OCCURRENCE OF FUNGAL INFECTIONS IN RUFIJI TILAPIA AND HYBRIDS OF FEMALE NILE TILAPIA AND MALE RUFIJI TILAPIA AT DIFFERENT SALINITIES

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ABSTRACT

Fungal diseases are prevalent in fish and have the potential of limiting productivity in aquaculture. This study aimed to isolate and determine fungal infections in hatchery with female Rufiji tilapia and their hybrids of male Rufiji tilapia and female Nile tilapia. An experimental study design was conducted to determine the occurrence of fungal infections and to characterize the isolates of *Aspergillus flavus* and *Aspergillus niger.* Fish samples were randomly collected from the hatchery at the Institute of Marine Sciences Mariculture centre in Pangani. In the sampled fish the gills, gastrointestinal tract and skin were collected. The morphological and physiological tests were employed to isolate and identify fungi using SDA. The lactophenol detached crystal cotton blue was used for characterization of fungi. The Rufiji tilapia stocked at different salinities were infected by *A. niger* and *A. flavus* at 72.4 % and 17.1% (n = 61) respectively. The prevalence of *A. niger* and A. *flavus* in water of tilapia and hybrids was 37.4 % and 10.6% respectively. Rufiji tilapia and hybrids were infected by *A. niger* and *A. flavus at* 37.9 % and 6.5 % (n = 62) respectively. For PCR based analysis, fragments of bp400 and 895 were detected for *A.flavus* and bp290 for *A.niger*. Despite the percentage variations for fungal isolates in some of the fish, overall, there was a significant ($P<0.05$) reduction of prevalence of fungal infections with increasing salinities. In the hybrids, an increase in salinity did not influence the prevalence of *Aspergillus* species ($P>0.05$). Increase in salinity has no influence on the growth of *A. flavus* in fish organs of Rufiji tilapia. Conventional methods are time-consuming and less sensitive; PCR methods provide more specification and high sensitivity of the target organism. Different salinities of 15, 25 and 35 are potential for mariculture since they cannot support the existence of *A. niger* unlike *A. flavus,* which showed significant difference in fish organs.

DECLARATION

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

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DEDICATION

This research work is dedicated to: My Mother Leocardia Bunzar and Uncle; Edward Igakamba for sending me to school. My sister and brothers, Zawadi Mtabika, Grace Mtabika, Bendachi Mtabika, Philipina Shayo and Elisha Mtabika for their prayer. My wife Asha Said Sendoki. My children: Christina Mtabika and Careen Mtabika, I love you.

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LIST OF ABBREVIATIONS, SYMBOLS AND ACRONYMS

♀	Female
♂	Male
^{0}C	Degree centigrade
A	*Aspergillus*
AFB2	Aflatoxin B two
AFBI	Aflatoxin B one
afID	Target gene for *Aspergillus flavus*
afIM	Target gene for *Aspergillus flavus*
aw	Water activity
bp	Base pair
DNA	Deoxyribose Nucleic Acid
Dr	Doctor of philosophy
et al.,	And others
EUS	Epizootic Ulcerative Syndrome
FCR	Feed conversion ratio
g	Gram
GIT	Gastrointestinal tract
h	Hours
IMS	Institute of Marine Science
n	Number of samples size
ng	Nano gram
NIGI	Target gene for *Aspergillus niger*
OHCEA	One Heath Central and Eastern Africa
OTA	Ochratoxins A

OTA2	Ochratoxin for *Aspergillus niger*
OTAI	Ochratoxin for *Aspergillus niger*
P	Probability
PCR	Polymerase Chain Reaction
pH	Power of Hydrogen
ppm	Parts per million
Ppt	Parts per thousand
ppt	parts per thousand
RNA	Ribose Nucleic Acid
rpm	Revolution per minute
SDA	Sabouraud Dextrose Agar
SAS	Statistical Analysis System
sec	Second
TAN	Tanzania
TBE	Tris- Bos EDTA buffer
TRAHESA	Training and Research in Aquatic and Environmental and Health in East and Southern Africa
UM	Ulcerative Mycosis
Uv	Ultravolet
V	Voltage
Vol	Volume
X^2	Chi-square
µl	Micro-litres

CHAPTER ONE

1.0 INTRODUCTION

Nile tilapias (*Oreochromis niloticus*) are an important and popular species for aquaculture. The species is cultured commercially in high stocking densities, which are accompanied by several risk factors leading to disease outbreaks. Tilapias have good resistance to poor water quality, diseases, and a wide range of environmental conditions. In addition, they have the ability of converting organic and domestic wastes into high quality protein efficiently (Faruk *et al*., 2017). Nile tilapia is the most commonly cultured fish species worldwide because of its high protein content, rapid growth rate, and good taste. Fish diseases are among major causes of important limitation in the production of fish including tilapias in Aquaculture. The common diseases for tilapia are skin infections, which are mainly caused by bacteria, parasites, and fungi (Cutuli *et al.*, 2015).

Fungal growths on the surface of eggs and larvae of tilapia fish can cause extensive mortalities. In incubating fish, eggs and dead eggs provide a fertile substrate for fungal growth. If dead eggs are not removed, the resultant fungal growths may cover adjacent healthy eggs and infect them (Meyer, 1991). There are more than 250 000 species of fungi, of which about 150 are pathogenic for animal and human. Fungal contamination of fish is considered the main cause of spoilage including flavour and unpalatable taste, which may constitute a public health hazard leading to economic losses (Ali, 2015). Fungal diseases are widely prevalent in fish and fish eggs in natural and artificial environments. Species of *Saprolegnia* usually affect fish and fish eggs (Pillai and Yvonne, 1983).

Although there are four orders within the Oomycetes, almost all the significant pathogens are within the family *Saprolegniacae*, the most important being the genera *Saprolegnia, Achlya, Aphanomyces* (Yagoub, 2004). The *Saprolegniaceae* cause many infections to fishes. The Oomycetes constitute the most important group of fungi, which are pathogenic to fish, and are widely distributed in the aquatic habitat. Conventional methods for the detection and identification of Saprolegnia are usually based on the patterns of asexual and sexual stages. Most fungi are eukaryotic and have hyphae that grow apically from the tips, and are completely heterotrophic (Yanong, 2003). Saprolegniasis is a disease of freshwater and brackish water species, which is caused by Saprolegnia, and thereby causing superficial disease with a mild inflammatory response (Yanong, 2003).

Saprolegnia is considered as the agent of secondary infection arising from bacterial infections, poor husbandry including poor water quality, and adverse water temperature. All of these factors increase the occurrence of saprolegnia infections (El-Atta, 2008). The asexual characteristics are the most often used in the identification of the mode of zoospore discharge (Zahran *et al*., 2017).

Another common fungal infection of biological importance is *Aphanomyces invadans* an invasive oomycete or water mould. In addition to phycomycetes, other fungi, such as *Aspergillus Penicillium aspergilla,* which are ubiquitous, are principally involved in saprophytic decay processes. Saprophytic oomycetes are known to be opportunistic pathogens and may be present as secondary invaders on the surface lesions caused by *A. invadans* (Huchzermeyer and Van der Wall, 2012). *Aphanomyces invadans* causes Epizootic Ulcerative Syndrome (EUS), which is characterized by the presence of ulcerative and dermal lesions in which invasive fungal hyphae elicit a granulomatous response.

Epizootic condition of freshwater and estuarine warm water fish have complex infectious aetiology, which is characterized by the presence of invasive *Aphanomyces* infection and necrotising ulcerative lesions typically leading to a granulomatous response (Baldock *et al*., 2005).

Contamination with different mycotoxin that produces fungal species is also common in aquaculture (Hashem, 2011). Fish in aquaculture farms are often fed with commercial diets containing different ingredients of plant origin. These diets include soybean meal and various cereal grains**,** all of which may be a source of mycotoxins. Most mycotoxins are thermostable and their processing has no influence on their effective concentration. As for *Aspergillus* species, these prefer warmer tropical areas and can grow on a wide variety of crops, including wheat, maize, and soybean (Matejova *et al*., 2017). Aflatoxins are a group of toxic metabolites, which are produced primarily by certain strains of fungi *Aspergillus flavus* under favourable temperature and humidity. The contamination of aflatoxins in food crops is common in subtropical and tropical areas (Deng *et al*., 2010).

Fungi in the genus Aspergillus, which can infect corn, peanuts, and other plants, produce aflatoxin B1 and related mycotoxins. These compounds can also reduce growth and suppress the immune system of fish (Tuan *et al*., 2002). *Aspergillus* spp. causes systematic diseases with high mortality rates in fish such infections mostly occur through contamination of fish feed (Mohamed *et al*., 2017).

Another category of mycotoxin is ochratoxins. Within their category, ochratoxin A was discovered as a metabolite of *A. ochraceus* in 1965, during a large screening of fungal metabolites that aimed at identifying new mycotoxins (Kumar *et al*., 2013).The most important mycotoxicoses in fish are caused by ochratoxins produced by *Aspergillus niger*.

Apart from being the most important contaminant of the environment, mycotoxins can enter organisms through ingestion leading to acute disorders and causing cancerogenic, mutagenic, and teratogenic effects in fish (Jackic-Dimic *et al*., 2005).

With increasing scarcity of fresh water for aquaculture, especially tilapia culture in arid regions, tilapia that tolerate high salinity would enable the expansion of wider geographical range of culture, and thereby increasing global production of tilapia. Cnaani and Hulata (2011) indicate that tilapias cultured in high salinity are more sensitive to handling and secondary infection than are those, which are cultured in fresh or low salinity water. Fungi that live in the sea are defined as obligate (Jaber *et al*., 2012). According to Yagoub (2004), many fungi are regularly parasites in fish and are conveniently divided into two groups, those with septate mycelium, and those with non-septate mycelium.

Aquatic fungi are often considered secondary invaders, which only become established on damaged or necrotic tissue resulting from mechanical trauma, primary bacterial, parasitic, or viral infection (Neish, 1977). One precondition appears to be the presence of bruises, wounds, or abrased surfaces, which provide the substratum for the growth of fungus (Roth, 1972). *Fusarium* spp infection is another cause of mass mortality of fish (Pillai and Yvonne, Fungal infections in tilapia may lead to mass mortalities of fish (Chauhan, 2014). Few previous studies also reported on mycotic infection in tilapia, including the role of environmental factors in mass mortality (Refai *et al*., 2010).

Aquatic diseases are the biggest constraint in aquaculture production. Several methods namely hygiene maintenance, the use of chemicals, and antibiotic shave been tried to control diseases of aquatic animal. High stocking densities and overfeeding become the cause of aquatic pollution. Furthermore, Sihag and Sharma (2012) reveal that the entry of

wild fish or restocking ponds with unhealthy or sub quality eggs, is another source of unhygienic condition in fishponds.

1.1 Problems Statement and Justification

Despite the fact that mariculture is growing fast in Tanzania, the sector is faced by lack of suitable species of fish. This necessitated research at Pangani Research station with the aim of acclimatizing fresh water fish that can adapt to marine environment. Hybrids of female Nile tilapia and male Rufiji tilapia that have shown the potential have been experiencing high mortalities. There have been mortalities in tilapia species resulting from microbial assemblages reported in fish culture units, which have been set at different salinities. The mortalities were associated with poor water quality parameters, poor feed storage, and lack of biosecurity measures in the culture units. While some studies indicated high mortalities due bacteria infections, there have been limited studies to demonstrate the contribution of fungal infections. Thus, the important step in preventing mortalities in fish farming is to identify the resident fungal populations and their possible virulence to the fish and establish mechanisms that prevent cultured fish from being invaded by harmful fungi.

1.2 General Objective

The general objective of the study was to determine the extent of fungal infections in Rufiji tilapia and hybrids of Nile tilapia♀ and Rufiji tilapia ♂ at different water salinity levels and confirm the isolates using DNA based techniques.

1.2.1 Specific objectives

Specifically, the study intended to;

i. Determine the prevalence of pathogenic fungi in water, and their infection in Rufiji tilapia and hybrids of Nile tilapia♀ and Rufiji tilapia ♂ at different salinity levels (2, 15, 25, and 35ppt).

ii. Confirm the identity of fungal isolates in Rufiji tilapia and hybrids of Nile tilapia♀ and Rufiji tilapia ♂farmed at different water salinity levels (2, 15, 25, and 35ppt) using DNA based techniques.

iii. Determine the composition of pathogenic fungal communities of tilapia raised at different water salinity levels (2, 15, 25, and 35ppt).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Classification of Aquatic Fungi

Saprolegniales is the best-known group of aquatic fungi, also referred to as water moulds. Members of this group are abundant in wet soil, lake margins, and debris; whilst some occur in brackish water (Yagoub, 2004). Saprolegniasis is a disease of fresh and brackish water species caused by *Saprolegnia*, although several other related species of Oomycetes such as *Achly*a, which are ubiquitous in soil and freshwater, can present clinical sign in a similar way. *Suprolegnia* spp are characterized by massive growth of white or grey thin threads, resembling tufts of cotton wool on the skin, gills, and/or fins (Singhal *et al*., 1987).

Saprolegniasis is a fungal disease of fishes and fish eggs. *Saprolegnia, Achlya* species and *Idctyuchus* species belonging to the family *Saprolegniaceae* are responsible for the disease (Eli *et al*., 2011). Water moulds can be found in the brackish water with salinity levels of up to 28 parts per thousand, though they occur primarily in fresh water. Initially, the affected areas may be small; however, the fungi can spread rapidly and cover most of the body. Water mould infections are the problem in intensively cultured fish during the cold season. The genera *Achlya, Aphanomyces, and Saprolegnia*are members of the family *Saprolegniaceae* in the class Oomycota are responsible for water mould infections in fish and the eggs of wild and farmed fish (Ahmed *et al*., 2012). *Saprolegnia* causes superficial disease with a mild inflammatory response whose lesions rarely go beyond the superficial musculature. These lesions, which often carry a free mass of fungal mycelium, have been recorded for over 250 years (Whisler, 1997).

Malnutrition, the presence of toxic substances in the water, damages of skin, fins, or gills, and stress can create room for secondary invasion of fish tissue by water moulds. Dead fish eggs are a good growth medium of fungi, whose mycelia mass are transferred from the dead fish eggs to the healthy fish thereby causing infections leading to fish mortality. This process increases the zoospores and infestation of fish eggs (Eli *et al*., 2011).

2.1.1 Pathogenic fungi

The first report of an oomycete infection was attributed to *Aphanomyces* species (Panchai *et al*., 2014). Difficulties in culturing *Aphanomyces*, verification of isolate pathogenicity, and debate about the speciation of the *Aphanomyces* involved in ulcerative mycosis were not resolved until they were confirmed by molecular studies. Thus, *Aphanomyces invadans* considered the primary pathogen of ulcerative mycosis (UM) (Sosa *et al*., 2007). *Aphanomyces piscicida* is responsible for many fungal associated diseases including epizootic ulcerative syndrome (EUS) in freshwater and brackish-water fish in Africa and Asia Pacific regions. The infected fish usually present dermal ulcers and loss of scales, haemorrhage, edema, and necrotic open ulcers on the body surface. The fungus has aseptate hyphae extending to the skeletal muscle within granulomas (Phadee *et al*., 2004).

Epizootic ulcerative syndrome is a disease that has affected wild and farmed fish in Asia and Australia over the past 30 years (Lilley *et al*., 2003). Furthermore, other typical water moulds that are often involved in the infections of wild and farmed freshwater fish, such as *Aphanomyces* spp and *Fusarium* spp, are considered important pathogens of marine fish and shellfish. Fusarial infections in marine fish include deep mycoses, ocular and skin lesions, fatal ulceration, and necro hemorrhagic dermatitis (Cutuli *et al*., 2015).

2.1.2 *Aspergilus* species

Aspergillus species cause systemic diseases with high mortality rates in fish. The infections mostly occur through contamination of fish feed. Today, more than 400 different mycotoxins are known, the most important of these is aflatoxins. The aflatoxins are highly carcinogenic agent causing haemorrhages, hepatotoxicity, nephrotoxicity, neurotoxicity, dermatitis, and immunosuppressive effects, through decreased antibody and cytokine levels. Many essential oils such as clove oil have antioxidant activity and anti-fungal properties (Mohamed *et al*., 2017).

2.1.3 *Aspergillus flavus*

Aspergillus species (i.e. *Aspergillus flavus and A. parasiticus*) produce secondary metabolites under favourable temperature and humidity. Therefore, the contamination with aflatoxins in food crops (especially the crops containing high starch and lipid content, such as peanuts, cottonseeds, maize, wheat, sunflower and soybean) is common in subtropical and tropical areas. *Aspergillus* species releases aflatoxin B1 that is the most prevalent and toxic to human, terrestrial animals, and aquatic organisms, mostly due to its strong carcinogenic, mutagenic, and teratogenic effects (Agag, 2004). The toxic signs in the majority of aquatic organisms exposed to *Aspergillus* spp that produces aflatoxin B1 (AFB1), include anorexia, yellowing of the body surface, weight loss, the reduction of feed conversion efficiency, liver dysfunction, and histological damages. In tilapia, decreased growth and feeding efficiency were reported in the short-term AFB1 exposure trial of less than 12 weeks (Deng *et al*., 2010).

Aspergillus flavus and *A. parasiticus* produce aflatoxin, a toxic compound that can be produced when fungi grow in the improperly stored feeds and feeds with inferior quality of ingredients (i.e. degraded protein resulting to ammonia and rancidity due to oxidation).

Aflatoxins area critical source of contamination in many foods and feeds produced from plants. Aflatoxin B1 is known to be the most significant form that causes serious risk to animal and human health. The carcinogenic effect of aflatoxin B1 has been studied in fishes such as salmonid, rainbow trout, channel catfish, tilapia, guppy, and Indian major carps (Cagauan *et al*., 2004).

2.2 *Aspergillus niger*

Species of the genus *Aspergillus* Section *Nigri* or the Black *Aspergillus* are widely distributed around the world and have the capacity of developing in a variety of substrates. Many species are able to cause deterioration of food although some of them are used in fermentation industries to produce organic acids, such as citric and gluconic acids, as well as hydrolytic enzymes such as lipases and amylases (Silva *et al*., 2011). Another important mycotoxin produced by *Aspergillus* spp includes ochratoxin A (OTA), which is produced by *A. ochraceus* and related species. OTA, a derivative of isocoumarin is linked to L-phenylalanine. It is also widely produced by *Penicillium* and *Aspergilus* species, particularly *P. verrucosum* and *A. niger*. Ochratoxin A *(*OTA) is a potent nephrotoxin, teratogen, and carcinogen whose inhalation can lead to renal failure (Sweeney and Dobson, 1998).

Fresh water edible fish species are susceptible to fungal infection by *A. niger* that causes Epizootic Ulcerative Syndrome infection (Podeti and Barnajee, 2016). The pathogenesis of *A. niger* has been reported in fresh water fishes (Pachade *et al*., 2014). Stressful environmental conditions and low temperature increase the fungal pathogens in fresh and marine water fishes. Pollution causes the EUS infection, which is common in India and throughout South Asia (Podeti and Barnajee, 2016). In substrates such as grapes, raisins and wine, the source of OTA contamination is considered to result from *A. carbonarius*.

Some species such as *A. niger*can produce a range of different toxic metabolites, which are used as a source of enzymes and organic acids for food processing, despite the reported ability to produce OTA (Esteban *et al*., 2006).

2.2.1 Aflatoxin B1 (AFB1) effect on the immune system of fish

The Nile tilapia (*O. niloticus*) is an exceptionally sensitive and economically model species for the study on aflatoxin as it reduces phagocytic capacity of macrophages. The studies of chronic effects of such xenobiotic chemical have been usually limited to mammals. Several aquatic species exposed to different AFB1 concentrations show a variety of immune deficiencies, ranging from innate to adaptative or both as have been reported by (Rodríguez *et al*., 2010).

2.2.2 The *Aspergillus* mycotoxins

Mycotoxins, which are secondary metabolites that are produced by certain filamentous fungi, can be produced in foods as a result of fungal growth. The most mycotoxins are thermostable whose processing has no influence on their effective concentration (Matejova, 2017). They cause a toxic response, termed a mycotoxicosis, when ingested by higher vertebrates. The four main naturally produced aflatoxins are B1, B2, G1, and G2; with B usually being the aflatoxin found at the highest concentration in contaminated food and feed. Aflatoxin B is regarded the most potent liver carcinogen for a wide variety of animal species including humans (Sweeney and Dobson, 1998). The reduction in growth is one of the major reported negative effects of aflatoxin B1 contamination. Several studies reported reduced growth rates in channel Nile tilapia. In addition, mortality rates of 17 percent were reported in diets with 0.2 ppm AFB1 fed to Nile tilapia (Santos *et al*., 2010). One way of preventing fungal contamination of feeds and subsequent mycotoxin formation or aspergillomycosis development is the maintenance of good husbandry and

adequate and rapid drying of feeds from water content from which fungal growth cannot occur (Olufemi, 1985).

2.2.3 Impact of aflatoxins in aquaculture

Aflatoxins are mycotoxins produced by *Aspergillus flavus* and *A. parasiticus*. Among the most toxic and common contaminants of cottonseed, peanut meal, and corn, and other feed components that can harbour aflatoxins include soybeans, fishmeal, wheat, and sunflowers. Four major aflatoxins (i.e. AFB1, AFB2, AFG1, and AFG2) have been identified, with AFB1considered as one of the most potent carcinogens. Aflatoxins can also degrade vitamins A, C, and B1 (Yanong, 2003). The presence of aflatoxins in and their impact on farmed aquatic species are still undervalued and Santos *et al.* (2010) have reported few studies on the toxicity of AFB1 to cultured aquatic invertebrates fed with artificially contaminated diets as.

Nevertheless, aflatoxicosis might potentially be serious in both cultured aquatic vertebrates and crustaceans. The replacement of marine fishmeal with vegetable feedstuffs might reduce costs significantly in aquaculture and could provide a sustainable alternative source of fish feed. However, there are feed ingredients of plant origin with higher incidences of aflatoxin contamination that could have adverse effects on fish. Feeding fish with contaminated feed increases the risk of the onset of disease especially in intensive rearing systems where natural food is completely replaced with pelleted feed (Santacroce *et al.*, 2008). The presence of mycotoxin in food can be observed during different stages of the plants cycle, such as the growing stage of grains and other vegetables, post-harvest, transport, processing, and storage.

Fungal growth and aflatoxin production can be influenced by environmental factors such as temperature, relative humidity, and interactions with other microbiota. Aflatoxigenic fungi have a viability temperature ranging from -3 ℃ to 40 ℃, with a pH tolerance of between 2.0 and 10.0 and a water activity (*a*w) for growth ranging from 0.77 to 0.99 as have been reported by Rodríguez *et al*. (2010). Aflatoxicosis in fish cause pale gills, liver damage, poor growth rates, and immune-suppression, several studies showed that AFB1 can cause poor growth, low digestibility, physiological disorders and histological changes mainly in the hepatopancreatic tissue of marine shrimps as have been reported by Santos *et al*. (2010).

2.3 Effects of Ochratoxin in Aquaculture

Ochratoxins are a group of secondary metabolites produced by fungi belonging to the *Aspergillus* spp and *Penicillium* spp. The most toxic and abundant mycotoxin is ochratoxin A (OTA). Ochratoxin A is generally associated with contamination of corn, cereal grains, and oilseeds and can affect animal performance by causing damage to the functioning of kidney. The reduction in body weight gain of diets with 0.2 ppm AFB1 fed to channel catfish for two weeks and 1ppm for eight weeks were observed (Santos *et al*., 2010), the reduced feed conversion ratio (FCR) was also observed in the same species with contamination levels of 4 and 8 ppm in rainbow trout. The pathological signs of ochratoxicosis, which included liver necrosis, pale, swollen kidneys, and high mortalities were observed (Santos *et al*., 2010).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was conducted at Bweni Village, Pangani District in Tanga Region, Tanzania (05° 26′ 0″S and 38° 58′ 0″E) (Fig. 1). Bweni village was selected because it is where the Institute of Marine Science Mariculture centre (IMS-MC) is located. The major economic activities of the communities of Bweni village include fishing, seaweed farming, and subsistence agriculture. The area is drained by Pangani River Basin bordered by the Kilimanjaro and Meru Mountains, and Pare and Usambara Mountain Ranges. The area is endowed with several species of terrestrial and marine ecosystems including mangroves. The Pangani River has a diversity of tilapia species including *O. pangani korogwe, O. pangani pangani, O.variabilis, T. rendalli and T. zillii*. Pangani is characterized by annual rainfall of above 1 000 mm with temperature ranging from 25 to 30 °C. The climate is generally warm and humid (Mapenzi *et al*., 2016).

The Rufiji tilapia♂ and Nile tilapia ♀brood stocks were collected from Rufiji River that drains its water into the Indian Ocean in Central Eastern Tanzania and from Lake Victoria in Mwanza Tanzania. Two replicates each of Rufiji tilapia ♂ and hybrids of Nile tilapia♀ and Rufiji tilapia♂ in the hatchery systems were sat at different salinities.

Figure 1: Map of Tanga region indicating the location of the Institute of Marine Sciences Mariculture Centre (IMS-MC) at Bweni Village, Pangani District, Tanzania.

3.2 Study Design and Setup of the Experiment

This was a complete randomized experimental study, in which fish were kept in tanks at different salinities levels (i.e. 2, 15, 25, and 35). The layout of the experiment included 16 tanks of about 31 m^3 each, which were set at different water salinity levels as illustrated in Appendices 4 and 5. About 288 fish fry were batch weighed prior to stocking and randomly distributed at 18 fish per tank. The initial weights ranged from 3 to 5 g per treatment. Furthermore, the Rufiji tilapia were stocked into the first eight tanks with different water salinity levels as shown in Appendix 4 followed by the hybrids of Nile tilapia ♀ and Rufiji tilapia ♂ as shown in Appendix 5.

3.3 Sample Preparation

3.3.1 Collection of fish samples

Forty eight (48) Rufiji tilapia and 48 hybrids of Nile tilapia♀ and Rufiji tilapia ♂ of 60 days old and a body weight of 15-20 g were randomly collected from the hatchery and

identified morphologically. Two replicates each of Rufiji tilapia ♂hybrids of Nile tilapia♀ and Rufiji tilapia ♂ were set at different salinities in the hatchery systems. The fish were collected from four levels of water salinity (2, 15, 25, and 35ppt) where the tanks of about 31 m^3were used to raise fish (grow out) which were fed two times a day. Water quality parameters such as salinity, dissolved oxygen, and PH were also measured.

Morphologically, Nile tilapia is reddish to white in colour with prominent bars on caudal fins and with white coloured strips on dorsal and anal fins (Mohamed *et al*., 2017). The fish were inspected for general body condition, clinical signs of diseases, and any apparent lesions and were declared healthy. The fish were dissected aseptically using forceps, scissor, surgical blades, alcohol, and gloves to obtain sample organs (gills, skin, and gastrointestinal tract) which were immediately transferred on ice packs using universal bottles to the laboratory at Sokoine University of Agriculture. The samples were subjected to mycological examination and transferred to the laboratory for microbiological analysis.

3.3.2 Isolation of fungi from fish samples

The collected fish samples did not show any clinical symptoms of fungal infection. The fish samples were scraped aseptically using sterile scissors, forceps, surgical blades, and alcohol. A small piece of fish organ cut into pieces of about 0.3-0.5 g each was inoculated onto Sabouraud dextrose agar (SDA) medium in the culture plates under aseptic conditions (Chauhan, 2014). The plates were sealed and incubated at 25 ^{o}C and then examined after three to seven days. The emerging fungal colonies were repeatedly sub cultured onto the fresh plates of SDA medium until pure cultures were obtained. The emerging hyphal tips were repeatedly transferred onto the fresh plates of SDA medium until pure cultures were obtained. The culture plate was labelled based on the type of specimen with a date of inoculation.

3.3.3 Water samples collection

The water samples were collected from culture tank systems. Water quality parameters were measured before and after the addition of seawater into the tanks. The pH and temperature were measured by HI8424 pH meter and salinity was measured by Digital Hand Held Marine Tester DMT-10 (Mapenzi *et al*., 2016) once a week from February to April 2018at the Institute of Marine Sciences Mariculture Centre (IMS-MC) at Bweni Village, Pangani District in Tanga Region, Tanzania. The approach to water sampling was done as reported by (Pham *et al*., 2015). Water samples were collected from all 16 tanks where 1 000 ml of water per tank was sampled. Furthermore, water sampling was done on monthly basis where in total 16 litres of water samples were collected. The water samples were filtered with the aid of Ultra-fine filter papers with the retention capacity of0.7 micrometer and the residues were kept in the universal bottles. The residues were collected once per month and were shipped in the ice packs to the College of Veterinary Medicine and Biomedical sciences, at Sokoine University of Agriculture for mycological analyses.

3.3.4 Isolation of fungi from water sample

Adopting the protocol of (Pham *et al*., 2015), with a slight modification, wet-mounts was done by inoculating small amounts of residues from water samples into Sabouraud dextrose agar (SDA) to check for the presence of fungal hyphae or conidia in the specimens. Small amounts of residues were taken and inoculated into culture medium using inoculating wire loop. The fungi, which were isolated from the water columns, were then cultured on SDA. The plates were incubated from three to seven days at 25^{o}Cand then a tuft of growing edges of the fungal colonies were sub cultured onto fresh plates for three subsequent times until when pure cultures were obtained.

3.3.5 Fungal characterization

The characterization of fungal isolates was carried out according to Sharma *et al*. (2013). A solution of lacto-phenol cotton blue was prepared by mixing 10g phenol detached crystals, 0.04 g cotton blue, 10 ml lactic acid, 20 ml glycerol, and 15 ml distilled water. The drop of lecto-phenol cotton blue was taken on the glass slide and fungal hyphae from the pure cultures were transferred aseptically onto it using an inoculation golf stick. The culture was then spread to an even thin film on the cover slide. The fungal hyphae were allowed to stain with lacto-phenol cotton blue for 2 minutes and a glass slide was prepared and observed under the microscope.

3.3.6 Fungal identification

Identification of the fungi was done based on cultural characteristics and colony morphology (shape, pigmentation, and arrangements) of the isolates as reported by Onyeze *et al*. (2013).

3.3.6.1 Identification of *Aspergillus* and *Rhizopus* species

The fungal species were identified based on macro-morphological and micro-morphological appearance according to Nakhchian *et al*. (2014). The texture of the fungi colour at the top and bottom of the cultured plate of all isolates of *Aspergillus* and *Rhizopus* spp were assessed when they were three and seven days of growth. The colonies appearances were observed by the naked eye while microscopic details were observed by the light microscope at x 10magnificationand were compared with the colour charts according to (Chehri and Hasani, 2017).

3.3.6.2 Molecular analysis

The conventional method of Polymerase Chain Reaction (PCR) was used for the identification of pathogenic *A. flavus* and *A. niger* from fungal isolates using specific strains of primers from *A. flavus* and *A. niger*.

3.3.6.3 Fungal cell preparation for DNA extraction

Fungal isolates were sub cultured in SDA to obtain pure culture. Five grams of fungal mycelium were harvested and then ground using a pestle and a mortar in the distilled water. The homogeneous solution was centrifuged for 15 minutes using micro-centrifuge as instructed by Quick- gDNA-MiniPre Kit (Zymo Research Corporation, Irvine, CA 92 614, United States). The volume of 200 µl of the supernatant was transferred to a new eppendorf tubes (Zymo Research Corporation, Irvine, CA 92 614, United States) and then Beta mercaptor-ethanol of 0.5 % (v/v) and genomic lysis buffer of 0.1 µl were added to optimize the performance of DNA extraction.

3.3.6.4 DNA extraction

DNA extraction was performed using Quick-gDNA-MiniPre Kit following manufacturer's instructions. The mixtures of supernatant solution (i.e. mercapto-ethanol and genomic lysis buffer) were incubated for 10 minutes at 27^{o}C. This was intended to break down the cells prior to the PCR process. Zymo spin tubes were arranged for DNA extraction therein DNA was trapped and pipetted in the incubated solution of supernatant, genomic lysis buffer and mercapto-ethanol. Hence, the mixture was then transferred once again to Zymo spin tubes, which were labelled equivalently to eppendorf tubes, the mixture in Zymo spin tubes was centrifuged with the aid of eppendorf 5 424 R for one minute, 0.2 ml of DNA pre-wash buffer was added in the mixture of genomic lysis buffer and mercapto-ethanol into Zymo pins. The mixture was centrifuged for one minute to separate the DNA,

followed by the removal of Zymo spin column, which had a membrane to trap the DNA. The volume of 15 µl elusion buffer was pipetted and transferred into Zymo spin column, and centrifuged for 30 seconds. The elutes were used for PCR detection of *aflD, aflM*, NIG 1 genes, and Tannase

3.3.6.5 Molecular detection of *Aspergillus* species

The detection of *A.flavus* and *A. niger* was done by PCR using specific pairs of primers (Tables 1) that amplified specific target genes (*aflD* and *aflM*, Tannase and NIG 1) for *A. flavus* and *A. niger*. Primers were reconstituted using ultra-pure distilled water and aliquoted into small vials and used consecutively.

Table 1: Primers of specific genes for *Aspergillus* species

Target species	Target Gene	Sequence 5'-3'	Product size(bp)	Reference
A.flavus	*aflD*	ACC-GCT-ACG-CCG-GCA-CTC-TCG-GCA-C GTT-GGC-CGC-CAG-CTT-CGA-CAC-TCC-G	400	Nagur *et al*., 2014
	aflM	ATG-TCG-GAT-AAT-CAC-CGT-TTA-GAT-GGC CGA-AAA-GCG-CCA-CCA-TCC-ACC-CCA-ATG	895	Nagur *et al*., 2014
A.niger	NIG1	GATTTCGACAGCATTT(CT/TC)CAGAA AAAGTCAATCACAATCCAGCCC	290	Kamal *et al*., 2016
	Tannase	TTCTGCTCTGGATCGCAATCTG ACTAGTGATTGATGGGGGAGAGG	950	Kamal *et al*., 2016

3.3.6.6 PCR Amplification

The targeted gene during amplification was NIG1and Tannase for *A. niger* (Fig. 2). The second gene was aflD and aflM (Figures 7and 8) for *A. flavus*. Amplification of fungal DNA was performed in the volume of 25 µl. The reaction mixtures consisted of 12.5 µl PCR master mix 2x (One Taq Quick Load 2 X Master Mix with Standard Buffer), 12.5 pmol of each primer, ±100 ng DNA template and nuclease free water, the amplification was as follows: Pre-denaturation for 5min at 94^{o}C, followed by 35 cycles of 30s at 94 ^{o}C for denaturation, 60s at 58 ^{o}C for annealing, 90s at 72 ^{o}C for extension, and 7minutes at 72 ^{o}C for the final extension using the TAKARA PCR thermo cycler machine

according to the manufacturer's protocol. Thus, the fragmented size of the PCR product to be amplified was 400 bp and 895 bp for specific genes of *A. flavus*. The PCR products were analyzed on 1.5 % agarose gel in 1x TBE buffer, stained with DNA marker and visualized under UV trans-illumination (Nagur *et al*., 2014).

3.3.6.7 Preparation of gel agarose

Agarose gel (1.5%) was prepared by measuring 1.2 g of gel agarose powder (Takara Bio Inc., Kusatsu City, Japan) into 100 ml 1X Tris-Boric-EDTA (TBE) buffer, following the manufacturer's instruction. The mixture was thoroughly dissolved by boiling in a conical flask until it became colourless, and then cooled to 60 ^{0}C. Ethidium bromide was added to the mixture and then poured into the casting trays to solidify. The gel was placed into electrophoresis tank containing 1X TBE. A total of 2 µl of loading dye were mixed with 5 µl of DNA and loaded into the gel along with DNA ladder positive and negative control following the instructions of the gel manufacturer. The gel was run at 100 volts for 45 minutes and thereafter, specific band visualization was done using trans-illuminator UV machine. The gel picture was taken for further analysis.The amplicon with the band size of 400 bp; 895 bp, and 290 bp were considered positive for *A. flavus* and *A. niger* following the Good Quality Control results.

3.3.6.8 The *aflD* and *aflM* genes

The genes were detected as reported by (Nagur *et al.*, 2014). The PCR reactions were performed using two pairs of species-specific primers for identification. The pairs of primers were used as shown in (Table 1) to amplify the fragments of 400 bp and 895bp respectively. The amplification of the fungal DNA was performed in the volume of 25 µl. The reaction mixtures contained 12.5 µl PCR master mix 2x (One Taq Quick Load 2X Master Mix with Standard Buffer), 12.5 pmol of each primer, ±100 ngDNA template, and

nuclease free water. The amplification was performed as follows: Pre-denaturation for 5 min at 94 ^{o}C followed by 35 cycles of 30s at 94 ^{o}C for denaturation, 60s at 58 ^{o}C for annealing, 90s at 72 ^{o}C for extension, and 7 min at 72 ^{o}C for final extension using TAKARA thermal cycler. The PCR products were analyzed on 1.5 % agarose gel in 1x TAE buffer, stained with DNA marker solution and visualized under UV trans-illuminator.

3.3.6.9 The NIG1 and tannase genes

The genes were identified (Kamal *et al*., 2014). PCR was performed with a 100 µl reaction mixture containing 10 mM Tris-Boric-EDTA (TBE) buffer (pH 9.0 at 25 °C), 10 mM KCl, 1.4 mM MgCl2, and 0.2 mM (each) deoxy nucleoside triphosphates (dNTPs), others include 0.1% Triton X-100, 50 pmol of each of the two primers, 0.2 U of SuperTaq DNA polymerase (One Taq Quick Load2X Master Mix with Standard Buffer), and 0.5 pg of DNA from each *Aspergillus* isolates which had been denatured at 94 °C for 5 min. Next, 30 cycles of amplification were performed by denaturing for 1 min at 94 °C, annealing the primer for 1 min at 42 °C, and allowing elongation for 3 min at 72°C. The pairs of Primers for NIG1 and tannase genes (Table 1) were designed from conserved regions of amino acids from an alignment of the translated sequences of genes from several sources.

The process of PCR degenerate was performed at 3min 94 ºC x1, 1min 94 ºC, 1min 48 ºC, 3 mins 72 ºC x 29, 1min 94 ºC, 1min 48 ºC, 7mins 72 ºC x1. The PCR products were analyzed on 1.5 % agarose gel in 1x TAE buffer, stained with DNA marker solution and visualized under UV trans-illuminator and PCR produced a clear product of ca 290 bp with *A.niger* genomic DNA, which was the size of product, predicted.However, tannase product of 950 bp using the same protocols did not produce any product after being visualized under UV trans-illuminator.

3.4 Statistical Analysis

The data in this study are continuous and data were cleaned prior to analysis. Data obtained were entered into Microsoft excel. Conditional logistic regression analysis was used to determine the prevalence of fungal infection in Rufiji tilapia and hybrids of Nile tilapia♀ and Rufiji tilapia ♂ at different water salinities. All values were not significantly different $P > 0.05$ except the values for fish organs were statistically significant $P<0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1 Isolation of Fungal Species from Fish Samples

Ninety-six (96) fish (48 fish Rufiji tilapia and 48 hybrids of Nile tilapia ♀and Rufiji tilapia♂) were sampled. Their gills, GIT, and skin were assessed for fungal infection. Seventy two point four percent (72.4%) of all fish were infected with *A. niger* and 17.1% with *A. flavus.* The results show that the fungal species isolated from Rufiji tilapia♀ and hybrids of Nile tilapia♀ and Rufiji tilapia♂ were about 34.9% *A.niger*, 6.5% *A.flavus* for Rufiji tilapia, 37.4% as *A.niger,* and 10.6% as *A.flavus* for the hybrids of Nile tilapia♀ and Rufiji tilapia♂as shown in (Tables 2 and 3). *Aspergillus niger* were the most prevalent fungi of all the isolates. *Rhizopus spp* was isolated from Rufiji tilapia♂ and hybrids of Nile tilapia♀ and Rufiji tilapia♂ with a varying percentage of 6.5 % and 2.4 % (Tables 2 and Table 3). The statistical test was not done for the samples collected from water due to small sample size; the data were thus translated by description.

Table 2: Fungal species isolated from Rufiji tilapia (n=61)

		Salinity (ppt)				Total
Organ	**Fungal spp**	2	15	25	35	
	A.flavus	0	0	0	0	0
	A.niger	4(6.6%)	4(6.6%)	4(6.6%)	1(1.6%)	13(21.3%)
GILLS	*Rhizopus sp*	1(1.6%)	1(1.6%)	0	0	2(3.3%)
	A.flavus	2(3.3%)	2(3.3%)	2(3.3%)	0	6(9.8%)
	A.niger	4(6.6%)	3(4.9%)	6(9.8%)	8(13.1%)	21(34.4%)
GIT	*Rhizopus sp*	1(1.6%)	0	0	0	1(1.6%)
	A.flavus	2(3.3%)	0	0	0	2(3.3%)
	A.niger	5(8.2%)	4(6.6%)	1(1.6%)	1(1.6%)	11(18.0%)
SKIN	*Rhizopus sp*	0	3(4.9%)	1(1.6%)	1(1.6%)	5(8.2%)
Number of isolates		19(31.1%)	17(28.0%)	14(22.9%)	11(18.0%)	61(100%)

Sample organs of48 fish samples of Rufiji tilapia were isolated and identified by macro and micro-morphological characterizations regarding *Aspergillus* spp *as* the most abundant fungi in Rufiji tilapia (Table 2).

The distribution of the three fungal species in the three organ systems is shown in Figure 2.

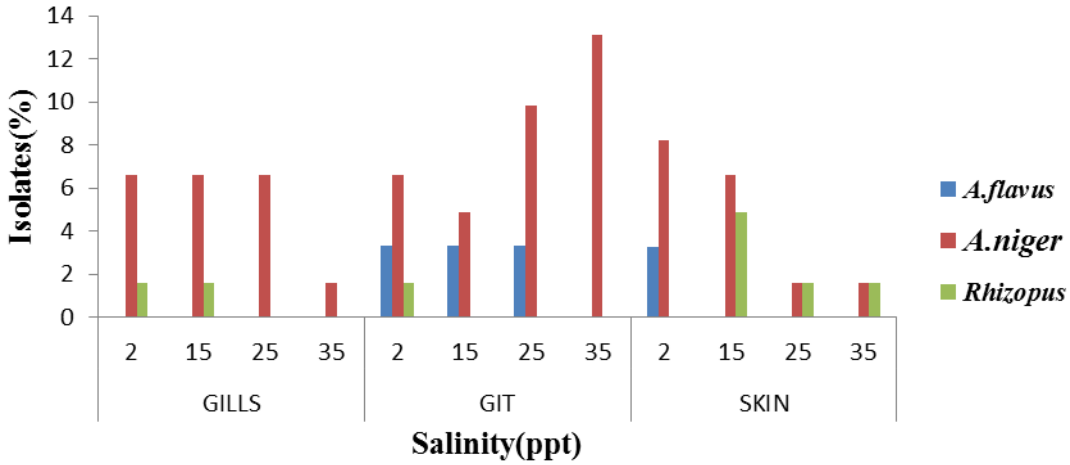


Figure 2: **Fungal species isolated from Rufiji tilapia at different salinity levels**

Figure 2 indicates an increasing percentage of positive samples for *A. niger* in gills with increasing salinity but a decrease in percentage in skin with an increase in salinity, *A. flavus* shows arising then a declining trend in gills, skin, and gastrointestinal tract (GIT) with an increase in salinity.

A logistic regression analysis was used to estimate the effect of salinity on the presence of *A. niger* in gills. The results showed no statistical difference among salinity levels (P>0.05 for all; P=0.217 for 35) compared with2 (Appendix1). Salinity of 2 is the baseline against which the different salinities are compared and thus not indicated as shown in Table 5. The number of positive samples in different organs and salinities for hybrids are shown in Table 3.

Table 3: Fungi isolated from the hybrids of Nile tilapia ♀ and Rufiji tilapia ♂stocked at different salinity levels (n=62)

		Salinity (ppt)				
Organ	**Isolate**	**2**	**15**	**25**	**35**	**Total**
	A.flavus	3(4.8%)	1(1.6%)	2(3.2%)	2(3.2%)	8(12.9%)
	A.niger	4(6.5%	6(9.7%)	4(6.5%)	4(6.5%)	18(29.0%)
GILLS	*Rhizopus* sp	0	0	0	0	0
	A.flavus	3(4.8%)	0	0	0	3(4.84%)
	A.niger	5(8.1%)	1(1.6%)	5(8.1%)	5(8.1%)	16(25.81%)
GIT	*Rhizopus* sp	0	0	1(1.6%)	1(1.6%)	2(3.22%)
	A.flavus	0	0	1(1.6%)	1(1.6%)	2(3.22%)
	A.niger	3(4.8%)	2(3.2%)	4(6.5%)	3(4.8%)	12(19.4)
SKIN	*Rhizopus* sp	1(1.6%)	0	0	0	1(1.61%)
Total no. of isolates		19(30.6%)	10(16.1%)	17(27.4%)	16(25.8%)	62(100%)

Samples from the hybrids were characterized morphologically and the most dominant fungi were *Aspergillus* spp as shown in Table 3. The same findings are shown in the as bar chart in Figure 3.

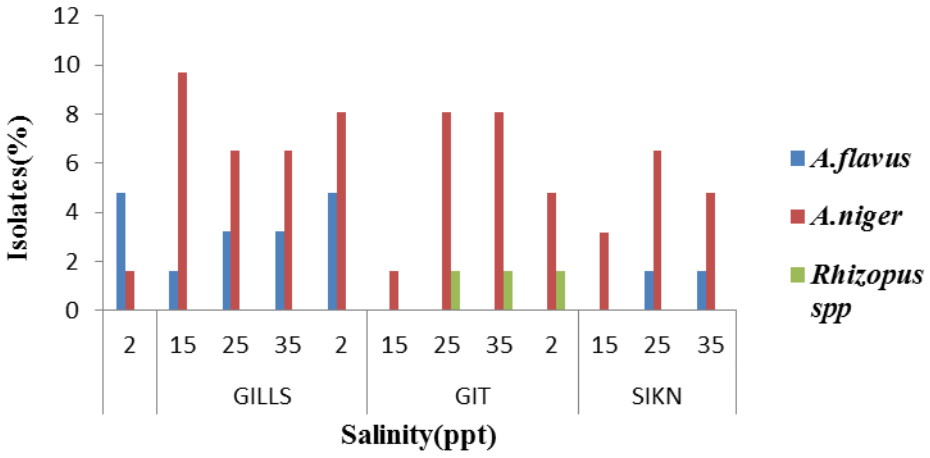


Figure 3: Fungal species isolated from the hybrids of Nile tilapia ♀ and Rufiji tilapia ♂at different salinity levels

Fig. 3 shows the proportion of different fungi for the different organs and salinities for the hybrids of Nile tilapia ♀ and Rufiji tilapia ♂. There are declining percentages of positive samples for *A. niger* in gills, variable in gastrointestinal tract (GIT) and skin with an increase in salinity. *A. flavus* shows a declining trend in gills but an increasing trend in

skin. A logistic regression analysis was carried out to examine *A. niger*, *A. flavus,* and *Rhizopus* spp. for each of these, there was no any significant difference for different salinity and organs.

4.2 Isolation of Fungal Species from Water Sample

Two *Aspergillus* species were isolated using SDA in culture plates incubated at 25 ^{0}C for 3 to 7 days. Macro- and micro-morphologically were identified on lactophenol detached crystal cotton blue. In isolating *Aspergillus,* species from eight samples of water from which Rufiji tilapia were stocked. *Aspergillus flavus* and *A. niger* were absent (Table 3). The only fungal spp, which was isolated in all water samples, was *Rhizopus* species.

The analysis was performed to detect the prevalence of *Aspergillus* and *Rhizopus* species in the water of which the hybrids of Nile tilapia ♀ and Rufiji tilapia ♂were stocked. Thus, out of 8 isolates, 7 were *Aspergillus* species, which were detected from the salinity of 15, 25, and 35. Fourteen (14) % was detected as low quantity for *Aspergillus flavus*. Despite the low number of isolates, the majority of fungi (15, 25 and 35ppt), which were detected in salinity water were reported as high quantity as 85.7 % for *Aspergillus niger* (Table 5).

Table 4: Fungal growth distribution from fish skin and gill samples from Rufiji tilapia

Organs	Fungal isolates	% fungal isolates	p<z	Level of significance (α)
Gills	15	24.6	0.009	0.05
Skin	18	29.5	0.008	0.05

Statistical analysis (logistic regression analysis) for the effect of salinity and organs (Appendix 2) shows that there was no any significant difference between salinity of 2 compared to other organs. The GIT (GL) and skin (MS) have significantly lower numbers

than gills (P = 0.009 and P = 0.008, respectively). Tests were also run for *A. flavus* and *Rhizopus* spp and no statistical differences were found (not shown) at P>0.05 (Table 4).

Table 5: Fungi isolated from water in which hybrids of Nile tilapia ♀ and Rufiji tilapia ♂were stocked at different salinity levels (n=8)

	Salinity(ppt)				
Isolates	**2**	**15**	**25**	**35**	**Total**
A. flavus	0	1(14.3%)	0	0	1(14.3%)
A. niger	2(28.6%)	1(14.3%)	1(14.3%)	2(28.6%)	6(85.7%)
Rhizopus sp	0	0	0	0	0
Total	2(28.6%)	2(28.6%)	1(14.3%)	2(28.6%)	7(100%)

The numbers of positive samples are relatively few with the highest number being found for *A. niger*. No statistical analysis was performed due small sample size as shown in Table 5.

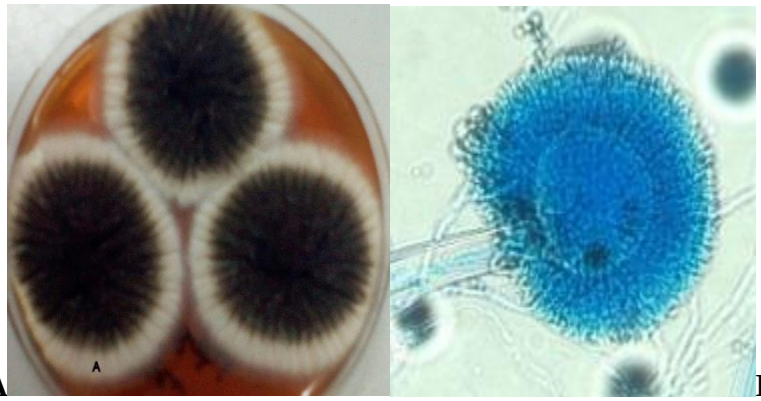
Table 6: Logistic regression analysis for fungal isolates between different salinity levels and fish organs of the hybrids Nile tilapia ♀ and Rufiji tilapia ♂

Salinity(ppt)	**Fungal isolates**	**% fungal isolates**	**P>z**	**Level of significance (α)**
15	10	16.1	0.205	0.05
25	17	27.4	0.864	0.05
35	16	25.8	0.125	0.05

Statistical analysis (logistic regression analysis) for the effect of salinity and organs for Nile tilapia ♀ and Rufiji tilapia ♂, shows that there is no difference between salinities (2ppt compared to higher) for the organs namely, GIT, (GL), and skin (MS) (Table 6), P>0.05. Similar analyses were carried out for *A. flavus* and *Rhizopus* sp. and no statistical differences were found between 2 ppt increasing salinity (P>0.05) as shown in Table 6.

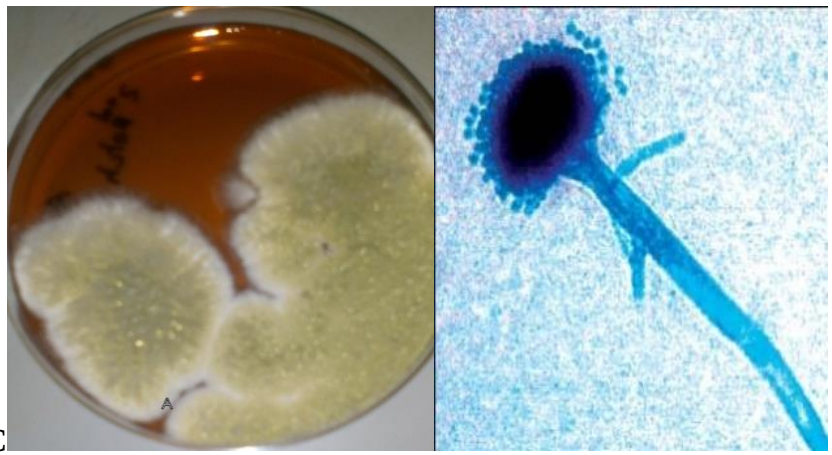
Table 7: Fungal growth distribution from fish skin and gill sample organs (tissues) (hybrids Nile tilapia ♀ and Rufiji tilapia ♂)

Organ	**Fungal isolate**	**%fungal isolates**	**P>z**	**Level of significance**
Gills	24	41	0.880	0.05
Skin	21	33.8	0.319	0.05



A **B**

Figure 4: **Macro-morphology (A) on SDA and micro-morphology (B) of *A. niger* on lactophenol detached cotton blue as seen under light microscope. *A. niger* colonies on SDA, which was incubated at 25 ^{0}C for 5 days, appears with white mycelia for the first three (3) days. With age, the colonies on SDA change to black conidial heads, they are biseriate, globose conidia heads and vesicle as shown in (Fig. 4) (B).**



C **D**

Figure 5: **Macro-morphology (C) on SDA and micro-morphology (D) of *A. flavus* on Lactophenol detached cotton blue as seen under light microscope, *A. flavus* colonies on biseriate conidia heads incubated at 25 ˚C for 5 days on SDA. On SDA, the colonies were yellow green with white mycelia at the edges, formed sporulation rings, and the conidia were rough (Fig. 5).**

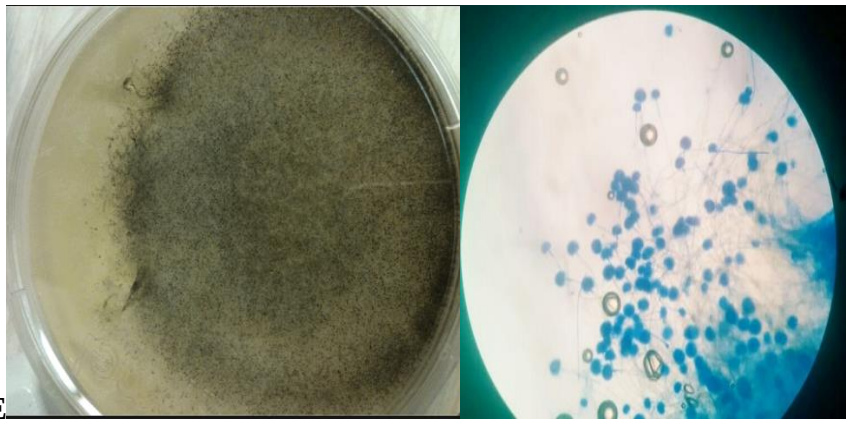
E  F

Figure 6: **Macro-morphology (E) on SDA and micro-morphology (F) of *Rhizopus species* on lactophenol crystal detached cotton blue as seen under light microscope. Rhizopus species colonies are fast growing and cover an agar surface with a dense cottony growth; it was incubated at 25 ^{0}C for 5 days on SDA, which was at first white becoming grey or yellowish brown with sporulation as shown in (Fig. 6).**

4.3 Molecular analysis for the detection of *Aspergillus flavus* and *Aspergillus niger*

Thirteen (13) isolates were subcultered from 123 fungal isolates of both Rufiji tilapia and hybrids of female Nile tilapia and male Rufiji tilapia to obtain pure culture for molecular analysis. Subsequently, five isolates of *A. flavus* and eight isolates of *A. niger* as shown in Tables 2 and 3 were collected as pure culture for DNA extraction. Two out of five *A. flavus* isolates and one *A. niger* out of eight isolates was taken independently for confirmation using PCR based technique (Tables 1). *Aspergillus flavus* and *A. niger* were confirmed based on AflD, AflM, and NIG1 target genes. Furthermore, the gene specific primer for *A. niger* (Tannase) was amplified though yielded PCR negative results. Therefore, *A. flavus* and *A. niger* were confirmed on target genes of aflD, aflM, NIG1, and tannase using the PCR.

4.3.1 PCR for aflD gene

All positive samples were suspected for aflD target gene. The gene was detected between 300 bp and 500 bp at 400 bp (Fig. 7). The positive sample organs were collected from both Rufiji tilapia♂ and hybrids of Nile tilapia♀ and Rufiji tilapia♂.

Figure 7: The *aflD* gene suspected in positive isolate of sample organs, samples tested from Rufiji tilapia and the hybrids. M represents Marker for aflD gene, +VE represent positive control and –VE represents Negative control, Lane 1-9 is *A. flavus*

4.3.2 PCR for aflM gene

All positive samples were suspected for aflM target gene. The gene was detected between 800 bp and 1000 bp at 895 bp (Fig. 8). The positive sample organs were collected from both Rufiji tilapia ♂ and hybrids of Nile tilapia ♀ and Rufiji tilapia♂.

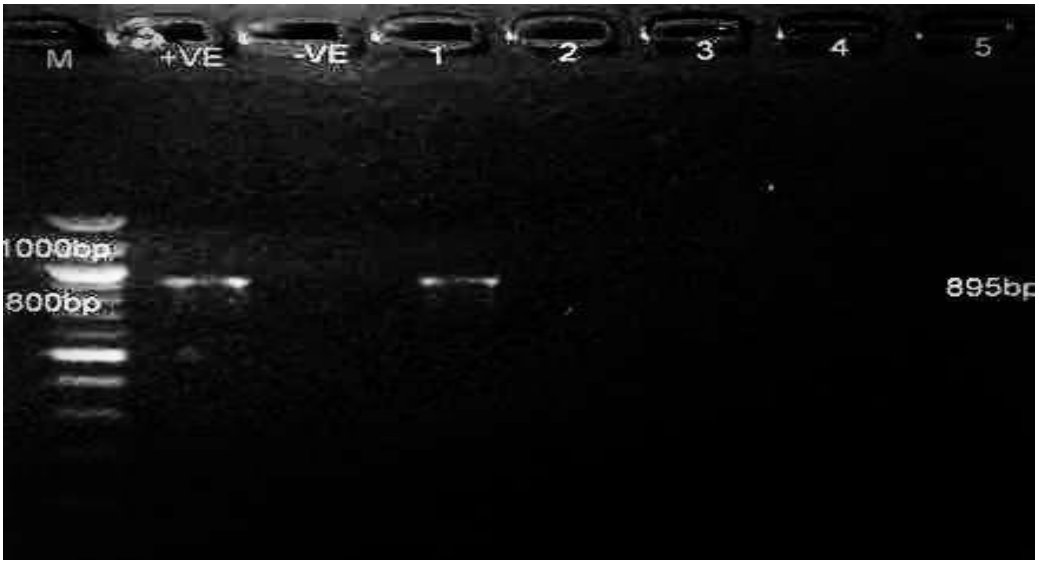


Figure 8: Positive samples detected *A. flavus* for a target gene aflM similar to M bands. M represents Marker for aflD gene, Lane 1-5 is *A. flavus,* +VE represent positive control and –VE represents Negative control

4.3.3 PCR for NIG1 gene

All positive sample organs were suspected for *A.niger*. The gene was detected between 200 bp and 500 bp at 290 bp (Fig. 9). The positive sample organs were collected from both Rufiji tilapia ♂ and hybrids of Nile tilapia♀ and Rufiji tilapia.

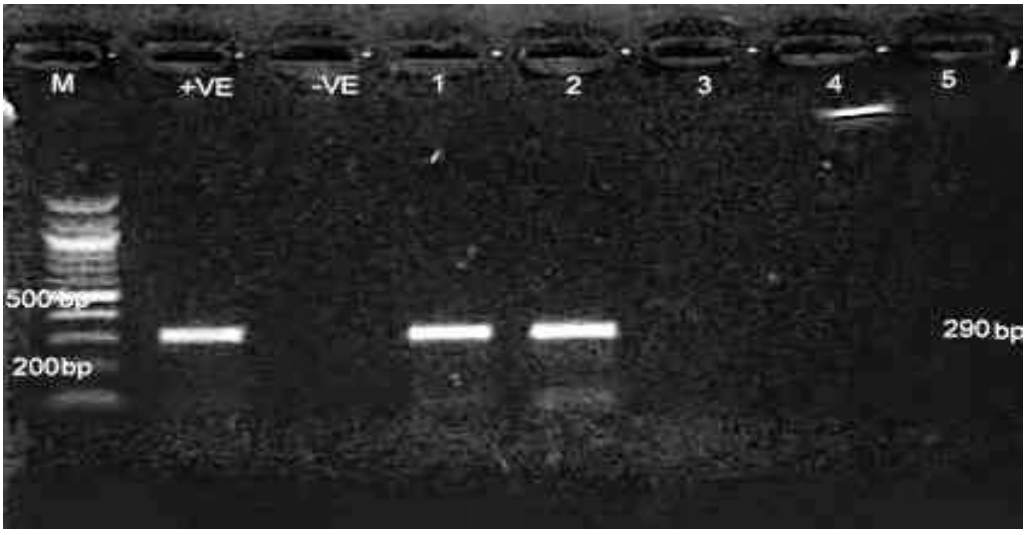


Figure 9: Positive samples detected for a target gene NIG1had M bands. M represents Marker (positive control) for NIG1 gene, Lane 1-5 is *A. niger,* +VE represents positive control and –VE represents negative control

CHAPTER FIVE

5.0 DISCUSSION

Generally, tilapia spp were more infected by *A. niger* than were infected by *A. flavus*, as it has been indicated on either of the tilapia spp cultured on different salinity levels. These finding simply showed that *A. niger* is more infectious than is *A. flavus* (Ellis *et al*., 2000). Isolated fungi from the Rufiji tilapia and hybrids of tilapia species at the fish hatchery in Tanga region were identified as *Aspergillus* and *Rhizopus* species. The occurrence and levels of *A. flavus* and *A. niger* in fish sample organs showed that there was higher prevalence of fungal isolates of *A. niger* in all tilapia species stocked at different water salinities than was for *A. flavus* (Ellis *et al*., 2000). However, Rufiji tilapia had lower prevalence of *A. niger* infection (Table 2) than was the case for *A. flavus*. This was contrary to the hybrids of female Nile tilapia and male Rufiji tilapia. Moreover, *Rhizopus* species had high prevalence of infection in Rufiji tilapia unlike the hybrids of female Nile tilapia and male Rufiji tilapia (Table 3) (Overy *et al*., 2014).

Many culture plates did not show any *Aspergillus* species colonies in water samples where Rufiji tilapias were stocked. However, from the same tank of water, *Aspergillus* species were readily isolated from samples of fish organs. The findings are similar to the observation made by Rao (2017). Furthermore, the intensive management of all water quality parameters unlike water clearness that was administered on weekly basis during the study period probably prohibited fungal sporulation in water. It has been reported that siphoning removes all fungal matrixes that are a favourable medium for fungal sporulation (Overy *et al.*, 2014).

The fungi isolated from Rufiji tilapias, which were stocked at different salinity levels, indicate that there were no significant differences across fungi found in Rufiji tilapia at different water salinity levels (Appendix 1). This finding implies that there was no significant difference between the fungal isolates at different water salinity levels, unlike fish organs (skin, gastrointestinal tract and gills) which had a significant difference at an increase in salinity (Table 4) which showed a decline in number of isolates from one sample organ to another. This is because the sampled organs have a good defence line that starts with tissue mechanisms, which provide the initial barriers against diverse pathogenic agents. These barriers are protected by a constant mucus secretion, which functions as a mechanical dynamic gate that also helps to maintain the inner stability. The mucus also contains biologically active substances such as antibodies lysozome, a complementary system, or C-reactive protein and hence reducing significantly the number of fungal isolates (Rodríguez *et al*., 2010).

The *Rhizopus* species are ubiquitous, in nature were not found, however, in the hybrids were isolates (Table 5). The occurrence of fungal isolates at different water salinity levels was not statistically different when compared with fungal isolates from the hybrids of female Nile tilapia and male Rufiji tilapia. The fungi isolated from low salinity water and the fungi isolated from high water salinities produced similar results. This implies that isolated fungi can survive in high salinity, freshwater and in brackish water (Chukanhom and Hatai, 2004). However, the fungal composition of *Aspergillus flavus* varied significantly from *Aspergillus niger* isolated from salinity water levels in which hybrids of female Nile tilapia and male Rufiji tilapia were stocked. Similarly, the numbers of fungal isolates from water salinities were significantly different.

The study focused on the possible pathogenic fungal infections causing diseases in tilapia species in the hatchery. It was observed that *A. niger* were relatively high in the hybrids of female Nile tilapia and male Rufiji tilapia in the hatchery than was the case with *A. flavus.* The *A. flavus* showed a declining trend in gills but an increasing trend in the skin. Therefore, in the hybrids, there is no significant difference across the fish organs as the hybrids are less tolerant against some fungal infections such as *A. flavus,* as opposed to *A. niger* that appeared to be abundant in the hybrids of female Nile tilapia and male Rufiji tilapia.

The population of fungi (i.e. *A. flavus* and *A. niger*) at different salinities had no statistical significant difference (Table 6) since the vegetative growth of fungi does not solely require the presence of seawater. This confirmed that the abundance of fungi in Rufiji tilapia stocked at different salinity water levels were not favoured by high concentration of salt content except for *Aspergillus flavus,* which is a permanent inhabitant in marine water (Overy *et al*., 2014).

The *Aspergillus* spp isolated from fish organs showed a clear decrease with an increase in salinity from 2, 15, 25 and 35 ppt respectively. Moreover, fish organ (gills, skin, and gastrointestinal tract) demonstrated a reduced number of fungal isolates in the gills and skin at an increase in salinity levels. Therefore, findings in Table 7 show that there is no significant difference across the fungal isolates found in fish organs at different salinity levels. Fungal isolates were found at high prevalence in gills and skin than in gastrointestinal track of fish, particularly the *A. niger*.

However, *A. flavus* colonizes the skin through the influence of environmental temperature, PH, and oxygen. *Aspergillus niger* that *naturally* inhabits leguminous plants, groundnuts,

and maize (Susca *et al*., 2007) were found to infect fish through feeding and sporulating in the gastrointestinal tract. These findings are in agreement with the findings previously reported whereby the majority of fungal isolates were found in fish organs than in water (Susca *et al*., 2007). The findings suggest that the water quality parameters, especially pH value, temperature, and oxygen were suitable for the distribution and multiplication of the fungi. Generally, fungal infestation occurs when fish are immunocompromised or when water quality changes (Pham *et al*., 2015).

The difference between morphological and physiological tests and Polymerase chain reaction (PCR) method during isolation is the application of culture media (SDA) (Olufemi, 1985). This always prolongs the process of identification for a specific fungal spp whereby PCR method uses primers of specific genes for rapid detection and confirmation of genes that carryout strains of specific target genes (aflD, aflM and NIG 1, Tannase) (Nagur *et al*., 2014; Kamal *et al*., 2016).

Regarding PCR detection method *A. flavus* and *A.niger* were confirmed using primers of specific genes that guarantee high sensitivity than morphological and physiological tests (Dao *et al*., 2005). In this case, all samples were analysed by morphological and physiological tests to obtain pure culture of *Aspergillus* species, then a pair of designed specific primers on 400 bp and 895 bp (Fig. 7 and Fig. 8) was amplified and specific gene target of aflD and aflM *for Aspergillus flavus* were used for detection. Similarly, the set of primers NIG1 and Tannase were used to detect specifically *A. niger* by PCR method using a product of 290 bp (Fig. 9) to amplify it on genomic DNA. In the present study, Tannase a target gene during amplification did not successfully detect a specific gene for *A. niger*.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

There is no statistical significant difference in the hybrids of female Nile tilapia and male Rufiji tilapia across water salinities; therefore, the increase in salinities has no effect on *Aspergillus* species in the organs of Rufiji tilapia. Similarly, it was observed that an increase in salinity does not influence an increase of either *A. flavus* or *A.niger*.in the hybrids of female Nile tilapia and male Rufiji tilapia.

There is an exponential increase of *A. niger* on the fish gills but a decrease of fungal spores on the fish skin as the salinity of water increases. Therefore, an increase of salinity of water influences the growth of *A. niger* on the gills and not on the skin. In addition, an increase in salinity has no influence on the growth of *A. flavus* found in gills, skin, and gastrointestinal tract of Rufiji tilapia. There was a similar observation in the hybrids where an increase of salinity does not contribute to the increase in *Aspergillus* species in the gills, gastrointestinal tract, and the skin.

There is a statistical significant difference ($P< 0.009$ and $P< 0.008$) in the sample organs (GIT and skin), which were collected from fish samples stocked at different salinity water levels. Thus, the salinity water differences have positive effect on the increase of *Aspergillus* species in farmed Rufiji tilapia. This means the hybrids have different information based on the increase of salinities that does not attribute to the increase of *Aspergillus* species in the gills and skin since there is no statistical significant difference between fish organs and fungal isolates.

Aspergillus species are not present in different salinity water levels; which indicates that the water medium does not support proliferation of pathogenic fungi in which Rufiji tilapia were stocked. In addition, the water in which hybrids were stocked has fewer fungal isolates; this means that salinity increase does not favour an increase of fungal multiplication. Morphological and physiological tests are time-consuming and often, mycological expertise is necessary. In the present studies, PCR-based methods provide more specification, sensitivity, and rapid detection of the target organism.

6.2 Recommendations

i. Enough water samples should be collected to increase the sample size for statistical test, hence it increases the number of isolates of fungal species.

ii. It is recommended that different salinities of water (15, 25 and 35) are potential for mariculture since the suggested salinities cannot support the existence of *A. niger* unlike *A. flavus,* which showed significant difference among the fish organs at different salinities.

iii. The identification of fungi by PCR method is more challenging: therefore, using molecular techniques such as PCR method cannot identify and detect fungal specific strain; however, PCR method deemed to operate to species level.

iv. With the existing challenges associated with marine and terrestrial fungi, *A. flavus* is referred to as fungi that inhabits either in marine or terrestrial environment, which is the most pathogenic in aquaculture, thus, it is worth to understand which fungal strain is toxigenic to fish and human being so that precautionary approaches have to be outlined to avoid the infections.

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APPENDICES

Appendix 1: Logistic regression analysis for fungal isolates from different salinity levels (Rufiji tilapia)

```
. logistic an i.salinity if org==1

Logistic regression                             Number of obs     =         41
                                                LR chi2(3)        =       1.88
                                                Prob > chi2       =     0.5975
Log likelihood =  -27.46641                     Pseudo R2         =     0.0331
```

an	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
salinity						
15	1.125	1.125	0.12	0.906	.1584714	7.986455
25	1.5	1.299038	0.47	0.640	.2747446	8.189425
35	3	2.66927	1.23	0.217	.5245165	17.15866
_cons	.6666667	.4303315	-0.63	0.530	.1881311	2.362419

Note: _cons estimates baseline odds.

Appendix 2: Logistic regression analysis for fungal isolates between different salinity levels and fish organs (Rufiji tilapia)

```
. logit an i.sal i.org if sp==1

Iteration 0:   log likelihood = -85.522084
Iteration 1:   log likelihood = -80.605387
Iteration 2:   log likelihood = -80.566804
Iteration 3:   log likelihood = -80.566798

Logistic regression                             Number of obs     =        134
                                                LR chi2(5)        =       9.91
                                                Prob > chi2       =     0.0778
Log likelihood = -80.566798                     Pseudo R2         =     0.0579
```

an	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
sal						
15	.0471071	.5391947	0.09	0.930	-1.009695	1.103909
25	-.417287	.5251938	-0.79	0.427	-1.446648	.612074
35	-.5296603	.5350277	-0.99	0.322	-1.578295	.5189748
org						
GL	-1.076052	.4594604	-2.34	0.019	-1.976578	-.1755259
MS	-1.256088	.4719396	-2.66	0.008	-2.181072	-.3311031
_cons	.3189848	.4541772	0.70	0.482	-.5711862	1.209156

Appendix 3: Logistic regression analysis for fungal isolates between different salinity levels and fish organs (hybrids of Nile tilapia)

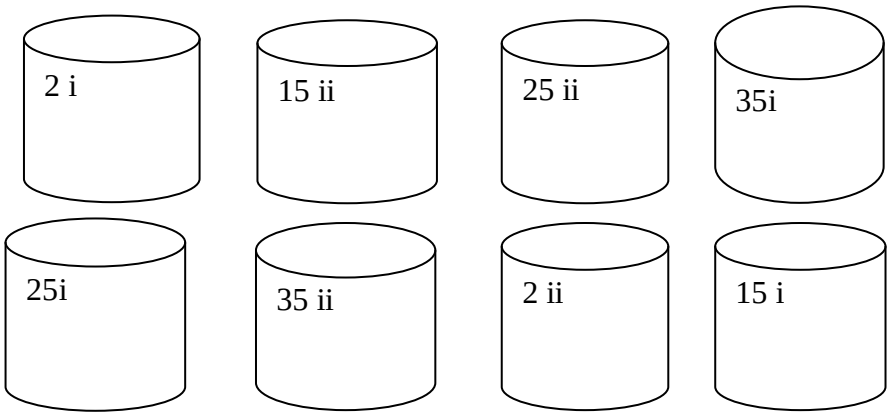
```
. logit an i.sal i.org if sp==2

Iteration 0:   log likelihood = -84.069231
Iteration 1:   log likelihood = -81.442187
Iteration 2:   log likelihood = -81.417328
Iteration 3:   log likelihood = -81.417318
Iteration 4:   log likelihood = -81.417318

Logistic regression                             Number of obs     =        136
                                                LR chi2(5)        =       5.30
                                                Prob > chi2       =     0.3799
Log likelihood = -81.417318                     Pseudo R2         =     0.0315
```

an	Coef.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
sal						
15	-.6559973	.518012	-1.27	0.205	-1.671282	.3592876
25	-.0843829	.4922667	-0.17	0.864	-1.049208	.8804422
35	-.88125	.5750424	-1.53	0.125	-2.008312	.2458125
org						
GL	.0679616	.4507328	0.15	0.880	-.8154585	.9513818
MS	-.4678049	.4690483	-1.00	0.319	-1.387123	.4515128
_cons	-.3236693	.4227595	-0.77	0.444	-1.152263	.504924

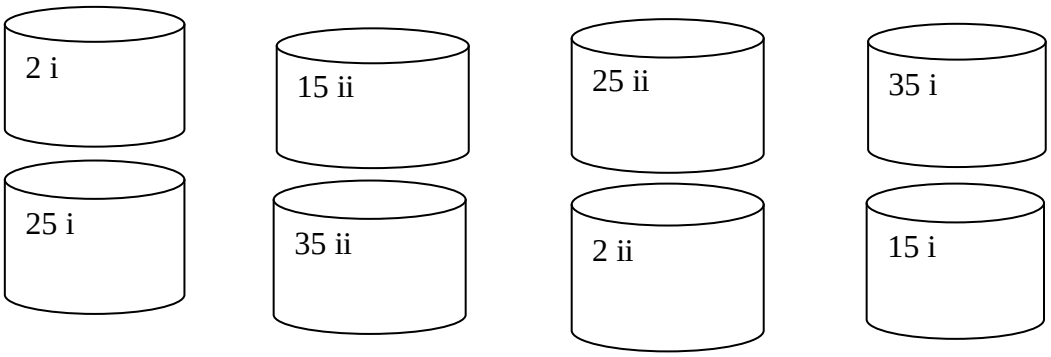
Appendix 4: Experimental set up for Rufiji tilapia stocked at different salinity levels at the IMS-MC Pangani, Tanga



Description for the experimental set up

Out of 8 tanks two replicates were used for each salinity levels. Stocking of fish was conducted through randomization process. Randomization involved catching and stocking the same number of fish serially**.**

Appendix 5: Experimental set up for hybrids of Nile tilapia ♀ and Rufiji tilapia ♂



Description for the experimental set up

Out of 8 tanks two replicates were used for each salinity levels. Stocking of fish was conducted through randomization process. Randomization involved catching and stocking the same number of fish serially**.**