design

A cross-sectional type of observation was performed in the study.

3.3 Sample Size

Sampling was as such of an outbreak investigation. In line with this, according to (OIE, 2003), sampling to enable detection at 95% confidence level of infected animals must involve case of clinical infection whichrequires sampling of 10 diseased fish; combined to form pools of a maximum of 5 fish each and detecting asymptomatic carriers that include samples combined in pools of no more than 5 fish per pool. According to Midlyng *et al.*(2000) the recommended minimum numbers of adult fish (>150g) to be sampled for outbreak investigation, is a sample size of 5.

Sampling frame of 5 cages/pools; 5 fish from each cage

Total Sample Size= 25 fish

Adjusted Sample Size= 29 fish; 9 organ samples from each fish

3.4 Sampling Technique

A total of 29 fish were captured during feeding time using a disinfected scoop-netfrom five different cages on the Lake Kariba. The subtotal of 25 ‘sick’ fish andfour ‘healthy’ fish were selected purposively from four of the nine cages experiencing disease cases and an increase in mortalities.Fish were initially thoroughly examined for external lesions,

measured (total length and circumference) using standard measuring tape and weighed using an electronic balance. Field data was recorded in designed Field Necropsy Sheet as shown in Appendix 4.

Swabs were collected from the abdomen, spleen, liver, brain, eye, gonads,blood and skin lesions present of each of the 29 fish. The swabs from these organs were then inoculated onto freshly prepared, appropriately labelledNutrient agar and 10% Sheep Blood agar (HiMedia Laboratory Pvt, Mumbai, India) plates by streaking using sterile disposable loops, on-site. Individual organ samples were then collected and stored in 10% buffered formalin, and blood smears were also prepared. Culture plates and samples were then stored at room temperature for 24hrs, and then transported to the laboratory under icepacks-4°C. The organisms were initially grown on Nutrient and Blood Agar media (HiMedia Laboratory Pvt, Mumbai, India) for primary isolation. Culturing was done on-site by streaking with sterile inoculating loop.

3.5 Isolation and Classificationof Bacteria

3.5.1 Culture of bacteria

Samples were processed at the School of Veterinary Medicine, Department of Paraclinical Studies, University of Zambia(Woodland, 2004). Isolation involved three stages; firstly, the different primary bacteria isolates were individually subcultured on Nutrient and Blood agar (HiMedia Laboratory Pvt, Mumbai, India) to ensure that possible contaminants are absent, and incubated at room temperature (25°C) for 24hrs. Then, based on colony morphological appearance, all different bacteria colonies from all organs of each sampled fish were each noted and labelled clearly.

3.5.2 Gram stain and morphology characteristics

The pure colonies produced were then Gram-stained to determine Gram-positive or Gram-negative nature and microscopic morphological appearance. Each individual pure colony was first emulsified in sterile normal saline on a well-labelled, clean, dry glass slide. This was then air-dried and fixed under a Bunsen burner. Thereafter the slides were then stained with Crystal Violet solution for 45seconds, and then washed under gently running water. Thereafter the slides were flooded with iodine solution for 45seconds then washed under gently running water. The slides were then decolourised with 70% alcohol solution, followed by gentle washing under running water. Finally, the slides were counter-stained with safranin solution for 45seconds, followed by gentle washing under running water. The slides were then air dried and viewed at X100 magnification under oil immersion. The microscopic morphology characteristics and Gram stain were then viewed and noted (Buller, 2014).Thereafter the following biochemical tests were performed to confirm the suspected isolates: Simmon's Citrate, SIM(Sulphur, Indole, Motility) test, Triple Sugar Iron test, Urease and carbohydrate 'sugar' utilization tests(Esculin, Galactose, Raffinose, Salicin, Maltose monohydrate, Xylose, Mannitol, Trehalose, Insulin, Sorbitol, Lactose monohydrate and Glucose).

3.5.3Phenotypic Identification of Bacterial Isolates

3.5.3.1 Sulphur Indole Motility Test

The SIM media was first prepared according to the manufacturer's protocol(HiMedia Laboratories, India).It was distributed in short tubes and autoclaved for 121°C for 15minutes. The tubes were allowed to solidify for 24 hrs, producing a clear, light-yellow coloured media.The pure isolates grown on nutrient agar were then collected using a sterile straight-wire loop, and inoculated into the media by stabbing once, under Bunsen burner to maintain sterility. This was followed by incubation for 24hrs at 37°C. After 24hrs, the media was viewed for motility(cloudy appearance) and production of sulphur

gas(blackening). This was followed by addition of two to three drops of Kovac's reagent to the suspension using a dropper, and waiting briefly for 5-10seconds for the reaction to take place. The formation of a pink-coloured ring that rises to the surface indicated a positive Indole result. The presence of no pink-coloured ring meant a negative Indole result.

3.5.3.2Simmon's Citrate Test

The Simon's citrate agar was prepared according to the manufacturer's protocol(HiMedia Laboratories, India).The agar was prepared according to the manufacturer's protocol. It was distributed in long tubes and autoclaved for 121°C for 15minutes. The tubes were then allowed to solidify for 24 hrs, producing a clear, crystal green coloured media. Pure isolates of the organisms were then collected from nutrient agar and inoculated into the agar using a sterile straight-wire loop by gently streaking the slant of the media, under Bunsen burner to maintain sterility. The media was then incubated for 24 hrs at 37°C. The Citrate agar was green in colour before inoculation. A positive result waswhen the colour changes to blue; meaning that the citrate was utilized, and in a negative result there was no colour change and the media remained green.

3.5.3.3 Triple Sugar IronTest

The TSI agar was prepared according to the manufacturer's protocol (HiMedia Laboratories, India). It was distributed in long tubes and autoclaved for 121^{o} C for 15minutes. The tubes were allowed to solidify for 24 hrs, producing a light-orange coloured media. The pure isolated colony was picked with a sterile,straight-wire loop followed by first stabbing of the agar and then followed by gently streaking the surface of the slant, under Bunsen burner to maintain sterility.It was then incubated at 37°C for 24hrs. Results were read and interpreted according to Table 2.

3.5.3.4 Urease Test

The Urease media was first prepared according to manufacturer's protocol(HiMedia Laboratories, India). It was distributed in short tubes and autoclaved for 121°C for 15minutes. The tubes were allowed to solidify for 24 hrs, producing a yellowish-orange clear media.The pure isolates grown on nutrient agar were then collected using a sterile, straight-wire loop, and inoculated into the media by stabbing once, under Bunsen burner to maintain sterility. This was followed by incubation for 24hrs at 37°C. After 24hrs, the media was viewed for colour change; a brick-red colour meant a positive result, whilst a yellowish-orange colour was a negative result meaning the media was not utilized by the bacteria.

3.5.4 Identification using sugars

The different strains were tested for biochemical reaction using 12 sugars and alcohol; disaccharides (maltose monohydrate, trehalose), hexoses (glucose, mannose and galactose), pentose (xylose), polyhydric alcohols (mannitol, sorbitol, inulin and salicin), trisaccharides (raffinose) and Esculin (HiMedia Laboratories, Mumbai, India), were prepared according to manufacturer's protocol. This wasperformed in a tube of phenol red broth, containing either one percent of sugar or alcohol, followed by inoculation with a single bacterial isolate using a sterile straight wire. The broth tubes were incubated at 30±0.5 ^{o}C for 48 hrs and the results were recorded positive if the production of acid condition induced a change in the phenol red indicator, from pink to yellow.

3.6 Antibacterial Susceptibility Test of Bacterial Isolates

The Kirby-Bauer disc diffusion antibacterial sensitivity test method was used to test antibacterial resistance of the bacterial isolates. A total of 9 antibacterial agents belonging to 5 antibacterial classes(Beta-lactam Penicillins, macrolides, tetracyclines,

aminoglycosides, and sulphonamides) were used to determine antibiograms of the isolates. Ten antimicrobial drugs were used. These included; Penicillin-G (P 10µg), Amoxiclav (AMC 30µg) and Amoxicillin (AMX 10µg) (HiMedia Laboratory Pvt, Mumbai, India),selected for being readily available and efface against Gram-positive bacteria. Cefotaxime (CTX 30µg),Ciprofloxacin (CIP 5µg) and Norfloxacin (NX 10µg) (HiMedia Laboratory Pvt, Mumbai, India) have known efficacy against Gram-negative bacteria, while Tetracycline (TE 30µg), Erythromycin (E 5µg), Co-trimoxazole (COT 25µg) (HiMedia Laboratory Pvt, Mumbai, India) are broad in their effect(Thompson MICROMEDEX, 2003).

Mueller-Hinton agar was prepared according to the manufacturer's protocol (HiMedia Laboratory Pvt, Mumbai, India). The organisms were purified on nutrient agar. Using a sterile, round-wire inoculating loop, a loop-full of the bacterial colonies was collected. This was then streaked onto the surface of the Muller Hinton agar plate until its surface was thoroughly covered, under Bunsen flame to ensure a sterile environment. Using a pair of sterile forceps, the antibiotic discs were removed from the dispensers, and thengently placed on the agar, making sure each disc was fixed on agar surface. The discs were placed equidistant from each other with only five antibiotic discs placed per plate to ensure clarity of results. The plates were then placed upside down and incubated for 24hrs at room temperature(NCCLS, 2000).

The sensitivity of each isolate was then read by measuring the clear, circular diameter around each disc. These results were recorded in millimetres and later classified as susceptible, intermediate and resistant.

3.7Data Analysis

Microsoft excel 2010 was used for storage of data and computation of prevalence. The relationship between weight and disease severitywas tested by Chi-square at 95% confidence interval.

CHAPTER FOUR

4.0 RESULTS

Field Observations of Sampled Fish

Based on appearance and behaviour, observations indicating the presence of disease included abnormal swimming; positioned in dorsal or lateral recumbency and swimming in circles. The skin was abnormal withhighly pigmented or black external appearance. The ulcers were present around the mouth and body with eroded fins. The most affected fins were dorsal and tail fins. The eyes were blind (appearing white) and exophthalmos or protruding eyes (Appendices 14-17).

4.2 Bacterial Isolates

Primary isolation was performed on-site. Out of the initial 462 plates inoculated, 98 plates did not have any growth, and 364 plates had growth; 179 isolates on Blood Agar and 185 isolates on Nutrient Agar. These were subcultured in the laboratory on Nutrient and Blood agar giving pure colonies (Fig. 8). Gross morphology on both media was then recorded.

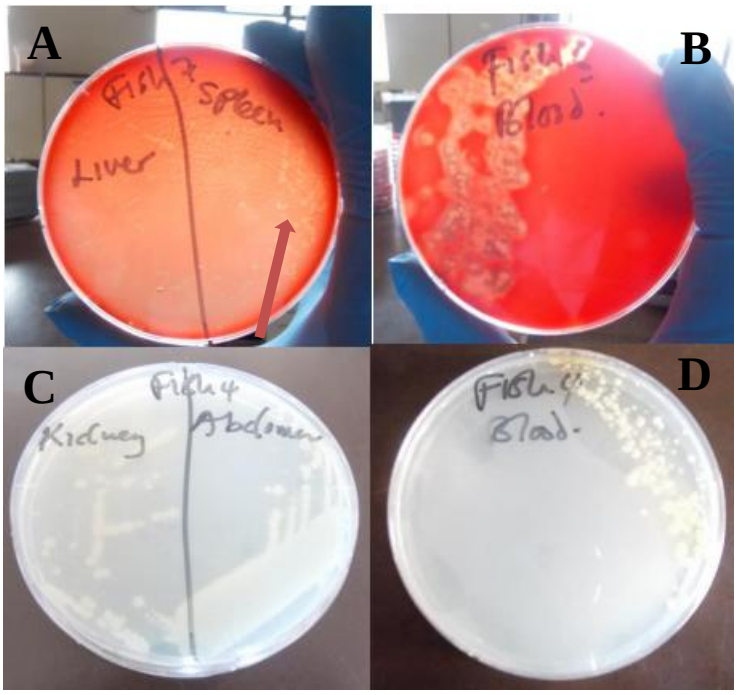


Figure 8: Labelled bacterial isolates a. Liver and spleen isolates of fish 7 showing haemolysis on blood agar, b. Blood isolates of fish 6 showing haemolysis on blood agar, c. Kidney and abdominal isolates of Fish 4 on nutrient agar, d. Blood isolate of fish 4 on nutrient agar.

Table 2:Gross pathologies observed in the organs of sick fish during post-mortem

Sites Sampled	Frequency of Observed Pathologies (n=31)	Percentage (%)
Brain	3	9.7
Eye	9	29.0
Abdominal cavity	4	12.9
Spleen	7	22.6
Liver	6	19.4
Gonads	0	0
Kidney	2	6.5
Blood	0	0

n: Total number

4.3 Chi-square Test

χ^2calculated value = 0.529

χ^2 expected value = 3.841

P (α) = 0.05; χ^2 Calculated value < 3.841 reflectsno statistical significance

H_O has been accepted

4.4 Gram Staining and Morphology

The subcultured (purified) colonies were then Gram-stained and viewed under oil emersion at X100 magnification. Bacteria were classified based on gram-staining and morphology characteristics.

Based on gross colony description and Gram stain, 78 bacterial isolates were selected and segregated as being representative of all different isolates present from each individual fish. Of these, 56 were Gram-positive and 22 Gram-negative (Appendix 7).

4.5 Biochemical Tests

A total of 16 biochemical tests were performed on representative varied bacterial colonies from each fish. To identify the bacterial colonies, the biochemical test results were then compared to that stated in literature(Buller, 2004).

Table 3: Interpretation of Triple Sugar Iron Agar reaction slants

Appearance	Reactions
Acid butt: yellow, alkaline; slant: red	Glucose fermented
Acid throughout medium: butt and slant yellow	Glucose, and sucrose and/or lactose fermented
Gas bubbles in butt and medium frequently split	Gas production
Butt shows blackening	Hydrogen sulphide produced
Unchanged or alkaline butt and slant: medium red throughout	None of the three sugars fermented

Source: Carter (1984)

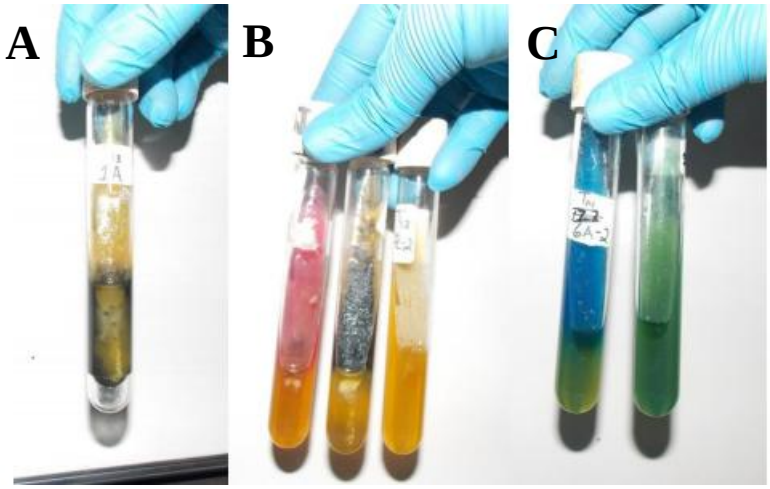


Figure 8: Biochemical test results of labelled bacterial isolates a. TSI illustration of acid throughout medium, hydrogen sulphide production (blackening) and gas production, b. TSI result, from left; acid butt and alkaline slant, middle; acid throughout medium and hydrogen sulphide production (blackening), right; acid throughout medium (Table 2), c. Simmon's citrate result, left; blue slant positive test result, right; no colour change negative test result.

Table 4: Prevalence of Bacterial Isolates at genus level based on morphological characteristics, Gram-staining and biochemical test screening

Identified Bacterial Isolates (Genus)	Number of Isolates (n=78)	Prevalence %
Aeromonas spp.	9	11.5
Aequorivita spp.	1	1.3
Enterococcus spp.	2	2.6
Serratia spp.	1	1.3
Lactococcus/Streptococcus spp.	36	46.2
Citrobacter spp.	1	1.3
Corynebacterium spp.	6	7.7
Edwardsiella spp.	2	2.6
Acinetobacter spp.	1	1.3
Bacillus spp.	2	2.6
Klebsiella spp.	1	1.3
Staphylococcus spp.	3	3.9
Norcardia spp.	1	1.3
Carnobacterium spp.	1	8.0
Rhodococcus spp.	1	1.3
Unidentified Bacteria	5	6.4

n: Total number , %: percentage

Table 5:Frequency of Bacteria Identified at genus level in Fish Sampled

Bacteria Identified (Genus)	Number of Fish Associated with the Bacteria
Aeromonas spp.	8
Aequorivita spp.	1
Enterococcus spp.	2
Serratia spp.	1
Lactococcus/Streptococcus spp.	25
Citrobacter spp.	1
Corynebacterium spp.	3
Edwardsiella spp.	1
Acinetobacter spp.	1
Bacillus spp.	2
Klebsiella spp.	1
Staphylococcus spp.	3
Norcardia spp.	1
Carnobacterium spp.	6
Rhodococcus	1

Table 6: Frequency of bacteriaisolated from the internal organs of diseased *O. niloticus* at genus level

Identified Bacterial Isolates	Brain	Eye	Abdominal cavity	Spleen	Liver	Gonads	Kidney	Blood
Aeromonas	-	3	1	1	1	-	2	1
Aequorivita	-	-	-	-	-	1	-	-
Enterococcus	-	1	-	1	-	-	-	-
Serratia	1	-	-	-	-	-	-	-
Lactococcus/Streptococcus	4	4	3	6	4	3	4	4
Citrobacter	-	-	1	-	-	-	-	-
Corynebacterium	2	-	-	-	-	-	3	1
Edwardsiella	-	-	1	-	1	-	-	-
Acinetobacter	-	-	-	1	-	-	-	-
Bacillus	1	-	-	-	-	-	-	1
Klebsiella	-	-	-	-	-	-	-	1
Staphylococcus	-	1	1	-	-	1	-	-
Norcardia	-	-	-	-	-	1	-	-
Carnobacterium	-	-	2	1	-	1	-	2
Rhodococcus	-	1	-	-	-	-	-	-
Total	**8**	**10**	**9**	**10**	**6**	**7**	**9**	**10**

4.6 Antibiograms

Appendix 11 illustrates a summary of the resistance profiles of bacteria isolates to the selected antibacterials.

CHAPTER FIVE

5.0 DISCUSSION

The interest and significance of this study has been fuelled mainly by reports of disease outbreak in commercially cultured *O. niloticus* on the Lake Kariba, Zambia. These outbreaks have been recorded from as far back as 2014, thus the need to do a thorough study of bacterial organisms that may be associated with sick fish.

The sick fish were first observed within their natural habitat for abnormal features and behaviour. Upon careful observation 'sick' fish were singled-out. This was based on clinical signs observed including abnormal swimming; positioned in dorsal or lateral recumbency, and swimming in circles, skin that was highly pigmented giving an almost black external appearance, eroded fins; dorsal and tail fins visible, ulceration around the mouth and body, blind eyes(appearing white) and exophthalmos or protruding eyes. These signs were confirmed as being some of the most commonly observed in fish that are bacterially diseased,(Austin and Austin, 2007; Noga, 2010; Parker,2012).

The clinical signs that were observed in the cultured *O. niloti*cus have been associated with infection by bacteria, namely;*Aeromonas*(Belém-costa *et al.*,2006), *Pseudomonas*(Amutha and Kokila, 2016), *Edwardsiella*(Amal and Zamri-Saad, 2011; Dong *et al*., 2016),*Flavobacterium*(Al-harbi *et al.,* 2005; Huicab-pech *et al*., 2016) and *Streptococcus*(Iregui *et al.*, 2004; Musa *et al.*, 2009; Anshary *et al.,* 2014; Pretto-giordano *et al*., 2015) species. This is in warm water regions similar to climatic conditions in Zambia.

It has been said that waters with a high organic load, which favour the multiplication of bacteria, rapidly changing temperatures, overcrowding, trauma and transportation are the most commonly encountered environmental stress factors which predispose to clinical disease in fish. Intensive fish culture systems such as that on the Lake Kariba are particularly likely to give rise to these factors (Roberts, 2012; Huicab-pech *et al*., 2016). As fish grow within the cage, biomass in form of their body size increases. The farm management reported that the sick fish originated from cages that had delayed harvest by 2-3 months. A market size of 700g is attained in about 4 months according to Anonymous (2016), though market traders, restaurants and the public generally require varying weights on a regular basis. In Zambia, fish are harvested around the ideal harvest size of 400-500g of weight. Therefore based on this, it can be said that the sick fish's delay in harvest resulted in an increased biomass beyond that which the caged environment could support. This in-turn resulted in a stressful condition that opened the window to infection by opportunistic organisms.

Chi-square test was performed to determine any correlation between weights of the sick fish and severity of infection. Mild infection was characterised by petechial haemorrhage and fin erosion. Severe infection was characterised by clinical signs including blindness, open wounds, ulcers, and abscesses, among those presented in mild infection. Resultsrevealed no significant relationship between the aforementioned parameters; the weight of the sick fish was not related to the severity of outward clinical signs observed.

The organs with the most abnormalities included the eye, spleen and liver, with the percentages 29.0%, 22.6% and 19.4% in respective, descending order. The eye is one of the most sensitive organs of the fish,with the retina having among the highest oxygen demands of any tissue in the body(Helfman *et al*., 2009). With any homeostatic

imbalances, the eye is often one of the first to show signs of underlying disease.This was very clearly observed during examination with signs of blindness, opacity and protrusion (exophthalmos) in majority of the sick fish. The spleen is one of the organs primarily responsible for the immunity, along with the kidney, thymus, and gut. The liver is involved in maintenance of blood chemistry. It is perhaps for these reasons, that they were severely affected by the disease.

Based on bacterial culture, morphology, Gram-staining characteristics and a series of biochemical evaluation and classification, an overwhelming majority, 49.3% of these isolates were identified as being *Lactococcus* or *Streptococcus* species, 12.3% were identified as being *Aeromonas* species.Among the other 15 bacterial genera identified included*Aequorivita* 1.4%, *Enterococcus* 2.7%, *Serratia* 1.4%, *Citrobacter* 1.4%, *Corynebacterium* 8.2%, *Edwardsiella* 2.7%, *Acinetobacter* 1.4%, *Bacillus* 2.7%, *Klebsiella* 1.4%, *Staphylococcus* 4.1%, *Norcardia* 1.4%, *Carnobacterium* 8.0% and *Rhodococcus* 1.4%(Buller, 2004).Therefore, based on the study, the pathogens most likely to be present in diseased *O. niloticus* on Lake Kariba include *Streptococcus/Lactococcus*, *Aeromonas*, *Corynebacterium*,*Carnobacterium*and *Staphylococcus*species.

Streptococcus/Lactococcus(Belém-costa *etal*., 2006; Musa *et al*., 2009; Amal and Zamri-Saad, 2011; Abdelsalam *et al*.,2013; Ahmed, 2013; Haenen *et al*., 2013; Anshary *et al.*, 2014; Pretto-giordano *et al.*, 2015; Amutha and Kokila, 2016) and *Aeromonas*(Austin and Austin, 2007; Noga, 2010; Roberts, 2012; Huicab-pech *et al*., 2016) species have been some of the most widely implicated bacteria in disease outbreaks, mainly due to their opportunistic nature. They have theability to survive in the natural environment in a dormant state, and then invade host tissues once there is destabilization in the environment

and/or host. The *Streptococcus/Lactococcus* was the most isolated at all eight organ sampling sites.

The sick fish had manifested full-blown disease had due to stressful cage conditions, this was proven by the clinical signs and post-mortem lesions documented in fishes 1, 8 and 14. These lesions included fin, nasal and buccal erosion and ulceration, ascites, also under-belly and body petechial haemorrhages. All of which are lesions characteristic of *Aeromonas* infection (Austin and Austin, 2007; Ibrahim *et al.*, 2008; Woo, 2011; Roberts,2012).

Bacteria from the family Streptococcae are also found within the natural aquatic environment and known to be opportunistic in nature. Disease caused by these bacteria has been associated with poor husbandry and excessive stocking densities (Roberts, 2012). Specific causative agents of disease outbreak worldwide include *Lactococcus garvieae*(Woo, 2011; Roberts, 2012; Helmy and Atallah, 2015), *Streptococcus iniae*(Mcnulty *et al.*, 2003; Baiano and Barnes, 2009; Pretto-giordano *et al.*, 2015)and *Streptococcus agalactiae*(Iregui *et al.*, 2004; Jafar *et al.*, 2008).Disease outbreaks usually take place when fish have been exposed to stress including an increase in water temperature, suboptimal oxygen levels in the water or overcrowding for a long period of time. Streptococcosis, theoretically, affects all fish sizes; however, bigger fish (from 100g to market size) are usually most susceptible to the disease(MSD Animal Health,2006).*Lactococcus/Streptococcus* was isolated in the sick fish, averaging 547.8g. These fish exhibited clinical signs of abnormal swimming; positioned in dorsal or lateral recumbency, and spiral swimming and lesions including numerous haemorrhages all over the body, wounds and ulcers and hyperpigmentation. This is as documented in literature (Al-harbi *et al.*, 2005; Musa *et al.*, 2009; Noga, 2010; Roberts, 2012; Ahmed, 2013; Huicab-pech *et al.*, 2016)as being characteristic of Lactococcosis/Streptococcosis.

Besides skin wounds, the eye is also a major point of bacterial infiltration, being one of the most sensitive organs of the fish (Helfman *et al*., 2009).In particular, *Streptococcus agalactiae* is known to cause unilateral and bilateral ocular lesions and has tropism for the central nervous system (CNS) (Iregui *et al*., 2004; Fish; MSD Animal Health, 2006; Jafar *et al.*, 2008; Roberts, 2012). Results of the study revealed that nine (9) out of the twenty-five (25) fish from which *Lactococcus/Streptococcus* was isolated has either ocular lesions or both ocular and brain lesions. These findings point to the possible causative agent being *Streptococcus agalactiae*. Lactococcosis/Streptococcosis generally lead to inflammation and necrosis of the liver, spleen, kidney, eye and brain, and septicaemia as infection is haematogenous(MSD Animal Health, 2006; Roberts, 2012).This has been evidenced by the organs in which bacteria was isolated from the various fish.

The sick fish were overdue for harvest by 2months, and regular feeding was maintained. Caged fish have a relatively small surface area to volume ratio compared to ponds and raceways, coupled with a more restricted food supply (dispersing feed over a comparatively small area of the cage),results in greater competition and more pronounced disparity in food acquisition among individuals (Beveridge,2004). In this particular case, stocking of the $900m^3$ cages was on the upper limit, thus more contact between individuals. Competitive and defensive feeding behaviour such as high speed, jaw protrusion at biting, spreading of the fins (Helfman *et al.*,2009), coupled with a biomass beyond that which the cage is meant to support, would result in high incidence of injury, leading to open wounds. Disease such as Streptococcosis is transmitted horizontally from fish to fish (via cannibalism and skin injuries), and also from the aquatic environment to the fish(MSD Animal Health,2006).

Among the bacteria isolated in the fish included *Bacillus*,which has been shown to have probiotic properties as a lactic acid producing bacteria. The same can be said about *Carnobacterium* and *Rhodococcus;*which havealso been discovered to have the same probiotic properties(Takyi *et al*.,2012). Bacteria including *Norcardia* and *Citrobacter* are known to be commensals in the aquatic environment and surrounding soil (Roberts, 2012; Takyi *et al*., 2012). *Staphylococcus*, *Serratia*and *Klebsiella* are opportunistic bacteria, and may manifest in heavily stressed fish, evidenced by the study.These bacteria may pose some public health risks, as is so with some *Enterococci* species,which are associated with human pollution(Marcel and Sabri, 2013).*Aequorivita* is a genus under the Flavobacteriaceae family. The *Flavobacteria* are mostly associated with living and dead phytoplankton present within the natural environment (Bowman and Nichols,2017).*Edwardsiella* septicaemias and ulcerative conditions have been documented in various fish species worldwide(Austin and Austin, 2007; Roberts, 2012; Huicab-pech *et al.*, 2016). Bacterial species under the*Corynebacteria*genera have been implicated in wide spread disease conditions affecting the kidney, in fish species other than*Oreochromis* species(Woo, 2011; Buller,2014). *Acinetobacter* species have been labelled as emerging fish pathogens in other fish species. These strains have been commonly known as microorganisms transmitting the antibiotic resistance genes, and therefore, may have a great impact on the resistance transfer in aquaculture (Kozińska *et al*., 2014).

In the eve of stemming disease outbreaks in Zambia in *O. niloticus* cage aquaculture, the active surveillance of disease pathogens has been a priority. Disease causing pathogens that have been isolated and confirmed at molecular level include *Streptococcus iniae*, *Lactococcus garvicae* and *Aeromonas hydrophila* (Hang'ombe and Ndashe, 2015).

The bacteria isolated were tested for sensitivity against some commonly used antibacterial agents in aquaculture. Some antibacterials documented to have been used worldwide include ROMET 30® or ROMET TC ® (sulphadimethoxine and Ormetoprim), Aquaflor® (Florfenicol) and Terramycin® in feed formulations (Sekkin and Kum, 2011; Kelly, 2013).Intensification and advent of disease outbreaks have left room for the development of resistant bacterial strains. The study looked at nine (9) commonly used antibacterial compounds,which were meant to give an overview of the profiles of antibacterial classes on the marketas treatment options in fish health. According to the research findings, the thirty-six (36) *Lactococcus/Streptococcus* expressed varying levels of antibacterial resistance, with one isolate having total resistance to all the antibacterials tested. The results also showed a set of two having the same resistance patterns, and another set of three having the same pattern. This could be an indication of them being the same strain. The most effective antibacterial compound was Tetracycline, with the bacterial isolates showing the lowest resistance of 13.9%.Co-trimoxazole was the most effective against the *Aeromonas spp.* (33.3%). Two isolates from two different fish had the same resistance profile, indicating that these could be one in the same strain. All six (6) isolates ofthe *Corynebacteria*expressed different levels of resistance, with Norfloxacin being the most effective against it (16.7% resistance)*Carnobacteria* was the most sensitive to Amoxicillin (33.3% resistance), and *Staphylococcus* was most sensitive to Co-trimoxazole, Amoxicillin and Norfloxacin (all 33.3% resistant).

Multiple resistances have been expressed towards antibacterials commonly used worldwide in aquaculture practices. These results otherwise suggest the possibility of undocumented and/or unregulated use of antibiotics by aquaculture communities on the lake. To date there have been no records of antibacterial use by fish farmers on Lake Kariba (Personal Communication, 2015). Contrary to this, results from the antibiogram

profiling of the isolates revealed multiple resistance to the majority of the antibacterial agents. Potential sources of this resistance include antibiotics flushed into the lake from surrounding human settlements and animal husbandry practices. Bacterial populations within the water and the fish may have gained resistance through mutation upon exposure to these antibacterials. Alternatively, the bacteria within the ecosystem may have innate resistance to the selected antibacterials. These are questions that may only be answered by further in-depth antibacterial screening and testing of the lake and surrounding environment.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In this study, fifteen bacterial generawere isolated from diseased *O. niloticus* on commercial cage fish farms on Lake Kariba. It reflected the vast number of ubiquitous, opportunistic bacterial organisms present within the aquatic environment. This profile is of relevance to the future of aquaculture establishments on the lake Kariba, as intensification practices advance.

The bacterial isolates all expressed varying levels of resistance to commonly used and available antibacterials, which ought to be revered in the different practices on the lake and could also be a potential public health concern.

6.2 Recommendations

1. There is a vast array of opportunistic bacterial species within the Lake Kariba aquatic environment, therefore, management of cage culture establishments must invest in acquiring knowledge on these potential pathogens and take appropriate measures and precautions to maintain an optimal environment of least stress for the fish.
2. A study of human settlements and animal husbandry practices as a source of the isolated bacteria found in the study should be considered.
3. Added scientific research in the area of antibacterial agents is needed so as to investigate the source of the resistance of the bacteria within the Lake. This would include testing of the lake water along with fish in different habitats within the

ecosystem, and added screening of bacterial isolates to a genetic level to determine their source.

4. The Veterinary department should work in conjunction with the department of fisheries to monitor aquaculture activities, with special attention to possible use of antibacterials as an area of public health concern.

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APPENDICES

Appendix 1: Identification features of bacteria pathogens associated with *Oreochromis niloticus*

Bacteria	Clinical Signs	Characteristics	References
Aeromonas hydrophila	Fin-rot and hemorrhagic septicaemia	Nutrient media; white to buff, circular convex colonies, Gram negative Straight, fermentative rods which are motile by polar flagella Voges Proskauer reaction positive, oxidase positive	(Austin and Austin, 2007).
Streptococcus iniae, Streptococcus agalactiae, Lactococcus garvicae	Darkened skin, lethargy and erratic swimming, spine displacement and unilateral or bilateral exophthalmia and abdominal distension	Blood agar; small round white solid colonies, alpha or beta hemolysis, short to long cocci chains. Gram positive Voges-Proskauer reaction negative, catalase negative, oxidase negative	(Iregui *et al*.,2004; Baiano and Barnes, 2009;Musa *et al*.,2009; Amal and Zamri-Saad, 2011; Anshary *etal*., 2014; Pretto-giordano *et al*., 2015;)
Edwardsiella tarda	Necrotic abscesses in the muscle that emits a putrid odour when incised	Nutrient media; small round whitish colonies, Straight, small motile rods, Gram negative, oxidase negative, catalase positive	(Woo and Bruno, 2011; Clavijo, Conroy, Santander,2014; Huicab-pech *et al*., 2016)
Pseudomonas aeroginosa	Septicaemic hemorrhage in the mouth region, opercula and ventral side of the body	Nutrient media; small pigmented circular colonies, straight or slightly curved rods, motile by polar flagellae Gram negative, oxidase positive	(Roberts, 2012; Amutha and Kokila, 2016)

Appendix 2: Daily observation records on A Fish Farm

Week of _____

Observation	Date	Time	Loc#1	Loc#2	Loc#3
DO level					
pH					
Total alkalinity					
N					
Total ammonia N					
Unionized ammonia					
Chloride					
Total hardness					
Temperature					
Observed signs of disease					
External parasites					
Feeding behaviour					
Number of mortalities					

N: Nitrogen, Loc#: Location number

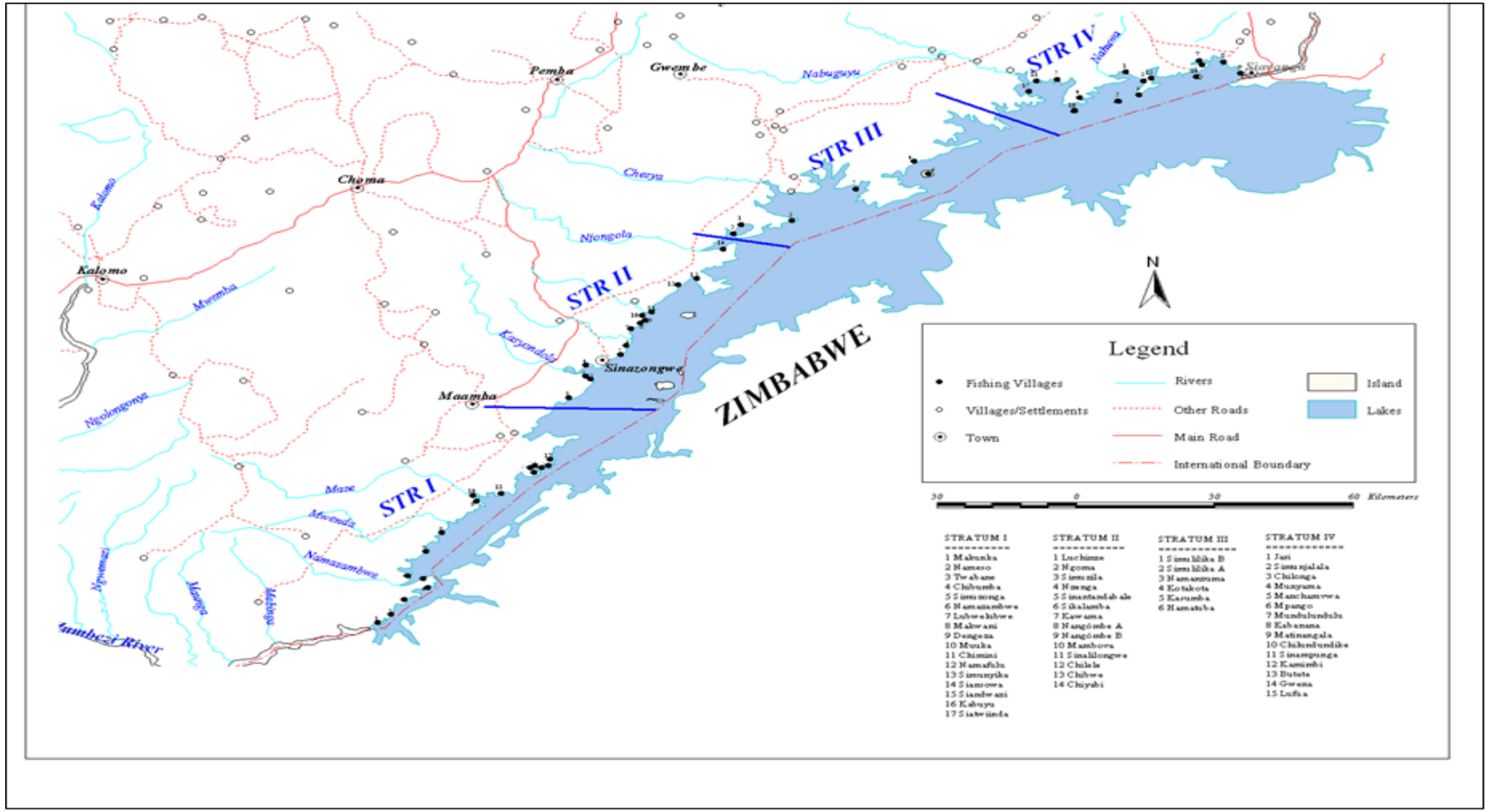
Appendix 3:Vaccines Developed Worldwide for Commercial use

Disease Vaccine	**Causative Agent**
Vibriosis	*Vibrio anguillarum*
Coldwater Vibriosis	*V. salmonicida*
Winter ulcer disease/ wound disease	*Moritella viscosa*
Furunculosis	*Aeromonas salmonicida subsp. Salmonicida*
Atypical *A. salmonicida*	*A.Salmonicida*
Yersiniosis	*Yersenia ruckeri*
Piscircickettsiosis	*Piscirickettsia salmonis*
Bacteria gill disease	*Flavobacterium branchiophilum*
Flavobacteriosis	*Flavobacterium psychrophilum*
Columnaris	*F. columnare*
Enteric septicaemia	*Edwardsiella ictaluri*
Edwardsiellosis or putrefactive disease	*E. tarda*
Bacterial kidney disease	*Renibacterium salmoninarum*
Lactococcosis	*Lactococcus garvieae*
Pasteurellosis	*Photobacterium damselae subsp. Piscicida*
Streptococcosis	*Streptococcus iniae*or *Streptococcus phocae*
Wound disease or winter ulcer disease	*M. viscosa*
Streptococcosis/Lactococcosis	*S. iniae* and *Lactococcus garvieae*

Appendix 4: Field Necropsy Sheet

FISH SAMPLE	PHYSICAL OBSERVATIONS	WEIGHT	LENGT TL	HS C	SEX	ORGANS							
						BRAIN	EYE	ABDOMINAL CAVITY	SPLEEN	LIVER	GONADS	KIDNEY	BLOOD

Appendix 5: Map of the study site; located at latitude 16°S 28.318', longitude 28°E 38.52', on the shore of the Lake Kariba within Strata IV (Ministry of Livestock and Fisheries, 2017)



Appendix 6: Individual fish physical observations and measured parameters

FISH SAMPLE	PHYSICAL OBSERVATIONS	Wt (g)	TL (cm)	C (cm)	SEX
1	Eroded fins, distended abdomen, nasal and buccal erosion, under-belly reddening	672g	32	26	F
2	Ulcerated fins(dorsal, pectoral and operculum), haemorrhagic dorsum, congested gills	1211g	40	30	M
3	Ulcerated dorsal fin, discolouration of skin, gills necrotic, haemorrhage of dorsum, necrotic gills	491g	27.5	22.5	M
4	Haemorrhages on lateral aspect of body, fin ulceration, pale gills	412g	27.5	25	M
5	Discolouration of entire body(fins inclusive)	613g	33.75	30	M
6	Discolouration of skin present	300g	25.5	22	F
7	Ulcerations; mouth and fins, pale gills, missing left eye	280g	24	9	M
8	Ulcerations on fins, haemorrhages, pus in left eye	800g	31.5	28	M
9	Ulcerations, abscesses and wounds present, gills congested	668g	34	26	M
10	Ulcers, abscesses present	315g	26	20	F
11	Ulcer on dorsum of mouth and pectoral fin	295g	26	20	F
12	Ascites, ulcer proximal to pectoral fin	353g	27	20	F
13	Ascites present	347g	29	20	M
14	Ascites, Ulcers around mouth, wounds on skin (haemorrhages)	611g	30	26	F
15	Haemorrhages all over body	604g	31	25	F
16	Abscesses on operculum, ventrum and around mouth, slightly distended abdomen	750g	36	29	M
17	Abscesses around mouth, pus in right eye	530g	31.5	23.5	M
18	Pale skin colour	1053g	35.5	31	M
19	Abscess around mouth	658g	32	27	F
20	No abnormalities observed	431g	28.5	23	M
21	Bilateral corneal opacity	543g	31	38	M
22	Bilateral corneal opacity	470g	27.5	23	M
23	Ulcers on ventrum of mouth	329g	27	21	M
24	Abscesses around mouth present	470g	28	23	M
25	No abnormalities observed	498g	27.5	25	M
26	Bilateral corneal opacity	489g	29	24	F
27	Abscesses present	531g	29	27	M
28	No abnormalities observed	289g	21	24	M
29	No abnormalities observed	513g	30	26	M

Appendix 7: Individual fish internal organ pathologies

	ORGANS							
FISH ID.	**BRAIN**	**EYE**	**ABD. CAVITY**	**SPLEEN**	**LIVER**	**GONAD**	**KIDNEY**	**BLD**
1	-	-	Fluid and excessive fat	Reactive	Fatty, enlarged gall bladder	Congested	-	
2	-	Opaque	Fluid present	Highly reactive	Pale and inflamed	-	-	-
3	-	-	-	Reactive	Enlarged	-	-	-
4	-	-	-	-	Friable	-	-	-
5	Jelly-like consistency (liquefying)	Left ocular opacity	Fluid present	Enlarged, Congested	-	-	-	-
6	Haemorrhage	-	-	-	-	-	-	-
7	-	No left eye	-	-	-	-	-	-
8	Liqufactive	Opaque with pus	-	-	Fatty liver	-	Congestd	-
9	-	-	-	Reactive, congested	-	-	-	-
10	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-
14	-	Blind right eye	-	Reactive	-	-	-	-
15	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-
17	-	Blind right eye (pus)	-	-	-	-	-	-
18	-	-	Fatty	Reactive	Enlarged	-	-	-
19	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-
21	-	Blind in both eyes	-	-	-	-	-	-
22	-	Blind in both eyes	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-
26	-	Blind left eye	-	-	-	-	-	-
27	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-

Appendix 8: Representative Bacterial Isolates gross colony description, Gram stain and morphology

ID	Media		Gram stain	Morphology
#	**Nutrient Agar**	**Blood Agar**		
1S-2	Medium, round, smooth, moist, white	Pinpoint ,round, smooth, white	+	Cocci in chains
1A	Medium, cream, mucoid	Pinpoint ,round, smooth, white	-	Rods
1L-2	Cream, mucoid, large	Cream, mucoid, large	-	Rods
1G	Yellow, mucoid, medium	Dark-grey, mucoid, large	-	Rods
1E	White, pinpoint, moist	Clear, pinpoint, smooth	+	Cocci in chains
2S-1	White, pinpoint, smooth	N/A	+	Cocci in clusters
2B-1	Cream, moist, mucoid, Haemolytic ,cream, large	_	Rods	
2E- 2	Cream, moist, medium	Haemolytic ,cream, moist	+	Cocci in chains
2S-2	Cream, moist, medium	Haemolytic, cream, medium, mucoid	+	Cocci in clusters
3A-1	N/A	Haemolytic, mucoid, white, large, colonies	+	Cocci in chains
3A	White, pinpoint, moist	Clear, pinpoint, moist	-	Short rods
3K-2	Cream, mucoid, raised, medium colonies	Cream, mucoid, round, medium colonies	+	Rods
3L-1	White, pinpoint, moist, round	Light-grey, pinpoint, moist	+	Cocci in chains
3Bd-2	Cream, mucoid, raised, large colonies	Haemolytic, cream, large, moist	+	Very short rods
4G	Cream, pinpoint, moist, colonies	Light-grey, pinpoint, moist	+	Cocci in chains
4B-2	Cream, mucoid, small colonies	Haemolytic, mucoid, cream, small colonies	-	Rods
4B-1	Cream, mucoid, small colonies	Light-grey, pinpoint, moist	+	Rods
4K- 2	Cream, mucoid	Cream, mucoid	+	Rods
5L-2	Cream, mucoid	Haemolytic, mucoid, cream-yellow colonies	-	Rods
5L-1	White, pinpoint, moist	Grey, pinpoint, moist	+	Cocci in chains
5E -1	Clear, pinpoint, moist	Light-grey, pinpoint, moist	+	Cocci in clusters
5S-1	Cream, white, mucoid	Haemolytic	+	Cocci in chains
5K-1	Clear, pinpoint, moist	Light-grey, moist	+	Rods
5A-2	Clear, pinpoint, moist	Light-grey, pinpoint, moist	-	Rods
6A-1	Clear, pinpoint, moist	Clear ,pinpoint, moist	+	Cocci
6S-2	Cream, yellow, mucoid	Dark-grey, mucoid	-	Short rods

ID	Media Nutrient	Blood Agar	Gram stain	Morphology
6A-2	Cream, pinpoint, mucoid	Grey, mucoid	-	Rods
6G-4	Clear, pinpoint, moist	Clear, pinpoint, moist	+	Cocci
7L-2	Cream, mucoid, large	Grey, mucoid, haemolytic	-	Rods
7G-1	White, pinpoint, moist	Clear, pinpoint, moist	+	Cocci
7B-2	Cream, mucoid, large	Haemolytic, mucoid, large	+	Rods
8B-1	Clear, pinpoint, moist	Grey, pinpoint, moist	+	Cocci
8Bd-2	Cream, mucoid, large	Haemolytic, cream, mucoid	-	Rods
8Bd-1	Clear, pinpoint, moist	Grey, pinpoint, moist	-	Rods
9K-1	Cream, mucoid, large	Haemolytic, cream, mucoid	-	Rods
9S-1	Clear, pinpoint, moist	Light-grey, pinpoint, moist	+	Cocci
9A-2	Yellow, pinpoint, moist	Crisp-white, pinpoint, moist	+	Cocci
10Abs-1	Clear, pinpoint, moist	Light-grey, pinpoint, moist	+	Cocci
10L-2	Cream, moist, large	Haemolytic, mucoid, cream	-	Rods
11A-1	Cream, moist, pinpoint	Light-grey, pinpoint, moist	+	Rods
11A-2	White, pinpoint, moist	Light-grey, pinpoint, moist	+	Cocci
12E -2	Cream, mucoid, large	Crisp-white, pinpoint, mucoid	+	Cocci
12B	Clear, pinpoint, moist	Light-grey, pinpoint, moist	+	Cocci
12G-2	White, small, dry, colonies	White, mucoid, colonies	+	Long, encapsulated, rods
13G-1	Yellow, mucoid, pinpoint	Yellow, mucoid, pinpoint	-	Short rods
13G-2	Cream, moist, pinpoint	White, pinpoint, moist	+	Cocci
13G -3	Cream, pinpoint, moist, colonies	Cream, pinpoint, moist	+	Rods
14S-1	Cream, small, mucoid	Cream, pinpoint, mucoid	+	Cocci
14B-2	Cream, pinpoint, moist, colonies	Pinpoint, clear, moist	+	Cocci
14S- 2	Clear, pinpoint, moist	Cream, mucoid, small	-	Rods
16Abs-1	Cream, mucoid, pinpoint	Light-grey, pinpoint, moist	+	Cocci
16Abs-2	Clear, pinpoint, moist	Haemolytic, cream, moist, small colonies	+	Rods
17E -1	Clear, pinpoint, moist	Light-grey, pinpoint, moist	+	Cocci
19K -1	Clear, pinpoint, moist	Light-grey, pinpoint, moist	+	Cocci
20L	Clear, pinpoint, moist	Light-grey, pinpoint, moist	+	Cocci
20Bd-1	Clear, pinpoint, moist	Light-grey, pinpoint, moist	+	Rods

ID	Media Nutrient	Blood Agar	Gram stain	Morphology
21K- 1	Clear, pinpoint, moist	Light-grey, pinpoint, moist	+	Cocci
21E- 2	Cream, mucoid, pinpoint	Haemolytic, mucoid, cream	-	Rods
21Bd-2	White, mucoid, pinpoint	Light-grey, pinpoint, moist	+	Cocci
22Bd-2	Cream, moist, small	Cream, mucoid, large	+	Rods
22E- 2	Cream, mucoid, pinpoint	Haemolytic, cream, mucoid	+	Very short rods
22Bd-1	Clear, pinpoint, moist	Light-grey, pinpoint, moist	+	Cocci
23Bd-2	Cream, moist, small	Haemolytic, cream, grey	+	Cocci
23K- 1	Clear, pinpoint, moist	White, pinpoint, moist	+	Cocci
24G- 1	Clear, pinpoint, moist	Light-grey, pinpoint, moist	+	Cocci
25K-1	Cream, pinpoint, moist, colonies	Light-grey, pinpoint, moist	-	Rods
26E- 1	Yellow, moist, pinpoint	Light-grey, pinpoint, moist	+	Cocci in chains
26E- 2	Cream, moist, small	Haemolytic, grey, mucoid	_	Rods
27K- 1	Clear, pinpoint, moist	Light-grey, pinpoint, moist	+	Cocci in chains
27K-2	Cream, mucoid colonies	Haemolytic, cream, mucoid	-	Small rods
28B- 1	Cream, mucoid, cream	Haemolytic, grey, mucoid	+	Cocci
28S- 1	Cream, moist, pinpoint	Light-grey, pinpoint, moist	+	Rods
28E- 1	Clear, pinpoint, moist	Light-grey, pinpoint, moist	+	Cocci
29Bd	Cream, moist, small	Haemolytic, mucoid, cream	+	Short rods
29L- 1	Clear, pinpoint, moist	Light-grey, pinpoint, moist	+	Cocci
29S- 1	Clear, pinpoint, moist	Light-grey, pinpoint, moist	+	Cocci in chains
29E- 2	Cream, mucoid, pinpoint	Light-grey, pinpoint, moist	-	Rods
29Bd-1	Cream, mucoid, small colonies	Haemolytic, grey, mucoid	+	Cocci

Appendix 9: Statistical analysis of weights of sick fish

	Positive (Severe symptoms)	**Negative (Mild symptoms)**	**Total**
High weight	**8**	**2**	**10**
Low weight	**10**	**5**	**15**
Total	**18**	**7**	**25**

High weight = ≥ 600g, Low weight = ≤ 600g

H_O: There is no significant difference between the severely and the mildly symptomatic fish

H_A: There is a significant difference between the severely and mildly symptomatic fish

χ^2calculated value = 0.529

χ^2 expected value = 3.841

P (α) = 0.05; χ^2 Calculated value > 3.841 reflected statistical significance

H_O has been accepted

Appendix 10: Biochemical test results of representative isolates

BIOCHEMICAL TESTS																					
SAMPLE #	**T_1**	**T_2**	**T_3**	**T_4**	**T_5**	**T_6**	**T_7**	**T_8**	**T_9**	**T_{10}**	**T_{11}**	**T_{12}**	**T_{13G}**	**T_{13S}**	**T_{13L}**	**T_{13g}**	**T_{14}**	**T_{15S}**	**T_{15M}**	**T_{15I}**	**T_{16}**	***PROBABLE ID***
1S-2	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	+	-	-	*Lactococcus*
1A	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+, H_2S	-	+	+	-	-	*Aeromonas*
1L-2	-	-	-	-	-	-	-	+	-	-	-	-	+	+	+	-	-	-	+	+	-	*Aeromonas*
1G	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	*Aequorivita*
1E	-	+	-	-	-	-	+	+	-	-	-	-	+	+	+	-	-	-	-	-	+	*Enterococcus*
2S-1	-	+	-	-	-	-	+	+	-	-	-	-	+	+	+	-	-	-	-	-	+	*Enterococcus*
2B-1	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	+	+	-	+	+	-	*Serratia*
2E- 2	-	-	-	-	-	-	-	+	-	-	-	-	+	+	+	+	-	-	+	-	-	*Lactococcus*
2S-2	-	-	-	-	-	-	-	+	-	-	-	-	+	+	+	-	-	-	+	+	+	*Lactococcus*
3A-1	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	+	-	-	+	+	+	*Lactococcus*
3A	-	+	-	+	-	-	+	+	-	-	-	-	+	+	+	-	-	-	-	-	-	*Citrobacter*
3K-2	-	-	-	+	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	+	*Corynebacteria*
3L-1	-	+	-	+	-	-	+	+	-	-	-	-	+	+	+	-	-	-	-	-	+	*Lacto/Strep.*
3Bd-2	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-	+	-	-	+	+	+	*Corynebacteria*
4G	-	+	-	+	-	-	+	+	-	-	-	-	+	+	+	-	-	-	-	-	-	*Lacto/Strep.*
4B-2	-	+	-	+	-	-	-	+	-	-	-	-	+	-	-	+	+	-	+	+	+	*Corynebacteria*
4B-1	-	+	-	+	-	-	+	+	-	-	-	-	+	+	+	-	-	-	-	-	-	*Corynebacteria*
4K- 2	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	+	+	*Corynebacteria*
5L-2	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-	+	+	-	+	+	+	*Edwardsiella*
5L-1	-	+	-	+	-	-	+	+	-	-	-	-	+	+	+	-	-	-	-	-	+	*Lacto/Strep.*
5E -1	-	+	-	+	-	-	+	+	-	-	-	-	+	+	+	-	-	-	-	-	+	*Lacto/Strep.*
5S-1	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	+	+	-	+	+	+	*Lacto/Strep.*
5K-1	-	-	-	-	-	-	-	-	-	-	-	-				+, H_2S	-	-	+	+	+	*Corynebacteria*
5A-2	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	+	-	*Edwardsiella*

SAMPLE #	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13G	T13S	T13L	T13g	T14	T15S	T15M	T15I	T16	*PROBABLE ID*
6A-1	-	+	-	-	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	*Lacto/Strep.*
6S-2	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	*Acinetobacter*
6G-4	-	+	-	-	-	-	+	+	-	-	-	-	+	+	+	-	-	-	-	-	+	*Lacto/Strep.*
7G-1	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	+	*Lacto/Strep.*
7B-2	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+	+	-	*Bacillus*
8B-1	-	+	-	-	-	-	+	+	-	-	-	+	+	+	+	-	-	-	-	-	+	*Lacto/Strep.*
8Bd-2	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	+	+	-	*Aeromonas*
8Bd-1	-	+	-	+	-	-	+	+	-	-	-	-	+	+	+	-	-	-	-	-	+	*Klebsiella*
9S-1	-	+	-	-	-	-	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	*Lacto/Strep*
9A-2	-	-	-	-	+	-	-	+	-	-	-	+	+	+	+	-	-	-	-	-	+	*Staph.*
10Abs-1	-	+	-	-	-	-	+	+	-	-	-	-	+	+	+	-	-	-	-	-	+	*Lacto/Strep.*
11A-1	-	-	-	-	+	-	-	+	-	-	-	-	+	-	-	-	-	-	+	+	+	*Carnobacteria*
11A-2	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	+	-	*Lacto/Strep.*
12E -2	-	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-	+	-	+	-	-	*Staph.*
12B	+	+	-	+	-	-	+	+	-	-	+	-	+	+	+	-	-	-	-	-	+	*Lacto/Strep.*
12G-2	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-	-(fg)	+	-	+	-	+	*Norcardia*
13G-1	+	-	-	-	+	-	-	+	-	-	-	-	+	-	-	-	+	-	+	-	-	*Staph. Aureus*
13G-2	+	-	-	+	+	-	+	+	-	-	-	+	+	+	+	-	+	-	+	-	+	*Lacto/Strep.*
13G -3	+	+	-	+	+	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	+	*Carnobacteria*
14S-1	+	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	*Lacto/Strep.*
14B-2	+	+	-	-	-	-	+	+	-	-	-	-	+	+	+	-	-	-	-	-	+	*Lacto/Strep.*
14S- 2	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	*Aeromonas*
16Abs-1	+	-	-	-	+	-	-	+	-	-	-	-	+	-	-	+	+	+	+	+	+	*Lacto/Strep.*
16Abs-2	+	+	-	+	+	+	+	+	-	-	-	-	+	+	+	-	-	-	-	-	+	*Carnobacteria*
17E -1	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-	+	*Lacto/Strep.*
19K -1	+	+	-	-	-	-	-	+	-	-	-	+	+	+	+	-	-	-	-	-	+	*Lacto/Strep.*
20L	+	+	-	-	-	-	+	+	-	-	-	-	+	+	+	-	-	-	-	-	+	*Lacto/Strep.*
20Bd- 1	+	+	-	-	+	-	+	+	-	-	-	-	+	+	+	-	-	-	-	-	+	*Carnobacteria*
21K- 1	+	+	-	+	-	-	+	+	-	-	-	-	+	+	+	-	-	-	-	-	+	*Lacto/Strep.*

SAMPLE #	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13G	T13S	T13L	T13g	T14	T15S	T15M	T15I	T16	*PROBABLE ID*
21E- 2	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	+	-	+	+	+	*Aeromonas*
21Bd-2	-	-	+	-	+	-	-	-	-	-	-	-	+	+	+	+	+	-	+	-	-	*Lacto/Strep.*
22Bd-2	+	+	-	+	-	+	+	+	-	-	-	-	+	-	-	+	+	-	+	-	+	*Carnobacteria*
22E- 2	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+	*Rhodococcus*
22Bd- 1	+	+	-	-	-	-	+	+	-	-	-	-	+	+	+	-	-	-	-	-	+	*Lacto/Strep.*
23Bd- 2	+	+	-	-	+	-	-	+	-	-	-	-	+	-	-	+	+	-	+	+	+	*Lacto/Strep.*
23K- 1	+	+	-	-	-	-	+	+	-	-	-	-	+	+	+	-	-	-	-	-	+	*Lacto/Strep.*
25K-1	-	-	-	-	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	-	*Aeromonas*
26E- 1	+	+	-	-	+	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	+	*Lacto/Strep.*
26E- 2	-	-	-	-	+	-	-	+	-	-	-	+	+	-	-	+	+	-	+	+	-	*Aeromonas*
27K- 1	+	+	-	-	+	-	+	+	-	-	-	-	+	+	+	-	-	-	-	-	+	*Lacto/Strep.*
27K-2	-	-	-	-	+	-	-	+	-	-	-	-	+	-	-	+	-	-	+	+	-	*Aeromonas*
28B- 1	-	-	+	-	+	-	+	-	-	-	-	-	+	-	-	+	+	-	+	-	-	*Lacto/Strep.*
28S- 1	-	-	-	-	+	-	-	-	-	-	-	_	+	+	+	-	-	-	-	-	+	*Carnobacteria*
28E- 1	+	-	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	*Lacto/Strep.*
29Bd	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	+	-	-	*Bacillus*
29L- 1	+	-	-	-	+	-	+	+	-	-	-	-	+	+	+	-	-	-	-	-	-	*Lacto/Strep.*
29S- 1	-	-	-	+	+	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	+	*Lacto/Strep.*
29E- 2	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	*Aeromonas*
29Bd-1	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	*Lacto/Strep.*

Appendix 11: Antibacterial Resistance profiles of bacteria isolates

Bacteria% Resistance															
A/B	**Aer**	**Aeq**	**Et**	**Ser**	**Lt/Stp**	**Cit**	**Cor**	**Ed**	**Acn**	**Bac**	**Kle**	**Stph**	**Nr**	**Carn**	**Rhd**
P 10µg	88.9*	-	100	100*	61.1	-	83.3	100*	-	100	100*	100	-	66.7	100
AMC 30 µg	100*	100*	100	100*	36.1	-	50.0	50*	-	100	50*	33.3	-	33.3	100
AMX 10 µg	100*	-	100	100*	72.2	-	83.3	50*	100*	100	50*	100.0	-	66.7	100
CTX 30 µg	44.4	-	100*	-	61.1*	-	50.0*	50	-	50*	100	66.7*	-	50.0*	-
TE 30 µg	55.6	-	50.0	-	13.9	-	33.3	50	-	100	50	-	-	33.3	-
E 5 µg	100	-	50.0	-	30.6	-	66.7	100	-	100	50	100.0	-	50.0	-
CIP 5 µg	55.6	-	100*	-	41.7*	-	33.3*	-	-	50*	50	33.3*	-	50.0*	-
COT 25 µg	33.3	-	100	-	75.0	100	50.0	50	-	50	100	33.3	-	66.7	100
NX 10 µg	44.4	-	100	-	69.4	100	16.7	-	-	50	100	33.3	-	66.7	-

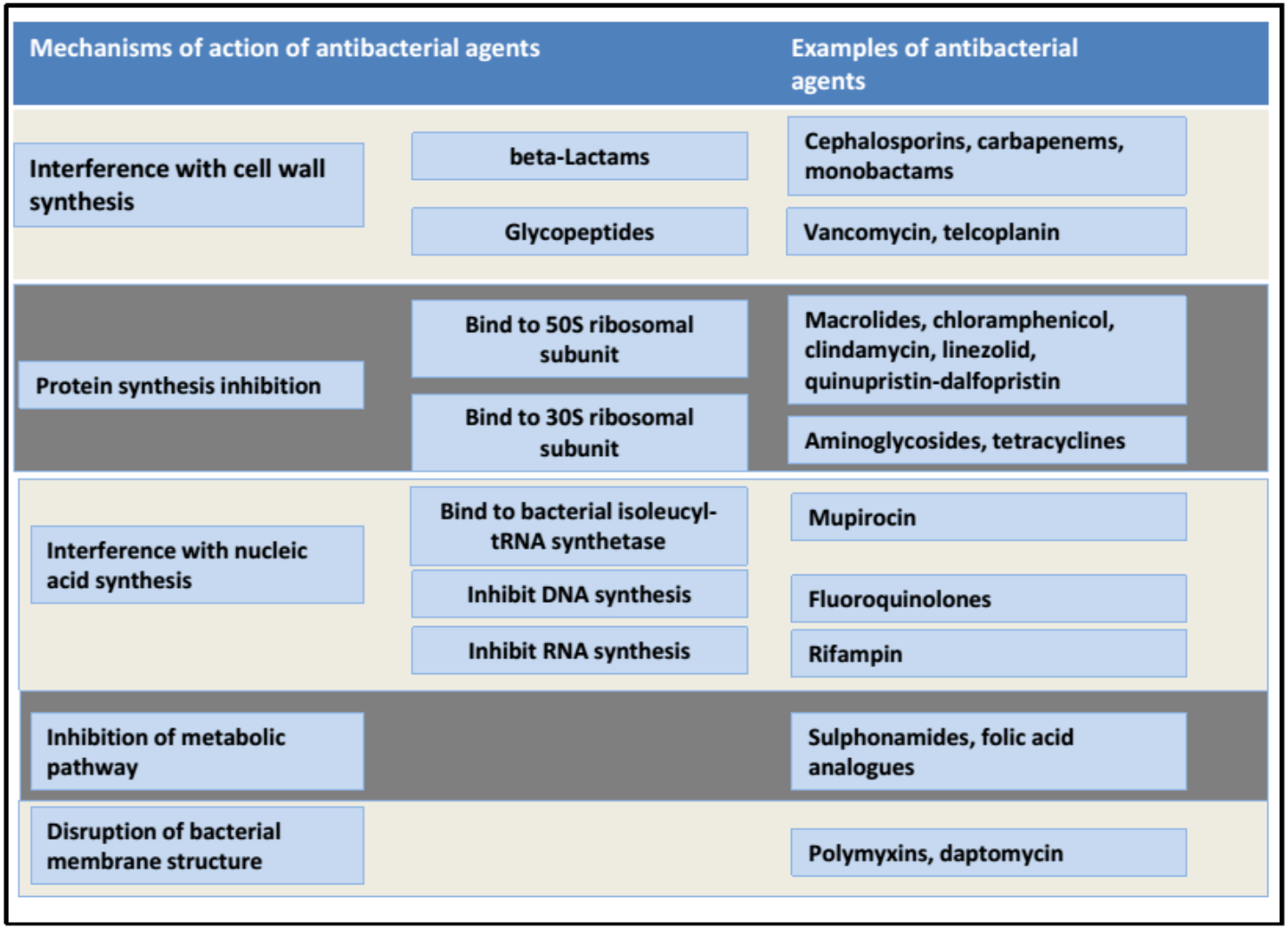
P : Penicillin, AMC :Amoxiclav, AMX :Amoxicillin, CTX : Cefotaxime, TE : Tetracycline, E : Erythromycin, CIP: Ciprofloxacin, COT: Co-trimoxazole, NX :Norfloxacin, A/B : Antibiotic, Aer : *Aeromonas*, Aeq : *Aequorivita*, Et : *Enterococcus*, Ser : *Serratia*, Lt/Stp or *Lacto/Strep*.=: *Lactococcus/Streptococcus*, Cit: *Citrobacter*, Cor : *Corynebacteria*, Ed : *Edwardsiella*, Acn :*Acinetobacter*, Bac : *Bacillus*, Kle : *Klebsiella*, Stph : *Staphylococcus*, Nr: *Norcardia*, Carn: *Carnobacteria*, Rhd: *Rhodococcus*, *Probable Natural resistance

Appendix 12: Detailed Antibiograms of Bacterial Isolates (CLSI, 2014)

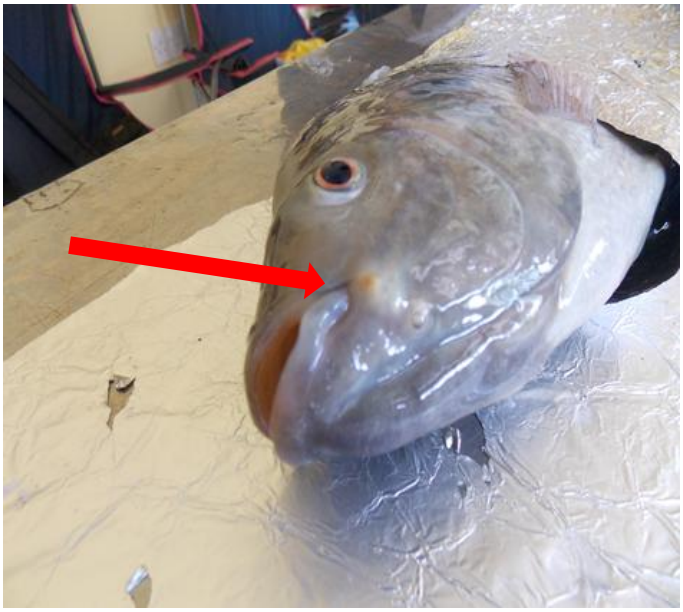
Sample ID	Bacteria Isolate	10 (P)	30 (AMC)	10 (AMX)	30 (CTX)	30 (TE)	5 (E)	5 (CIP)	25 (COT)	10 (NX)
6S-2	*Acinetobacter*									
1G	*Aequorivita*	I	R	I	S	S	I	S	S	S
8Bd-2	*Aeromonas*	R	R	R	R	R	R	R	I	I
1A	*Aeromonas*	I	R	R	S	R	R	I	S	I
27K-2	*Aeromonas*	R	R	R	S	S	R	I	S	I
26E- 2	*Aeromonas*	R	R	R	I	R	R	R	I	R
14S- 2	*Aeromonas*	R	R	R	R	R	R	I	R	R
1L-2	*Aeromonas*	R	R	R	R	R	R	R	R	R
29E- 2	*Aeromonas*	R	R	R	R	R	R	R	R	R
21E- 2	*Aeromonas*	R	R	R	I	I	R	R	S	S
25K-1	*Aeromonas*	R	R	R	I	I	R	S	S	S
29Bd	*Bacillus*	R	R	R	R	R	R	R	R	R
7B-2	*Bacillus*	R	R	R	S	R	R	I	S	S
20Bd- 1	*Carnobacteria*	I	S	I	R	S	I	I	R	R
16Abs-2	*Carnobacteria*	R	S	R	R	R	I	R	R	R
28S- 1	*Carnobacteria*	R	R	R	R	R	R	R	R	R
13G -3	*Carnobacteria*	I	I	I	I	S	I	S	S	R
22Bd-2	*Carnobacteria*	R	R	R	S	S	R	R	S	S
11A-1	*Carnobacteria*	R	I	R	I	S	R	S	R	S
3A	*Citrobacter*	S	S	S	I	S	S	I	R	R
4K- 2	*Corynebacteria*	R	R	R	R	R	R	R	R	I
3K-2	*Corynebacteria*	R	I	R	R	I	R	I	S	I
5K-1	*Corynebacteria*	R	S	R	R	R	R	R	R	S
4B-2	*Corynebacteria*	R	R	R	S	I	I	S	S	S
3Bd-2	*Corynebacteria*	R	R	R	S	S	R	S	S	S
4B-1	*Corynebacteria*	I	S	I	S	S	I	I	R	R
5A-2	*Edwardsiella*	R	S	S	S	R	R	S	R	S
5L-2	*Edwardsiella*	R	R	R	R	I	R	I	S	S
2S-1	*Enterococcus*	R	R	R	R	R	R	R	R	R
1E	*Enterococcus*	R	R	R	R	I	S	R	R	R
8Bd-1	*Klebsiella*	R	S	S	R	S	I	I	R	R
13G-2	*Lacto/Strep.*	R	S	I	R	S	R	S	I	I
4G	*Lacto/Strep.*	R	S	I	S	S	I	I	R	I
26E- 1	*Lacto/Strep.*	S	S	S	S	S	I	I	S	I
7G-1	*Lacto/Strep.*	I	S	S	S	S	S	I	S	I
5E -1	*Lacto/Strep.*	R	R	R	I	S	I	I	R	R
27K- 1	*Lacto/Strep.*	I	S	S	R	I	I	I	R	R
3L-1	*Lacto/Strep.*	I	I	R	R	S	I	I	R	R
5S-1	*Lacto/Strep.*	I	S	R	R	S	I	I	R	R
14B-2	*Lacto/Strep.*	I	S	R	R	S	I	I	R	R
20L	*Lacto/Strep.*	I	S	S	R	S	I	I	R	R
8B-1	*Lacto/Strep.*	R	R	R	S	S	I	I	R	R
23Bd- 2	*Lacto/Strep.*	R	R	R	R	I	R	I	R	R

Sample ID	Bacteria Isolate	10 (P)	30 (AMC)	10 (AMX)	30 (CTX)	30 (TE)	5 (E)	5 (CIP)	25 (COT)	10 (NX)
23K- 1	*Lacto/Strep.*	R	R	R	R	I	I	R	R	R
28E- 1	*Lacto/Strep.*	R	I	R	R	R	I	R	R	R
29L- 1	*Lacto/Strep.*	R	R	R	R	R	I	R	R	R
9S-1	*Lacto/Strep.*	R	S	I	R	S	I	R	R	R
5L-1	*Lacto/Strep.*	I	S	R	R	S	I	R	R	R
14S-1	*Lacto/Strep.*	I	S	R	R	S	I	R	R	R
19K -1	*Lacto/Strep.*	I	S	R	R	S	I	R	R	R
21K- 1	*Lacto/Strep.*	R	S	R	R	S	I	R	R	R
29S- 1	*Lacto/Strep.*	R	S	S	R	S	I	R	R	R
10Abs-1	*Lacto/Strep.*	R	R	R	S	S	I	R	R	R
22Bd- 1	*Lacto/Strep.*	R	I	R	R	I	R	R	R	R
28B- 1	*Lacto/Strep.*	R	R	R	R	I	R	R	R	R
12B	*Lacto/Strep.*	R	I	R	R	R	R	R	R	R
17E -1	*Lacto/Strep.*	R	R	R	R	R	R	R	R	R
29Bd-1	*Lacto/Strep.*	R	R	R	R	R	R	R	R	R
6G-4	*Lacto/Strep.*	I	S	S	S	S	I	S	R	R
6A-1	*Lacto/Strep.*	I	S	S	S	S	S	S	R	R
16Abs-1	*Lacto/Strep.*	R	S	R	R	S	R	I	I	S
11A-2	*Lacto/Strep.*	R	I	R	S	S	R	S	R	S
21Bd-2	*Lacto/Strep.*	R	R	R	S	I	R	S	S	S
2E- 2	*Lactococcus*	R	R	R	S	S	I	S	S	S
2S-2	*Lactococcus*	R	R	R	S	S	I	S	S	S
3A-1	*Lactococcus*	R	R	R	S	S	I	S	S	S
1S-2	*Lactococcus*	I	I	R	S	S	R	S	S	S
12G-2	*Norcardia*	I	S	I	S	S	I	S	S	S
22E- 2	*Rhodococcus*	R	R	R	S	I	I	S	R	I
2B-1	*Serratia*	R	R	R	S	S	I	S	S	S
9A-2	*Staphylococcus*	R	S	R	R	S	R	S	S	I
12E -2	*Staphylococcus*	R	R	R	R	I	R	R	R	R
13G-1	*Staphylococcus*	R	S	R	S	S	R	S	S	S

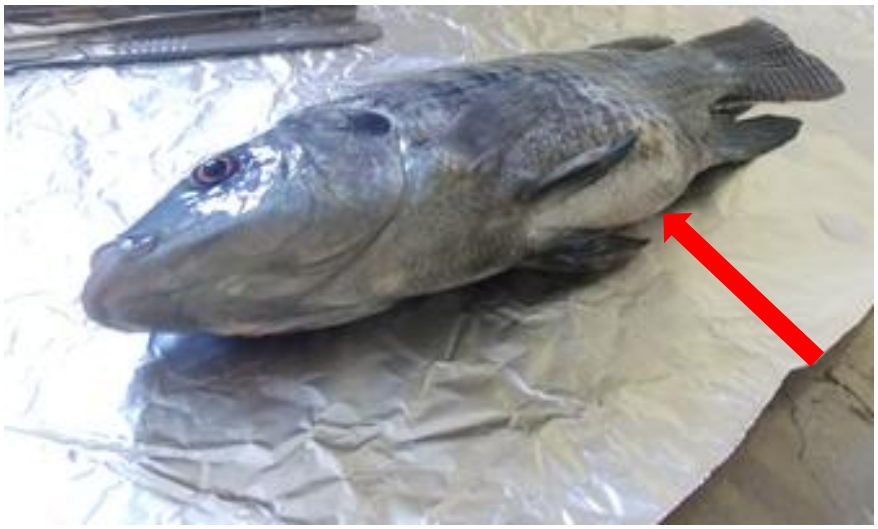
Appendix 13: The different mechanisms of action of antibiotics (Romero *et al.*, 2012)



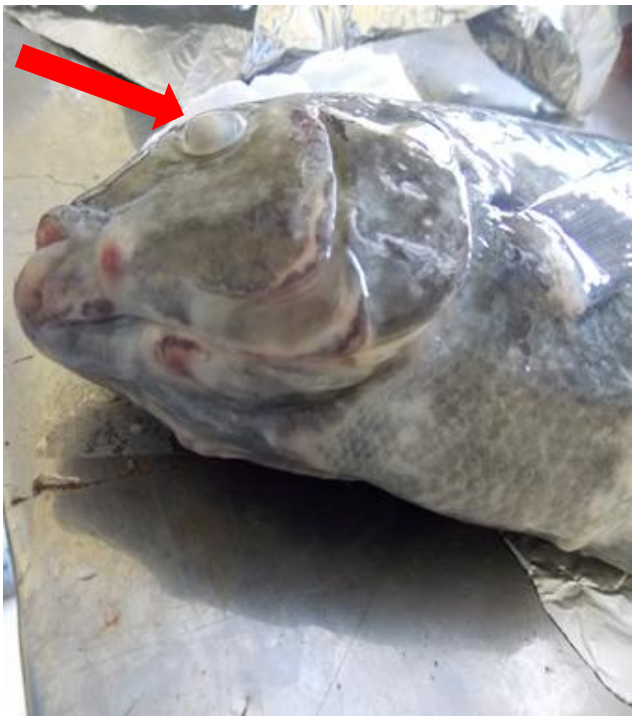
Appendix 14: Ulceration around the mouth



Appendix 15: Ascites (swollen abdomen)



Appendix 16: Ocular opacity (blindness)



Appendix 17: Hyperpigmentation (darkening) of the skin

Appendix 18: Erosion of pectoral fins