UNIVERSITY OF GHANA
COLLEGE OF BASIC AND APPLIED SCIENCES
SCHOOL OF BIOLOGICAL SCIENCES

INVESTIGATING PATHOLOGICAL AGENTS ASSOCIATED WITH THE RUSTY-BROWN SPOTS ON FARMED NILE TILAPIA (*Oreochromis niloticus* Linnaeus, 1758) IN GHANA

BY

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A THESIS SUBMITTED TO SCHOOL OF GRADUATE STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF MASTER OF PHILOSOPHY IN FISHERIES SCIENCE

DEPARTMENT OF MARINE AND FISHERIES SCIENCES

JULY, 2019

DECLARATION

This dissertation is the result of research work undertaken by Rose Ewura Adwoa Deho in the Department of Marine and Fisheries Sciences, University of Ghana under the supervision of Dr. Samuel Addo, Dr. Samuel Duodu, and Dr. Michael Wiafe-Kwagyan. I do hereby declare that the dissertation consists entirely of my own work and that no part of it has been previously published or submitted for a degree or diploma elsewhere.

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ABSTRACT

The outbreak of disease in fish farming has become a worldwide problem. The aquaculture industry in Ghana has seen a major rise in the rate of fish mortality in recent times with significant economic losses. New and emerging diseases maybe associated with these mortalities including the rusty brown spot recorded on cultured *Oreochromis niloticus* (Nile tilapia). However, there is no much information on the pathological agents associated with the condition. The rusty brown spots have been observed in tilapia cultured in earthen ponds and concrete ponds. This work looks at isolating and identifying the possible organisms that are likely to be the cause of the rusty brown spots. Preliminary culture of the fish skin on Tryptone soy agar (TSA) and Tryptic Yeast Extract salt agar (TYES) recorded the following bacteria species *Psuedomonas aureginosa, Klebsiella pneumoniae, Staphylococcus aureus, Staphylococcus chonii and Flavobactrium.* Data obtained shown that *Aspergillus niger* and *Flavobacterium* spp. were the primary pathogens associated with the rusty brown spots. However, molecular identification is yet t be done to determine the exact strain of *Flavobacterium* and *Aspergillus niger* and to confirm their virulence in a challenge infection experiment.

DEDICATION

To my husband Jewel for encourage ng me, my children Wilford and Alyson for letting me believe I was the best, and to my Mum and other family members, I am thankful for the support.

ACKNOWLEDGEMENTS

My sincere appreciation to my father in Heaven for the gift of life. I also appreciate a number of people whose immense contribution helped me finish this research.

My gratitude to Dr. Samuel Addo of the Department of Marine and Fisheries Science, University of Ghana, Legon, Dr. Samuel Duodu of the Department of Biochemistry, Cell and Molecular Biology and Dr. Michael Wiafe-Kwagyan of the Department of Plant and Environmental Biology who held my hands and taught me all I needed to know in their areas of specialty.

I am thankful to Professor Newman of the Department of Microbiology, School of Medicine and Dentistry, Korle-Bu. Mr George Akwetey of the Department of Plant and Environmental Biology, Mr. Amos Akumwena of the Department of Microbiology, Korle-Bu, who all took time to look at my work and guided me.

I also want to thank Mr. Kofi Anyan, Mr. Michael Kwame-Biney, Miss Genevieve Nuamah, and all staff of the Departments of Marine and Fisheries Science, Plant and Environmental Biology and Microbiology for the immense support and encouragement. I am also indebted to the Ashaiman Aquaculture Demonstration Centre for providing me with the fish samples used in this study

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LIST OF ABBREVIATIONS

FAO	Food and Agriculture Organization
MOFAD	Ministry of Food and Agriculture
ACDC	Aquaculture Development Centre
TYES	Tryptone Yeast Extract Salt
TSA	Trypticase Soy Agar
SDA	Sabouraud Dextrose Agar
PDA	Potatoes Dextrose Agar
AgGDP	Agriculture Gross Domestic Product
GDP	Gross Domestic Product
GSS	Ghana Statistical Service
PAC	Pilot Aquaculture Centre
LCD	Lethargic Crab Disease
EUS	Epizootic Ulcerative Syndrome
PCR	Polymerase Chain Reaction
WHO	World Health Organisation

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CHAPTER ONE

INTRODUCTION

1.1 Background

Tilapia production has gained increased importance in aquaculture throughout the world and is second only to carp by production volume estimates (FAO, 2012; Addo *et al.*, 2017a). Of the several commercially cultured species of tilapia, Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758) is the most abundant and important species. Global aquaculture production of tilapia has increased from around 200 000 metric tonnes in 1990 (FAO, 2012) to about 110.2 million metric tonnes in 2016 with 80.0 million metric tonnes used directly for human consumption (FAO, 2018). Aquaculture value as at 2016 was at USD 232 billion, and per capita consumption as of 2017 was estimated at 20.5 kg.

The fisheries sector in Ghana accounts for about 6.1% of Agricultural Gross Domestic Product (Ag GDP) and 1.1% of GDP (GSS, 2017). Total fish production in 2016 was 465,356 metric tonnes, 666,975 tonnes below the national fish requirement of 1,132,332 metric tonnes in the same year (FAO, 2018). This shows that only 41% of the annual fish requirement was met from local production. Fish is also an affordable source of animal protein, contributing to 60% of the total protein intake in the diet of the average Ghanaian (FAO, 2016). Thus, fish and fish products play a critical role in national food security and economy. However, Ghana is not self-sufficient in fish production and prospects for capture fisheries continue to decline. The high per capita fish consumption of 19.8 kg the deficit in fish supply, and high demand for Nile tilapia have given credence to a growing aquaculture industry (Rurangwa *et al,* 2015). Aquaculture production has seen a gradual

increase from 720 metric tonnes in the late 1990's to about 52,470 metric tonnes in 2016 (FAO, 2016; MOFAD, 2017) with the Nile tilapia representing over 80% of farmed fish production in Ghana (MOFAD, 2017). However, Verner-Jeffrey *et al.* (2017) attributed the low volume of tilapia production in the country in recent years to high mortality rates due to diseases.

The global perspective on fish culture is towards increased intensification and commercialization (Goncalves *et al.*, 2011), but disease remains a major inhibition to the growth of the aquaculture industry and severely hamper both economic and socio-economic progress in producer countries (Austin & Austin, 2007; Addo *et al.*, 2017b). With the rapid growth of the aquaculture industry, there has been a rise in conditions such as water pollution, disease outbreaks and the need for improved welfare of the fishes that are farmed (UNEP, 2010, FAOSTAT, 2013, Ghose, 2014). Fish diseases occur from variable sources. It is not a simple relationship between host, pathogen, and environment (Salah 2013).

Diseases can be categorized into infectious and noninfectious diseases. Infectious diseases are related to pathogens present in the aquatic environment and therefore there is a need for its control and treatment (Morse, 2001). Non-infectious diseases are associated with biotic and abiotic factors which can be dealt with by employing best management practices (Campos-Herrera *et al*, 2012).

Disease caused by microbial pathogens (bacteria, viruses, fungi) and parasites is responsible for mass deaths and annual losses. In Ghana, the most common pathogens that have been isolated from farmed tilapia include bacteria genera such as *Streptococcus,*

Pseudococcus, *Flavobacterium*, *Edwardsiella*; fungi such as *Saprolegnia* and parasites like *Trichodina, Chilodonela, Argulus, Ergasilus* and leeches among others (Austin & Newaj-Fyzul, 2017) . These have led to massive loss of revenue for some fish farmers and the collapse of some small-scale fish farms (personal communication with President of GAA). Industrywide losses to diseases of aquatic organisms are in excess of 6 billion dollars annually which loss is comparable to loses of livestock due to disease such as foot and mouth (World Bank, 2014).

1.2 Justification and Problem statement

In recent times, there have been reports of mass mortality of tilapia in Ghanaian fish farms across the country. The Ghana Fisheries Commission, National Disaster Management Organization, reported that over 100 tonnes of tilapia from various farms in the Eastern region have died. These included Amur fisheries at Asikuma, Tropo farms at Mpakadan, and West African fish farms on the Volta River among others **(**Fisheries commission, 2018; myjoyonline.com, 2018)

In October 2018, Fujian farms in the Shai Osudoku District of the Greater Accra Region lost over 18 tonnes of tilapia (graphic.com.gh, 2018). These deaths could be attributed to poor water quality or could be as a result of microbial infections. According to El-Deen et al. (2018), mass mortality of fish are usually due to infections.

In recent times, rusty brown spots have been reported on cultured Nile tilapia in Ghana. These spots present a problem because it reduces the fishe's appeal to the market, which then affects its market value thus causing loss of income to farmers. Since the aetiology of

the rusty brown spots found on cultured Nile tilapia in Ghana is not yet known, it was anticipated that this study will give insights to the causes and conditions under which these infections are formed. Findings from this present study will inform farmers on the best management practices to employ.

The presence of rusty brown spots on some pond cultured Nile tilapia has been reported by some farms in Ghana, however, there is no available scientific information on the cause of the spots in the country. Some scanty technical information by the Fisheries Commission have attributed it to the bacteria in the sediments of ponds where they are cultured. There has however, been observations of these brown spots on some tilapias also cultured in concrete tanks where there are no sediments. There have been undocumented reports from the Aquaculture Research and Development Centre (ARDEC), Akosombo, Aquaculture Demonstration Centre (ACDC), Ashaiman, Opoku Ginaye Farms, Kumasi .and the Pilot Aquaculture Centre (PAC) Kona-Odumase near Kumasi. Athough these rusty brown patches have been observed on cultured tilapia, there has not been any known published scientific information on its aetiology. According to reports from the ACDC, Ashaiman, the spots are only found on juveniles to adults. Undocumented reports further indicate that these spots are observed when fingerlings are transferred from one culture facility to another and on adults during harvest (personal communication with Joyce Lutterodt, 2017). Although in all observations and reports from farmers, these rusty brown patches have not been associated with any mortalities, it reduces the aesthetic value of the fish and do cause financial loss to the farmer as consumers will not buy such fish (Oladosu *et al.*, 1990).

1.3 Objective of Study

The main aim of this study was to investigate the pathogenic agents associated with the rusty brown infection on farmed Nile tilapia at the Aquaculture Demonstration Centre, Ashaiman as a prelude to ascertain the primary agent responsible for the condition.

The specific objectives were to isolate and characterise the:

1. Bacteriological agents associated with the rusty brown patches on infected fish.
2. Mycological agents associated with the rusty brown patches on infected fish.
3. Bacteria and fungi present in water and sediment from ponds with infected fish.

CHAPTER TWO

LITERATURE REVIEW

2.1 Aquaculture and Food Security

Aquaculture, which is the culturing of aquatic organisms for human and animal consumption, has become a driving force in with issues of global food security and nutrition. In a world of the growing demand for fish and declining wild stock, the contribution of aquaculture has been significant to world food security. Fish farming is regarded worldwide as the major way to supplement the declining wild fish catch (Naylor *et al,* 2000). Fish data indicates an increase in aquaculture production from about 57 million tonnes to about 106 million tonnes in the time period between 2005 and 2015 (Kobayashi *et al,* 2015). This can be deduced as about one hundred percent increase in farmed fish production in a 10-year period. Also, FAO's data on Global Aquaculture Output Production indicates a total of about 110 million tonnes in 2016. Nile tilapia which is the most cultured tilapia species in the world has a magnitude of 7% of the total global aquaculture production (FAO, 2018). This increment is as a result of some major breakthroughs in aquaculture production systems and species genetic modification, among others.

In Ghana, aquaculture production has grown since its introduction in the 1950s. Data organized by FAO indicates an increase in Aquaculture Production from 1,150 metric tonnes to 52,480 metric tonnes from 2005 to 2016 in Ghana. This represents about 98% increment within a 10-year period. However, in Ghana, the Nile Tilapia is the most propagated fish and accounts for 80% of total aquaculture production. In Eastern Africa,

in landlocked Uganda, its fish production has seen a rise sfrom 32,000 tonnes in 1997 to 51,000 tonnes in 2007, predominantly from the production of tilapia, *Oreochromis niloticus*, and catfish species (Akoll *et al.* 2012). Generally, in developing countries in Africa such as Ghana, Nigeria among others, most of the farmed fish come from small scale farmers. A greater percentage of fish products come from small-scale producers in developing countries and more than 80% of global aquaculture products are produced in fresh water (Frimpong & Adwani, 2015). The population of Ghana is increasing rapidly, and there is a corresponding demand for fish. A study conducted by (Hiheglo, 2008) stated that the country's demand for fish far outweighs the country's ability to supply creating a greater demand and supply gap year in year out.

However, fish is a delicacy in Ghana and a main source of protein. Ghana's per capita consumption of fish is 26kg higher than the 20kg world average and 10kg average for Africa (FAO, 2014, 2016). Moreover, the comparatively cheaper price of fish to that of other animal proteins means that the yearly short-fall in fish production will affect the protein intake of the poor and vulnerable in the society in the not too distant future if nothing is done about it (Hiheglo, 2008). There is, therefore, the need to intensify research into the limiting factors that stagnate efforts of exploring aquaculture in Ghana. Since the fish disease is an indispensable factor in fish farming, there is a need for intensive research into the identification and cause of disease in both cultured and wild stock (Walker & Winton, 2010).

2.2 Nile Tilapia

Nile tilapia, *Oreochromis niloticus* is a surface feeding omnivore fish belonging to the family Cichlidae. The Nile tilapia, *Oreochromis niloticus (Linnaeus, 1758)*, is one of the

first fish species to be cultured in the world. *Oreochromis niloticus*, is freshwater tropical and subtropical species. These filter feeders feed on both phytoplankton and zooplankton.

2.3 Fish Disease

A disease is a disorder of structure or function in a human, animal, or plant, especially one that produces specific symptoms or that affects a specific location and is not simply a direct result of physical injury, (Oxford English Dictionary). In the bid to provide food for a growing human population and produce supplementary food for animal farming, Aquaculture is in the spotlight in achieving these global goals of sustainable food production (Bizikova *et al*., 2013; Hixson, 2014). As a result, highly extensive production methods have been employed to meet this need.

Fish disease is a major problem in aquaculture as it is a determinate of the survival of cultured fish. It has been demonstrated that farmed fish are more inclined to pathogenic microbe infection in intensive farming. This basically is due to overcrowding in farms. The state of skin is an indication of the health state of the fish since the pathogens readily attack the skin. This is not only due to contamination of the pond but may also be due to the invasion of pathogenic microorganisms (Mahmoud *et al*., 2014). The microbial biota of fresh fish is usually a reflection of the environment in which it was harvested. Ponds and rivers may be the source of the microorganisms on the fish due to pollution of the water bodies by the dumping of excrement and runoffs during rainfalls (Laibu 2015; Emikpe 2011).

Disease control plays a major role in determining the economic viability of any aquaculture production (Godfray, 2010). There is, therefore, the need for proper health management in

fish propagation. The state of health of fish depends on the relationship between fish (host), environment and pathogenic parasite, (Plumb and Hanson, 2010). The host's inherent resistance and physiological and immunological status play an important role when exposed to pathogens. (Hooper *et al*., 2012). The host's vulnerability is influenced by factors such as species susceptibility, strain susceptibility, age of the host, nutritional status, stress, sexual maturation, an already existing infection and other co-factors. (St-Hilaire *et al.*, 1998). The health of fish depends on the interrelationship of six major components of the fish and the environment, where it lives (Plumb and Hanson, 2011). These factors include the physiological state of fish, nutrition availability, pathogenic parasites awareness and control, and basic husbandry.

The high stocking densities in intensive fish farming give rise to stress related problems that predispose them to diseases (Mohamed *et al.*, 2017).

There are a variety of factors that can contribute to disease breakout in fish (Hedrick,1998). Schäperclaus (1992) indicated that the cause of fish disease is rarely considered as a single factor but comprises the type of parasite, the physiological condition of fish and the environment. All these factors together play a role in the manifestation of the fish disease. Environmental factors causing fish disease in aquaculture basically include water quality and high stocking density (Boyed & Tucker, 2012). Infections are however difficult to identify and deal with due to lack of proper local structures and personnel to help rural fish farmers to deal with the adversity due to fish diseases. A study in Bangladesh, (Faruk *et al.*, 2004) stated that, most rural fish growers have limited understanding of the signs of diseases, and hence do not report the incidences of diseases. This makes room for the economic impact of diseases to be down played. To mitigate the full impact of disease

outbreaks, strategic and an all-inclusive management system should be set up (Subasinghe *et al.,* 2001; Bondad-Reantaso *et al.,* 2005; Akoll *et al.,* 2012). A study by (Faruk *et al.,* 2004) indicated that small size farms suffered from highest average loss (19.6%) than the bigger size farms (14% for medium and 11.2% for large farm).

Microbes that typically are not fatal but have the probability to become virulent to immune-compromised hosts are called opportunistic pathogens (Martinez, 2013).

2.4 Infections and Cost

Infectious diseases are those that are caused by bacteria, viruses or fungi. Cultured fish are faced with various infectious diseases. Globally, some diseases faced by fish include streptococcal infections, flavobacteriosis, myxobacterial skin lesions, renibacteriosis, mycobacteriosis (fish tuberculosis), gill rot, pseudomoniasis, aeromonasis, columnaris, and infection with intracellular bacteria, peduncle disease, white spot, saddleback, saprolegniosis, and rusty brown spot. In order to improve health conditions in the rearing of aquatic organisms, several alternatives such as improved husbandry, nutrition, and water quality; optimal stocking density; and use of vaccines, probiotics and immunostimulants have been proposed (Soosean *et al.,* 2010).

These aforementioned elements in fish health management demands for more technical know-how, continual research and sustainable management of both culturing and water holding units and the species involved. Notwithstanding the fact that aquaculture is the fastest growing food-production industry in the world, the sector is plagued by diseases.

The annual economic loss to the aquaculture industry through diseases is estimated to be billions of US dollars worldwide (Subasinghe, 2001). For example, Thailand lost $650

million to the white spot disease in the Penaeid shrimp. In Brazil, lethargic crab disease (LCD), has been reported to cause capacious population reduction in the mangrove land crab (*Ucides cordatus*) (Boeger *et al*., 2005). Many countries have therefore set up research funds to deal with the outbreak of diseases. For instance Norway allocated $50 million dollars in animal health research while China invested $6 million in the same research (Bondad-Reantaso *et al*., 2005).

2.5 Bacteria infections in fish

As the culturing of organisms is the paramount root of food security, their propagation cannot be without pathogenic bacteria and aquaculture is therefore not exempted. Bacterial diseases ia a mojor challenge to the agriculture industry globally. Some bacterial diseases include vibriosis, columnaris, aeromonasis, streptococcosis, edwardsiellosis, mycobacteriosis, renibacteriosis and flavobacteriosis among others.

The causal bacteria can be categorized into gram-positive and gram-negative bacteria. Gram-positive bacteria are those that retain the crystal violet dye and do so because they possess a thicker layer of peptidoglycan than gram-negative bacteria which do not retain the violet dye and are colored red or pink. Furthermore, studies have shown that the gram-negative bacteria are more resistant to antibiotics relative to the gram-positive (Azam et.al., 2012). *Columnaris, Aeromonas, Edwardsiella*, *Flavobacteria*, *Pseudomonas*, and *Vibriosis* are some gram-negative bacteria. Streptococcosis and mycobacteriosis are caused by gram-positive bacteria (Brown *et al*.,2015).

Some bacteria prevalent in warm water systems include *Streptococcus agalactiae, Streptococcus iniae, Mycobacterium fortuitum, Mycobacterium marinum, Vibrio*

vulnificus, and *Edwardsiella tarda* (Haenan, 2017). Columnaris disease has been fund to cause disease that affects the skin and gills of freshwater fish and is most commonly caused by *Flavobacterium columnare*. (Rattanachaikunsopon *et al.*, 2009).

Some infections cause fish to lose some of their protective scales. Columnaris is a well-known warm water disease. The disease can be chronic in which a small number of fish are affected with low mortalities. It can also be acute with explosive mortality reaching as high as 90%. No fish age group is totally immune as it can affect fish of all ages (Plumb, 1997). Dong (2015) reported that there are two strains of *F. columnar*. Under the microscope, the pathogenic strain has rhizoid morphology and the nonpathogenic one has no rhizoid morphology. The nonpathogenic strain is opportunistic pathogens and causes less mortalities of 0-20%.

Streptococcal infection is a major bacterial disease in mostly older fish that develops due to the infection of Streptococcus species. It has been reported to be found in various fish including trout and tilapia (Yanong & Floyed 2002). The disease which is a global fish disease is found in tropical, sub-tropical and temperate waters. The economic and health impacts of warm-water streptococcosis are especially noticeable in Mediterranean countries. The main pathogenic species that causes *Streptococcal* infections are (*Streptococcus parauberis, Streptococcus iniae, Streptococcus difficilis, Lactococcus garvieae, Lactococcus piscium, Vagococcus salmoninarum,* and *Carnobacterium piscicola*) (Bercovier *et al*., 1997; Eldar *et al.*, 1997,1999; Mata, 2004). *Streptococcus* causes more than 50% mortality in fish between 3-7 days after. However, some outbreaks are more chronic and some fish may not show any signs of infection. The outbreak of

streptococcus infection in fish ponds is stress related. Overstocking, poor handling and harvesting are some of the stressful situations (Southgate, 2010).

Pseudomonas infection has been named as the most common bacterial infection of fish and is seen to be a stress-related (Abowei & Briyai, 2011). It is considered as one of the most pathogenic bacteria affecting fishes such as *O. niloticus* and *O. mosambicus* (Thomas *et al*., 2014). The causative agents of pseudomonas septicemia infection has been noted to be *Pseudomonas fluorescens*, *Pseudomonas angulliseptica*, *Pseudomonas aeruginosa*, and *Pseudomonas putida* (Thomas *et al.*, 2014). The disease is expressed by petechial hemorrhage, darkned skin, detached scales, ascites on the abdomen and exophthalmia (Austin and Austin, 2007). Among the agents of fish diseases caused by bacteria, *Pseudomonas* and *Aeromonas* are known to be the most important fish pathogens. According to Paniagua *et al*. (1990), these microbes are responsible for diseases such as hemorrhagic septicemia, tail and fin rot, bacterial gill rot among others well known symptoms.

2.6 Fungi infections in Fish

Every fish in freash water is likely exposed to at least one species of fungus during its lifetime (Klinger & Floyed 1998). Diseases caused by fungi are the second most serious cause of losses in aquaculture (Meyer, 1991; Iqbal and Saleemi, 2013). Fungi have been found in all the life stages of fish (Hansen and Olafsen, 1999). Disease caused by fungi is a source of worry for cultured fish (Davenport *et al.*, 2009). Their spores are found in all fish ponds and are especially predominant in ponds with poor water quality which leads to increased infection of fungi (Daszak *et al*., 1999). In many cases, these fungi are ubiquitous

in the water but may become infective in injured fish and those that are immunocompromised (Noga, 1993; Pang *et al*., 2004). Gozlan *et. al*. (2014) stated that it is difficult to detect fungal infections in freshwater fishes although they have been found to be responsible for mass population loss, some of these diseases are chronic with no external symptoms for example, the rosette agent *Sphareothecum destruens*, which has been rapidly spreading all over Europe via an invasive healthy fish host carrier.

Several fungi infecting fish are opportunistic pathogens but increased susceptibility to fungal diseases occur secondary to viral and bacterial infection or due to loss of mucus from excessive handling (Mohamed *et al.*, 2017). Fungus has been reported to cause serious diseases in estuarine and freshwater fishes in Australia, Japan and throughout South Asia (Noga , 1993;Iqbal & Saleemi, 2013).

The oomycetes are often referred to as water molds, although the water-preferring nature which led to that name is not true of most species, which are terrestrial pathogens

The evolutionary lineage of oomycetes indicates that those that are pathogenic are capable of infecting all kinds of host ranging from algae to vertebrates, (Van West, 2006; Beaks *et al.*, 2012). Pathogenic oomycetes are usually neglected in spite of their significant economic importance. This is mainly due to inadequate study and lack of data (Dieguez-Uribeondo *et al.*, 2009). The outbreak of oomycotic diseases such as saprolegniasis and epizootic ulcerative syndrome (EUS) can result in a large scale loss in aquaculture and also threaten the ecosystem as stated by Mohammed (2014). According to Fernandez-Benitez (2008), *Saprolegnia* has been attributed to the loss of about 10% of all Salmonid hatchlings.

The most studied oomycetes in fish belong to the order *Achlya*, *Aphanomycetes*, and *Saprolegnia* (Ruthig, 2009, Steciow *et. al*, 2014). Pathogenic oomycetes of aquatic animals are called water mould, and they are said to be cosmopolitan organisms that can be found in a variety of aquatic habitats (Beakes & Thines, 2017). Water mould infections are usually found in tilapia (*O. niloticus*). They have been found to be as a result of pathogens that belong to the family Saprolegniaceae (Panchai, 2015). Water mold infections can be observed throughout the year (Panchai, 2015; Noga 1993).

However, *Saprolegnia parasitica* has been noted to be one of the strains that have caused a major upset in fish farming. Bricknell (2017) reported that it caused a 40-million-dollar loss in catfish farmed. Rucker (1944) suggested that oomycete infections of fish affect the epidermis and the epithelial cells, however, Mishra *et. al*.(2017) suggested that the infection may enter the body of the fish in areas where there is less scale. *Saprolegnia* pathogen becomes rampant during cold seasons or when the water body suffers a drop in temperature.

Apart from *Saprolegnia*, *Aspergillus* species is another fungus that is causing major concerns in the fish farm industries. Chauhan and Rekha (2014) stated that the most observed pathogenic species of the genus *Aspergillus* are *Aspergillus fumigatus, Aspergillus niger, Aspergillus sydowii,* and *A. flavus* on some freshwater fishes. *Aspergillus* species cause systemic diseases which are usually as a result of contamination through feed (Mohamed, 2017).

Fungal infection affects both temperate and tropical fish species. Fungal outbreaks among farmed fish stocks are frequently associated with poor water quality, injuries associated with handling and grading, temperature shock and spawning (Plumb and Hanson, 2010).

The problem of fungal growth in both wild and farmed fish can be prevented where there is a proper water quality monitoring or management in place (Meyer, 1991).

2.7 Co-infections

Co-infection refers to the simultaneous infection by separate pathogens. This condition occurs such that the organism has two or more infections at the same time, thus making two or more pathogenic agents active together at the same time. Kotob *et al.* (2016), stated that even though co-infections have a fundamental effect and can alter the course and the severity of different fish diseases, co-infection effects have still received restricted scrutiny in aquatic animals like fish and available data on this subject is still scarce. Some studies have shown that when two bacteria or viruses are cultured together, the interaction maybe a positive, negative or mutualistic.

A study by Hjerde *et al.* (2015) showed that two different bacteria growing in the same organism will both be affected as one will have a negative effect on the other. Most profound was the impact *Aliivibrio wodanis* had on *Moritella viscosa* growth; it is therefore likely that the relationship between the two species is of a competitive nature and that *A. wodanis* is better adapted when the two species occupy the same environment. This indicates that a fish co-infected by these pathogens are likely to thrive and manifest two different diseases.

In a pathological study (Kotob *et al.*, 2017) of Rainbow trout infected with *Myxobolus cerebralis* and *Tetracapsuloides bryosalmonae* showed hightened pathological changes which increased mortality. A severe kidney swelling and cartilage destruction coupled with displacement were seen when compared with the pathological changes in fish with single

infections with *T. bryosalmonae* or *M. cerebralis*. The study further compared the primary infection of *T. bryosalmonae* and co-infected by *M. cerebralis* showed pathological diseases but lower mortality rates.

2.8 Effect of Diseases on Fish Populations

Disease problem occurs in fish farming as well as in the wild like the case of land animal farming. A population of fish may increase or decrease based on different factors. A fish population increase may be influenced by recruitment or growth while on the other hand, it may decrease as a result of mortality. Mortality control is a major concern in aquaculture as it determines the viability of production. An infected fish is capable of transmitting infection which may wipe the entire farm population when in an acute stage. It is therefore important for the fish farmer to employ the services of a professional fish pathologist to clinically examine the health of the fish production area periodically. This is because the degree of spread of the disease in a farm site depends on three elements: the environment, the pathogenic parasite and the fish (host).

It is not ideal for the farmer to rely only on visual examination or symptoms, as this may be unreliable. Aquaculture regulatory institutions further advice that fingerlings for culture, if purchased, should be purchased from a registered or reputable hatchery, as disease or infections may be transmitted from source. Some parasitic or viral infection may not show any symptoms in an infected fish but may be transmitted from one fish to another, thereby posing a disturbing problem to the producer.

Viral infections are the most problematic since fishes that survive their infections can be carriers of the viruses and transmit them to non-infected animals, even if they present no

symptoms. The problem with the spread of diseases in fish farms is that they are usually caused by transfer of pathogens from other areas or farms (Pulkkinen *et.al.*,2009).

Some fish diseases are zoonotic. Although the infection of humans with fish pathogens is relatively unusual, it is a health risk that needs to be picked on by fish farmers, handlers and consumers (Chai, 2005).

The incidence of transmission of disease from fish to humans is dependent on some factors such as the type of organism, host susceptibility, and environmental factors (Brownstein *et. al.*,2002).

Most aetiological agents which can be transmitted from fish to humans according to Taylor *et al*. (2001) are bacterial. From their work, they identified *Aeromonas, Edwardsiella, Streptococcus iniae*, and many more as being zoonotic.

The body of fish is a haven for diseases hence it is important to put the safety of the consumer first. According to Mead *et al.* (1999) diseases caused by food were estimated to cause 76 million illnesses, 325,000 hospitalizations and about 5,000 deaths in the US yearly. Diseases from known pathogens were estimated to be about one-fifth of the cases. The Rapid Alert System for Food and Feed (RASFF) of the EU reported that the rejection of imported seafood was amounted to 15% of total products rejected in 2012 (Tahkapaa 2015). The major reason assigned for the rejection of products is the presence of bacterial pathogens (Ababouch *et al.*, 2005). Fish must be pathogens free such that aquaculture can reach its desired potential.

Apart from economic falloffs that come with diseases in the fishery sector, it could lead to a long term biological damage such as a reduction in fish growth, decrease nutritional values and also become zoonotic.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

The study was carried out at the Aquaculture Demonstration Center that is located at Ashiaman in the Greater Accra Region of Ghana.

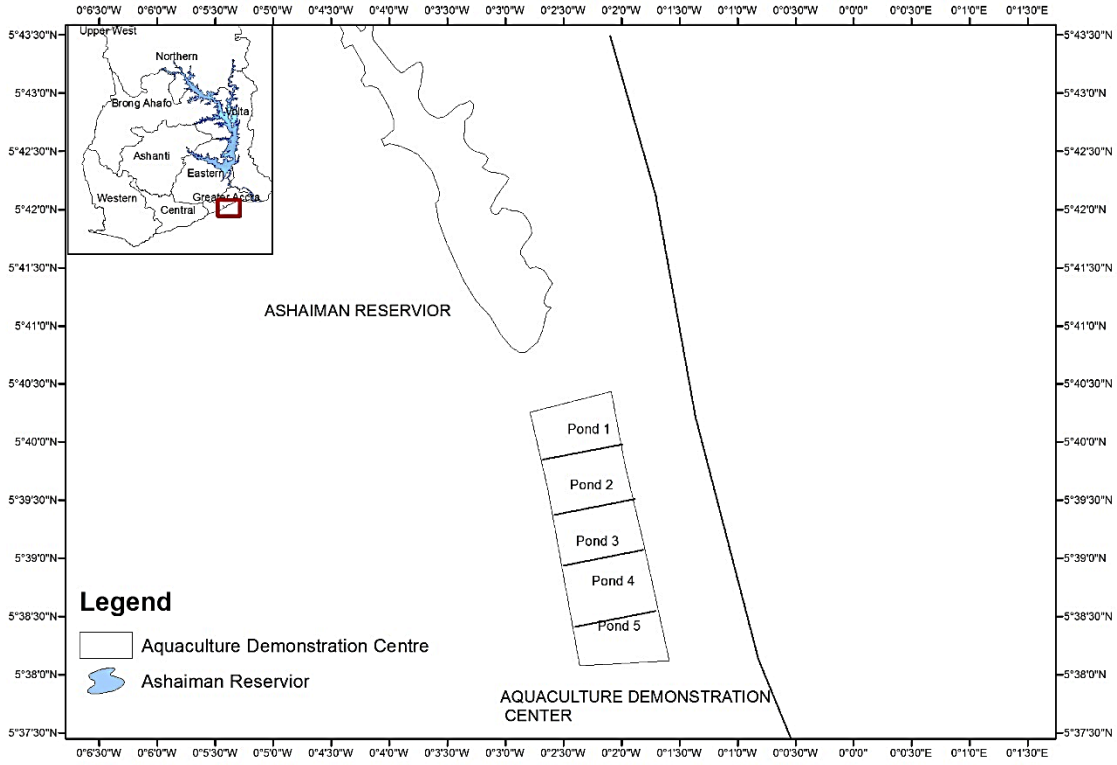

Plate 1. A map showing the location of ACD

It was set up 44 years ago under the auspices of the fisheries commission of Ghana. Its core mandate is to provide support for the growing aquaculture industry in Ghana by providing quality fingerlings to farmers at affordable prices. Their activities include the production of fingerlings of catfish (*Clarias gariepinus*) and the Nile tilapia, (*Oreochromis niloticus*). The facility is sited close to the Ashaiman irrigation dam which is its main source of water.

They have sixteen tanks and five earthen grow out ponds in which broodstock are grown. Also, on site is a hatchery from where the eggs are hatched.

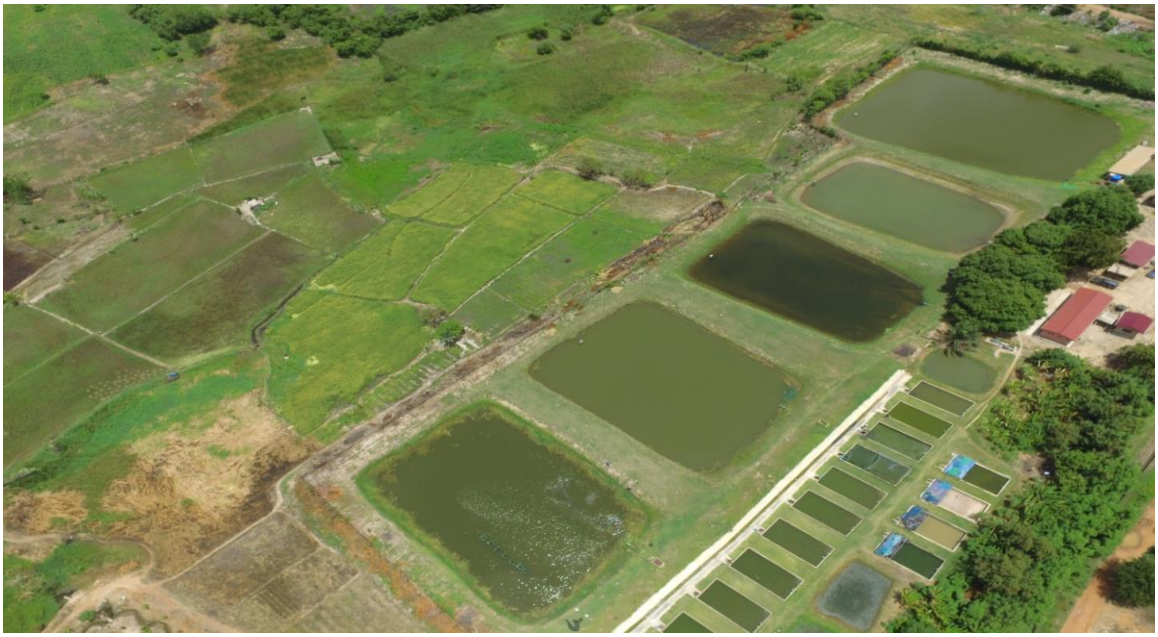

Plate 2: Aerial view of the earthen ponds at Ashaiman ACDC.

Credit: M.K Biney

3.2 Sampling

All the fish samples for this investigation were collected from earthen ponds that had tilapia at various stages of growth. Samples were collected randomly with a cast net and placed into sterile Ziploc bags and immediately placed on ice. Live sample were transported to the laboratory in polyethen bags with aerated water. Sampling was done from December 2018 to February 2019.

3.3 Media Preparation for fungal isolation

The potato dextrose agar (a general-purpose media) used in the culture of yeast and mould was prepared using 200 g of peeled potatoes, 20 g of glucose, and 15 g of agar mixed in

1000 ml of distilled water and heated till totally dissolved. Media was autoclaved at 121 °C for fifteen (15) minutes and then poured on petri dishes (plates) to cool.

Richard's Synthetic Agar is a highly nutritive media for aquatic fungi it has a high content of sucrose which serves as a carbohydrate source for the growing fungi. The formulation of Richards medium contained 10 g potassium nitrate, 5 g of potassium dihydrogen phosphate, 2.5 g of magnesium sulfate, 0.02 g of ferric chloride, 50 g of sucrose, 15 g of agar and 1000 ml of distilled water, boiled and autoclaved at 121°C for fifteen minutes.

Czapek-Dox medium was formulated by using 15 g of agar, 2.0 g of sodium nitrate,1.0 g of potasium dihydrogen phosphate, 0.5 g of magnesium sulfate, 0.01g of ferrous sulfate, 30 g of sucrose and 1000 ml of distilled water boiled till all particles dissolve and then autoclave at 121°C for 15 minutes.

Sabouraud Dextrose Agar (SDA) is a selective medium primarily used for the isolation of dermatophytes. It was formulated with 10 g mycological peptone (enzymatic digest of casein and animal), 40 g dextrose, 15 g agar, and 1liter purified water. The compounds were heated with frequent agitation till it completely dissolved. The media was autoclaved at 121°C for fifteen minutes.

3.4 Procedure for the Culture of Fungi

The skin of the fish was cleaned with 70% ethanol to reduce bacteria contamination of the plates. The plates were inoculated using direct plating method (2 mm of skin, muscles of the infected fish were then collected and inoculated unto the various media plated and incubated for 5-7 days and serial dilution method (1 g of the infected parts was also placed in 100 ml peptone water in Erlenmeyer flasks and then shaken in Gallenkamp Model

Orbital shaker at 140 rev/min for 30 minutes to make a stock). A volume of 1 ml of the stock was three times diluted and dispensed into plates.

The fungi were isolated by using a sharp scalpel to cut a piece of fish skin and plated on the surface of the Sabouraud Dextrose Agar. The inoculated plates were inverted to prevent condensation of vapour back onto the surface of the medium after which it was incubated at a temperature of 28°C for 5 days.

Fungal colonies were stained with plain lactophenol and lactophenol cotton blue, the slides were observed under microscope Leica at a magnification of x 400 and identification was done using their colour, mycelium, and conidia morphology. The standard identification manuals (Barnett and Hunter, 1998; Sampson and Reenen- Hoekstra, 1988) were used as a guide.

3.5 Preparation of samples for isolation

Fish samples were rinsed with chlorine-free tap water and gently placed in a clean sterile container. The skin sample was collected aseptically by cutting thin sections of the skin about 1cm in diameter of the specimen with the spots.

Preparation of Inoculum

Tissues obtained were immersed in 700µl sterile PBS containing glass beads and homogenized by mechanical agitation. A portion of the homogenate (20µl) was spread on TSA and TYES media for bacterial growth.

Sterile cotton-tip swabs were also used to collect samples by swiping the affected areas, and the tips cut into well-labeled tubes containing Brain heart infusion broth. This was then incubated overnight at 28◦C to resuscitate immobile organisms and increase cell numbers.

After the overnight incubation, samples were inoculated into both differential media (MacConkey,) and enrichment media (Blood agar, chocolate agar, Muller Hinton agar) and incubated for 24 hours at 28◦C.

The enrichment media was incubated in an oxygen deficient environment using a candle jar to improve the growth of anaerobic bacteria present, and the differential media was incubated in the incubator. Bacteria on the plate was sub-cultured and identified by, morphology and biochemical reactions

Gram Staining

A thin smear of bacteria on a microscopic slide was prepared from single colony cells suspended in a drop of distilled water. The smear was allowed to air dry and then passed over flame 2 to 3 times with the smear side up.

The heat-fixed smears were placed on a staining trays and gently flooded with crystal violet and allowed to stand for 60 s, and gently washed with distilled water using a wash bottle. The smears were subsequently flooded with Lugol's iodine for 60 s and then washed off with distilled water. Decolourisation was done rapidly with absolute alcohol and washed immediately with water. The smears were then covered with Safranin stain for 2 mins. Slides were blotted dry with tissue and observed under light microscope with oil immersion at x 400 (Gram, 1884).

Biochemical Techniques

Biochemical techniques used to differentiate the various Enterobacteriacae (*Klebsiella* and *Pseudomonas*) and commensal bacteria (*Staphylococcus* and *Streptococcus*) included

1. Citrate utilization test to identify organisms capable of utilizing citrate as a carbon source for metabolism.

2. Urea test to identify bacteria capable of hydrolyzing urea into ammonia and carbon dioxide using the enzyme urease.

3. Motility test to determine whether an organism is equipped with flagella and thus capable of swimming away from a stab mark.

4. Indole test to detects the ability of an organism to produce indole from amino acids tryptophan and in differentiating E-coli forms.
5. TSI: Triple sugar iron agar test to determine whether gram-negative bacilli utilize glucose and lactose or sucrose for fermentation and produce hydrogen sulfide (H_2S).

6. Catalase test to detect the presence of catalase in a given bacterium. It helps to differentiate Staphylococcus species from Streptococcus species using hydrogen peroxide.

7. Oxidase: The test is used to differentiate *Pseudomonas* spp from another gram-negative enteric bacterium.

CHAPTER FOUR

RESULTS

4.1 Fungi Isolated from Infected Fish

Thirty (30) infected Nile tilapia (*Oreochromis niloticus*) were sampled, and a total of thirty (30) fungi species, belonging to eleven (11) different genera, were identified. These were *Achlya, Aspergillus, Cladosporum, Fusarium, Mucor Penicillium Rhizopus, Saprolegnia Syncephalastrum, Trichoderma, and Yeast*. *Aspergillus* species was identified in all 30 fish sampled. *Penicillium, Trichoderma* and *Fusarium* occurred on 5 fishes. *Saprolegnia, Cladosporum* and *Rhizopus* were isolated from three fish samples while *Yeast, Mucor, Syncephalastrum* and *Achlya* were found on one fish sample (see Fig. 4.1).

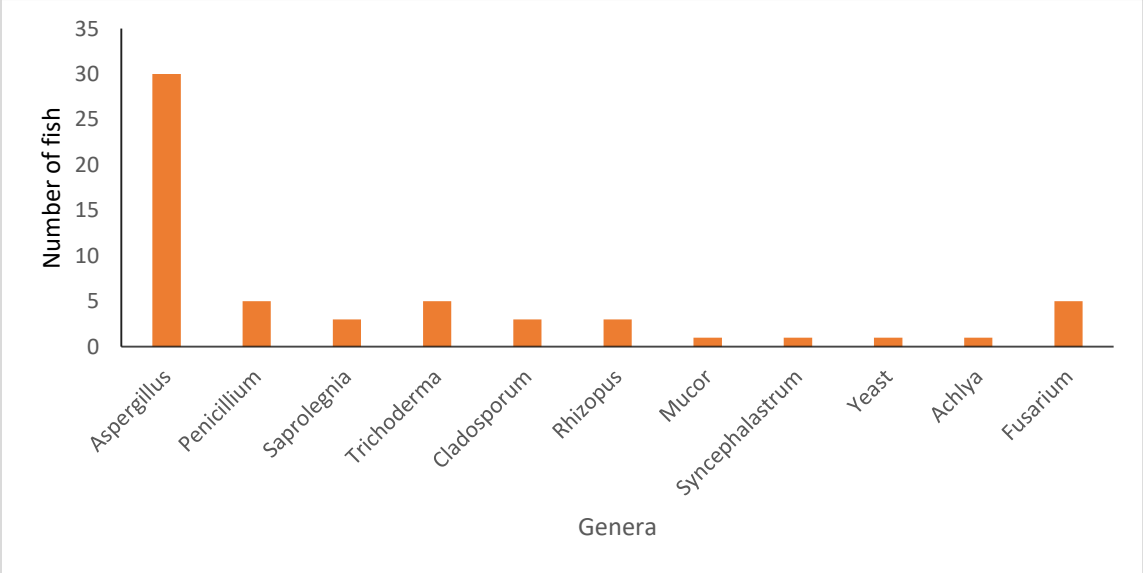

Figure 4. 4: Genera of fungi associated with the rusty brown spots on infected Nile tilapia.

The genus *Aspergillus* had the highest number of species (10) followed by *Penicillium* with 3 species. *Saprolegnia, Rhizopus* and *Fusarium* were represented by 2 species each. *Trichoderma, Cladosporum, Mucor, Syncephalastrum, Yeast* and *Achlya* had only one species each(Fig. 4.2). The type of fungi species identified within each genus have been provided in Appendix 1.

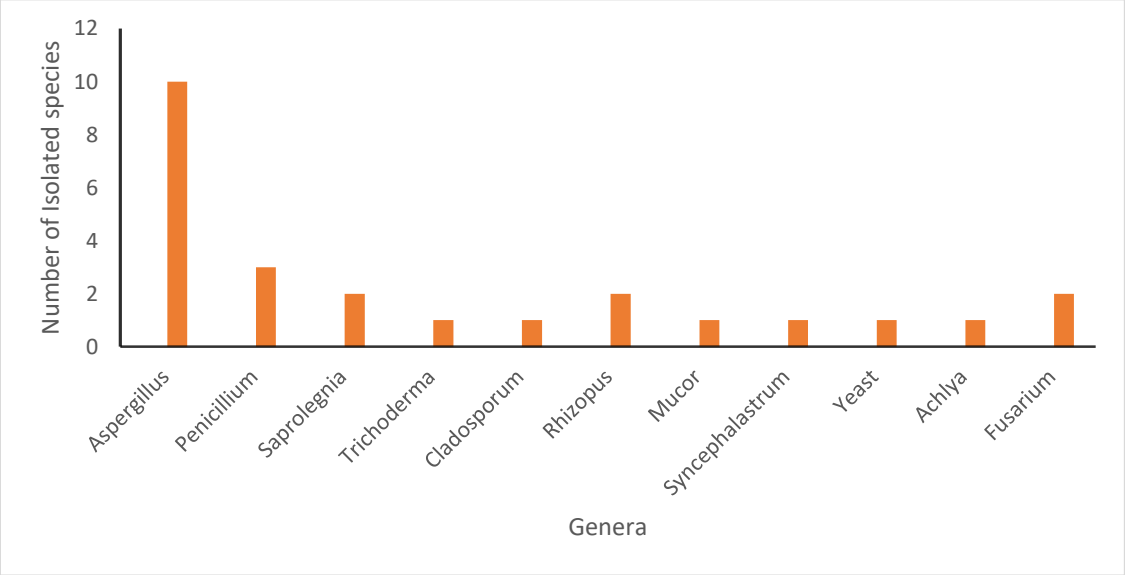

Figure 4. 5: Number of species per genus of fungi associated with the rusty brown spots on infected Nile tilapia.

Aspergillus species showed profuse growth on Richard's medium and were present in all the samples in this study. The most predominant species was *A. niger* which was recorded on all the four media used although it growth was better on Richard's medium. On the other hand the second most dominant *Aspergillus* species *A. flavus* performed better on Czapek-Dox agar.

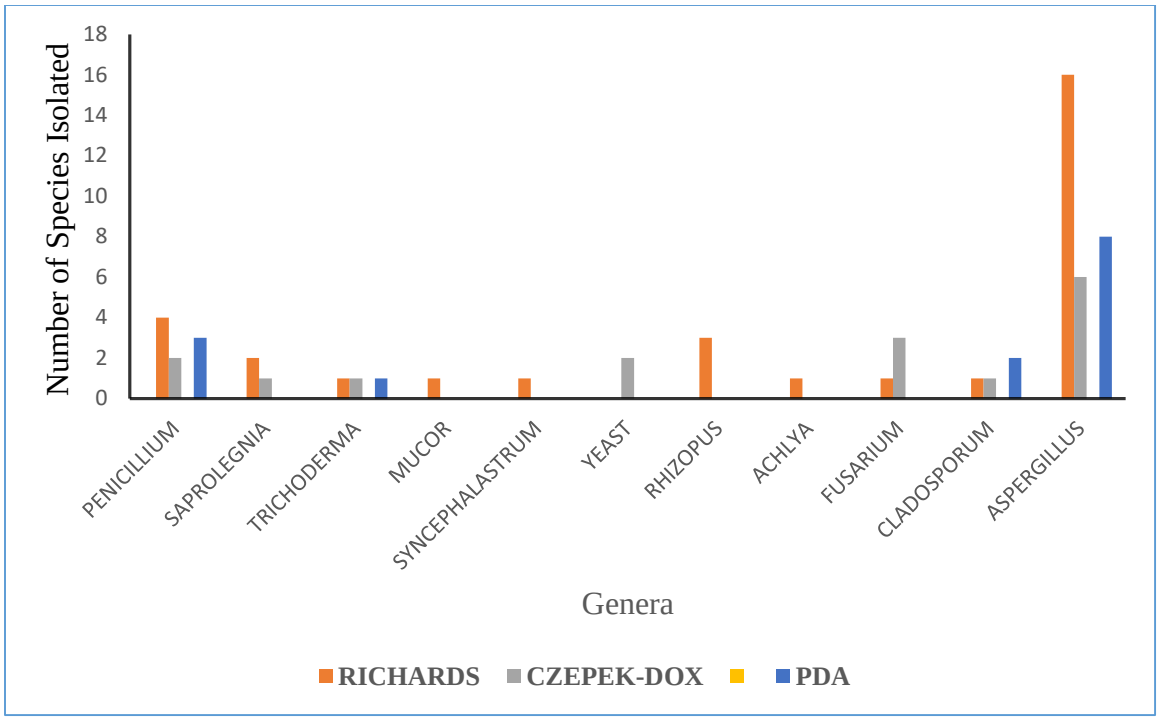

Figure 4. 6 Culture media selectivity for growth of fungi isolated from infected fish.

4.2 Fungi Occurrence in Feed Fed to Infected Fish

Four (4) fungal genera (*Aspergillus, Rhizopus Penicillium* and *Cladosporum*) were isolated from feed sample fed to infected Nile tilapia at the farm. *Aspergillus* species constituted 66% whereas *Rhizopus stolonifer*, *Penicillium cyclopium*, and *Cladosporum herbarum* made up approximately 11% each (Fig. 4.4).

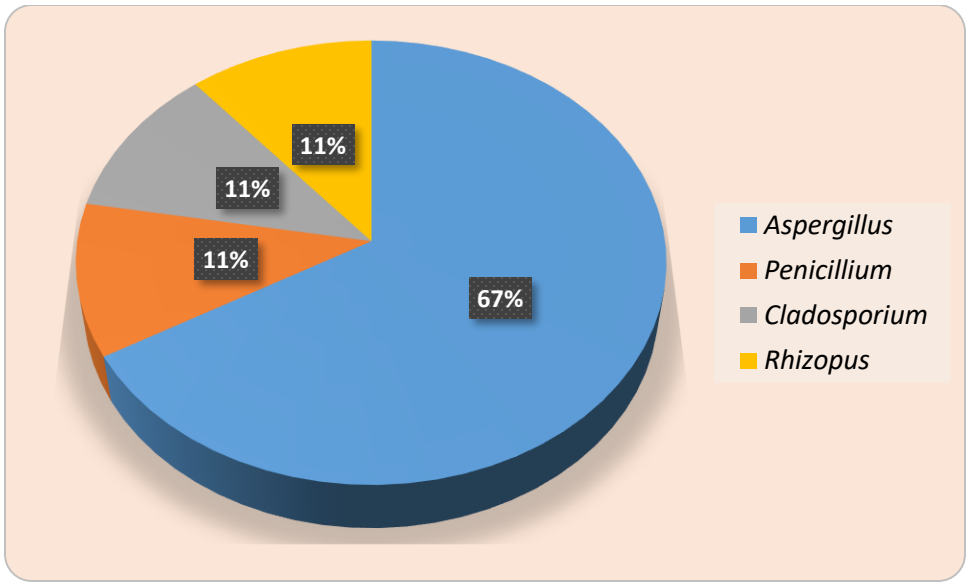

Figure 4.4 Percent occurrence of fungi genera isolated from fish feed fed to infected Nile tilapia.

4.3 Fungi Isolated from Water in Pond with Infected Fish

Seven genera of fungi were isolated from the pond water in which the infected fish were cultured. These were *Aspergillus, Cladosporum, Fusarium Paecilomyces, Penicillium, Saprolegnia, and Yeast. Aspergillus* made up 41.8% and *Penicillium* made up 16.66%. The rest had 8.3% occurrence each (Fig. 4.5).

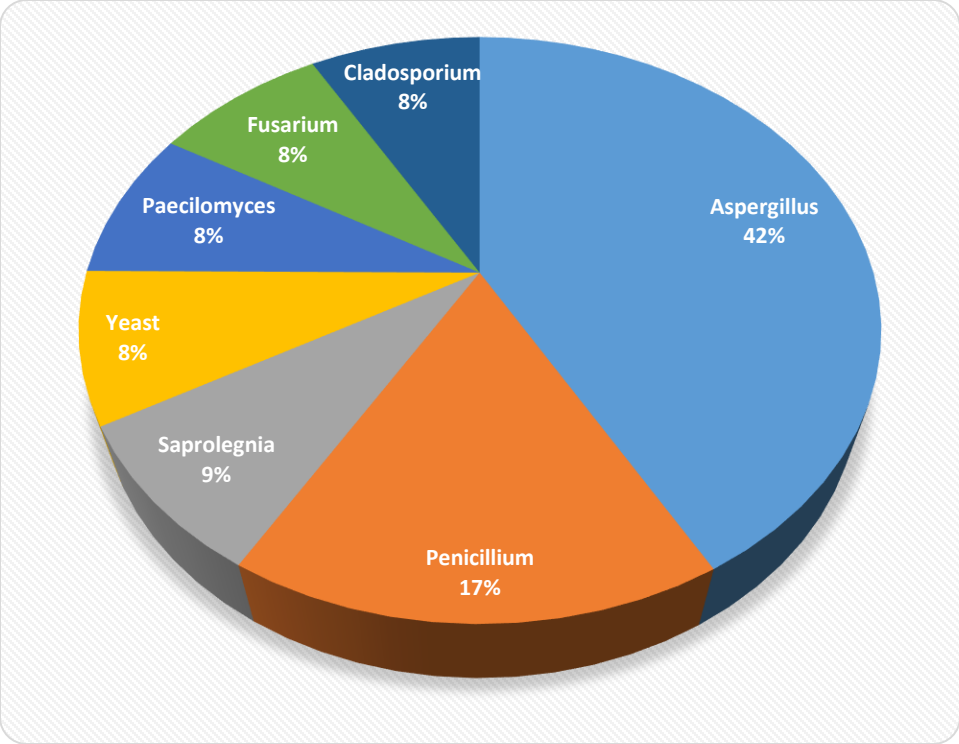

Figure 4.5 Percent occurrence of fungi genera in pond water in which infected fish were cultured.

4.4 Fungi Isolated from Bottom Sediment in Pond with Infected Fish

Aspergillus ,Fusarium, Paecilomyces and Yeast, and were isolated from the sediment from pond with the infected fish. The most abundant species was *Aspergillus* (57%) followed by *Fusarium, Paecilomyces* and *Yeast* exhibited 14.3% occurrence each (Fig. 4.6).

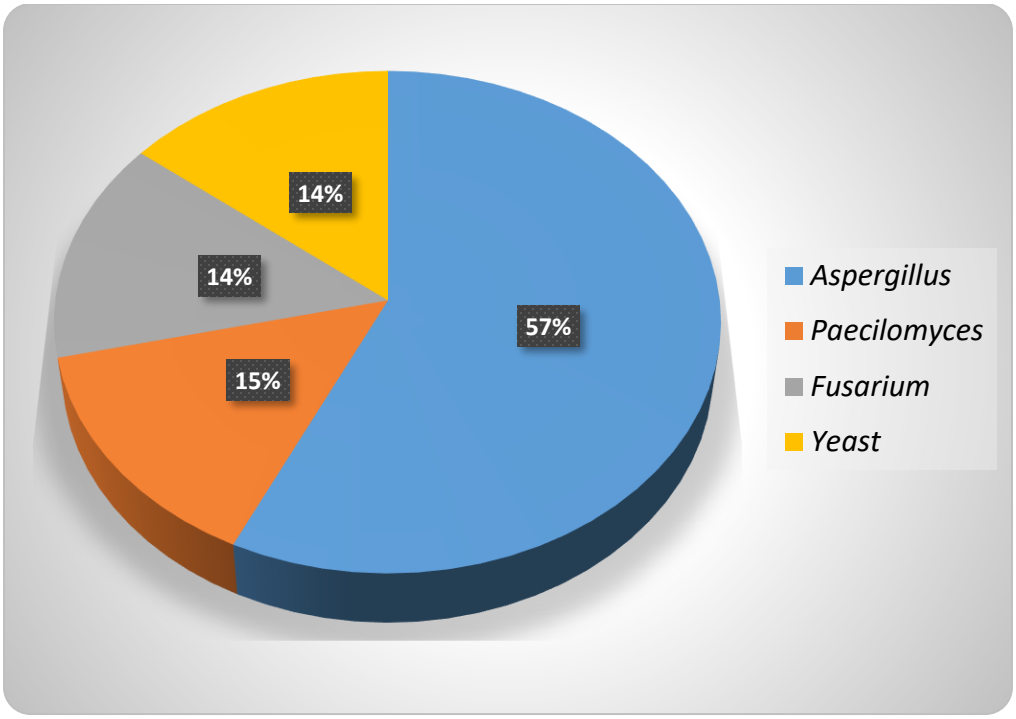

Figure 4.6 Percent occurrence of fungi genera in bottom sediment from pond with infected fish.

Data obtained shows that *Paecilomyces* was found to be present in both the water and sediment but was absent in the feed and the fish skin. *Achlya Americana, Aspergillus alutaceus, Aspergillus glaucus. Aspergillus oryzae, Mucor, Penicillium digitatum, Rhizopus oryzae, Saprolegnia Americana, Syncephalastrum, Trichoderma harzianum,* were found to be present on only the infected fish skin. *Aspergillus flavus* and *Aspergillus niger* were found in all the samples. *Fusarium solani* was isolated from the sediment and the skin of infected fish. *Rhizopus stolonifer, Aspergillus nidulans, Aspergillus suphereus,* and *Aspergillus versicolor* were isolated from feed, skin and water samples. *Saprolegnia parasitica* was isolated from the water sample and the fish skin (Fig. 4.5). It was observed that *Aspergillus* species was the most dominant occurring fungi in the fish skin with the rusty spots (Fig. 4.1).

Table 4. 1 The frequency of occurrence of* Aspergillus *spp. isolated from fish skin.

Species of Aspergillus	**Number of fish observed**
Aspergillus flavus	18
Aspergillus niger	18
Aspergillus glaucus	15
Aspergillus nidulans	12
Aspergillus parasiticus	9
Aspergillus tamari	9
Aspergillus versicolor	6
Aspergillus alutaceus	6
Aspergillus terreus	3
Aspergillus fumigatus	3
Aspergillus sulphereus	3

The identified *Aspergillus* species were also isolated from the sediment, feed and water samples. *A. niger*, and *A. flavu*s were present in all the samples (skin, feed, water, sediment. *A. suphereus* was isolated from the skin, water and feed. *A. nidulans* and *A. versocolor* were isolated from the skin and feed samples. *A. oryzae*, *A. tamarii*, *A. alutaceus* and *A. glaucus* were only seen to be present on the parts of the skin with the rusty brown spots on the fish sample. *A. parasiticus* was isolated from the skin, water and sediment samples. *A.*

wentii was observed in only the water sample and *A. terreus* was recorded only in the feed sample whereas *A. fumigatus* was also isolated from both sediment and water samples (Table 4.2).

Table 4. 2: Occurrence of *Aspergillus* spp. in infected fish, feed, water and sediment.

SPECIES OF *ASPERGILLUS*	SKIN	WATER	FEED	SEDIMENT
Aspergillus flavus	+	+	+	+
Aspergillud niger	+	+	+	+
Aspergillus fumigatus	-	+	-	+
Aspergillus wentii	-	+	-	-
Aspergillus sulphereus	+	-	+	-
Aspergillus nidulans	+	-	+	-
Aspergillus versicolor	+	-	+	-
Aspergillus tamarii	+	-	-	-
Aspergillus glaucus	+	-	-	-
Aspergillus oryzae	+	-	-	-
Aspergillus alutaceus	+	-	-	-
Aspergillus parasiticus	+	+	-	+

4.5 Bacteria Identified from Infected Fish, Feed, Water and Sediment

The preliminary culture of the skin,water and sediment samples on TSA and TYES showed the presence of bacteria with different appearances, on TYES, bacteria appeared as creamy

mucoid colonies, yellow mucoid colonies, creamy white dry colonies and pale yellow colonies, and TSA gave white mucoid colonies and creamy mucoid colonies (Table 4.3).

TABLE 4.3 Bacteria isolated from samples of fish, feed, water and sediments.

Sample	*Flavobacterium*	*Klebsiella*	*Staphylococcus*	*Pseudomonas*	Coagulase –ve *Staphylococcus*
Fish	+	–	–	+	–
Feed	–	–	+	–	+
Water	–	+	+	–	+
Sediment	–	+	+	+	+

(+) presence of bacteria in sample, (-) absence of bacteria in sample.

Presumptive identification with gram stain revealed Gram positive cocci in clusters, Gram negative cocci and Gram negative rods.

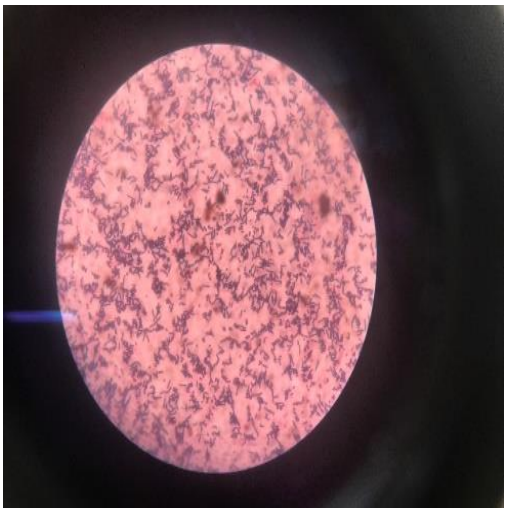

Plate 2: Gram positive cocci bacteria in clusters

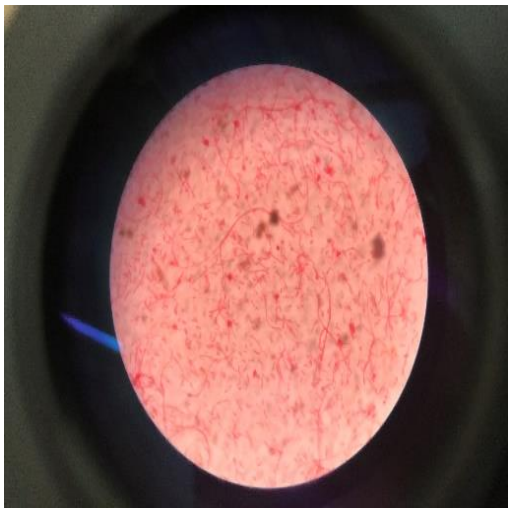

Plate 3: Gram negative baccilus.

Gram positive bacteria stained violet due to the presence of a thick layer of peptidoglycan in the cell wall. Gram negative bacteria stained red because of a thinner layer of peptidoglycan in the cell wall.

Plate 4 : *Pseudomonas aureginosa* on McConkey

Plate 5: Staphylococcus aureus on Muller Hinton agar.

Isolated pure cultures confirmed the presence of *Flavobacterium, Klebsiella, Pseudomonas, Staphylococcus,* and some coagulase negative *Staphylococcus* based on appearance on plates and gram reaction. The bacteria isolates when subjected to biochemical test gave the results in the Table 4.3.

Table 4. 4 Biochemical characteristics of bacteria isolated from Fish, Feed, water and Sediment samples.

Test	*Flavobacterium*	*Pseudomonas*	*Klebsiella*	*Staphylococcus*
Glucose	-	+	+	+
Lactose	-	-	+	+
Catalase	-	+	-	+
Oxidase	-	+	-	+
Motility	Gliding motility	Unipolar	-	-
Indole	+	-	-	-
Urease	-	-	+	+
Citrate	+	+	+	+

Confirmatory test (Bruker Maldi Biotyper) further confirmed the presence of the following pathogens; *Pseudomonas aeruginosa, Klebsiella pneumonia, Staphylococcus cohnii, Staphylococcus aureus and a Flavobacteria spp.*

CHAPTER FIVE

DISCUSSION.

5.1 Fungi Infections

The study brought to light numerous fungi genera associated with the rusty brown spots. These were made up of 12 different fungi genera and were a combination of ascomycetes, zygomyces, and oomycetes. The majority of fungi causing diseases in fish belong to Ascomycota and many genera have been implicated in fish diseases. They are opportunistic pathogens and are not exclusive fish parasites.

However, fungi are ubiquitous in the environment, and most are airborne hence can easily be introduced into the water. Iqbal *et.al*.(2012) indicated that, *Penicillium, Aspergillus,* and *Rhizopus* are all part of the normal flora of fish skin and hence its presence in high numbers in this study. Shukla & Shukla (2011), alluded that fungal spores in fish ponds create problems and can infect fish. Rao (2017) observed that *Aspergillus fumigatus, A. terreus, A. flavus,* and *A. niger* were mostly encountered in the air and are likely to be transferred unto the fish which can cause infection in fish. Most infections in fish caused by a fungus usually attack external tissues with only a few affecting internal organs (Meyer, 1991). Gozlan (2014) and Ibrahim (2016) confirmed that fish skin infections were caused by species of fungi belonging to the genus *Penicillium, Aspergillus,* and moulds that belong to the family saprolegniaceae. The culture of skin tissues and the isolation of the individual fungi form the Nile tilapia with the rusty brown spots confirmed that the presence of fungi on the fish skin has the potential to cause skin related infections.

Pitt & Hocking (2009) implicated *Aspergillus niger* in the deterioration of stored feed which suggests that the spores of *A. niger* which was found to be the most recurring fungal species in this study may have been introduced through the feed. Mousavi *et.al.*(2016) suggested that the easily dispersed conidia of *Aspergillus niger* makes it easy to find them in the environment and can be isolated from all substrates as this study has emphasized. The observation of the *Aspergillus* species identified on both skin, feed and sediment is an indication that many of these pathogens were introduced into the pond through their feeding, they may therefore not be the primary cause of the condition. Nevertheless, this study identifies *A. niger* as an opportunistic pathogen in the presence of pathogenic bacteria. According to Shehata *et.al*, (2018), the presence of *Aspergillus* species in the feed, pond and on the fish as observed in this present study is an indication of the poor state of the pond and their presence on the fish could be the result of ingestion. Van-West (2006) reported that fungi had a wide range of infections depending on the management of the pond and its immediate environment. The isolation of *Aspergillus, Penicillium,* and *mucor* are indicative of the poor state of the pond as a result of contaminations as reported by Al-Niaeem *et al.* (2015). Feed decomposition and the death of some fish within the pond may have added to the infection. However, according to Mendonca and Arkush (2004) some healthy fish can serve as carriers for certain diseases.

A study by Gozlan *et al.* (2014) encountered the following fungi *Aspergillus, Penicillium,* and *Saprolegnia* as pathogenic fungi and their presence associated with the rusty brown spots could indicate their possible role in the condition. Other documented fish fungi such as *Cladosporium* and *Paecilomyces* were also identified in this study. Liu (2016) suggested

that skin infections of fish were as a result of the interaction between fungi particularly oomycetes such as *Saprolegnia,* ascomycetes such as *Aspergillus* and Bacteria.

The current study did not conclude that the rusty brown spots are caused by these ascomycetes isolated and there is no literature to compare it with as there is no detailed work that has been done on this condition. The condition of the rusty brown spots on cultured Nile tilapia has only been reported on farms in Ghana in recent years.

The culture and isolation of the fish samples also revealed the presence of water moulds which are aquatic fungi. *Achlya, Saprolegnia, Aphanomyces* and *Pythium* are the most randomly encountered. Two out of these were discovered in the study. These were *Achlya americana, Saprolegnia americana* and *Saprolegnia parasitica*. In warm waters, the most commonly encountered fish pathogen is the *Saprolegnia parasitca* which causes Saprolegnianiasis in fish. The growth of the cultures was a mixture of pathogens which according to Austin (2011) means that the actual pathogen causing the disease will be masked or missed.

The emergence of the rusty brown spots on the Nile tilapia cultured in Ghana may lead to a serious disease condition which can lead to their death. Cano (2016), reported that puffy skin disease was an emerging disease in England and Wales in *Oncorhynchus mykiss* (rainbow trout). This condition was associated with low mortality but was anticipated to cause economic losses due to the downgrading of the carcass which reduces its economic value in line with the current study. However, according to Iqbal and Saleemi (2013), skin infections in fish are not considered dangerous, so much attention is not given to it. The isolated organisms from this study may be pathogenic but their role can better be understood if they are viewed as opportunistic fungi. This basically is due to the fact that

many of them possess virulence factors which enables them to cause disease when the condition for their growth is suitable as reported by Refai *et al.* (2004, 2010).

In a study by Mastan (2015), Phillips *et al.* (2003), they reported that Saprolegniasis gives the indication of small brown spots on fish skin at the onset of the infection and only become big ulcerative lesions when the condition is advanced and this present study agrees based on the isolation of two species of *Saprolegnia* which is *Saprolegnia american*, and *Saprolegnia parasitica*.

As the rusty brown spots on the cultured Nile tilapia is considered an emerging disease, the exact role of *Saprolegnia* and other pathogenic fungi are not certain but work done by Hjerde *et al.* (2015) indicates that the occurence of more than two pathogens is likely to cause a different expression of disease from their individual expressions.

5.2 Bacteria Infections

The result of microbial characteristic analysis shows that the gram-negative bacteria isolated from the rusty brown spots on the Nile tilapia outnumbered the gram-positive bacteria isolated. The current study isolated *Staphylococcus chonii*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Pseudomonas aurigenosa*, and *Flavobacteria spp.* *Staphylococcus spp* isolated is gram-positive while the other isolated bacteria were gram negative. The isolated bacteria consist of Enterobacteriaceae which is *Klebsiella pneumoniae.* Bacteria that belong to Enterobacteriaceae are part of the natural flora of fish, however, some of them are of potential threat to humans (Macfarlane & Dillon, 2007).

Klebsiella is a common bacterium which is be found in almost every environment. According to Octavia & Lan (2014) it has been cultured from water, soil, vegetation,

animals including fish and in human. It causes conditions such as urinary tract infections in people, pneumonia, septicemias among other notable disease conditions. They are normally found in the gastrointestinal tract of its hos. However, they become opportunistic pathogens when found outside the body of the host and so become infective quickly thereby causing outbreaks. In a study by Adeshina *et al*. (2016), it was reported that *Klebsiella* in fish is usually present in fish tissues such as the skin, gill, and muscle. This is of great concern because these organs are consumed by human and therefore can be a medium to transmit pathogens to the consumer. Also their presence on the fish raises concern in the handling of the fish.

The *Staphylococci* are Gram-positive cocci with their primary habitat in the skin, glands and mucous membranes of warm-blooded animals including humans. *Staphylococcus* species are one of the most important foodborne opportunistic bacteria in fishes. It has been implicated in severe food poisoning in humans (Bujjamma & Padmavathi, 2015). The species of Staphylococcus identified were *Staphylococcus aureus* and *Staphylococcus cohnii*. Their presence on the fish agrees with the findings of Bujjamma& Pasmavathi (2015). This is an indication of contamination from handlers. Their association with the cultured *O. niloticus* is a cause for concern in the safety of handling of the fish and subsequent consumption. This according to Garg (2017) is because it has been mentioned in relation to meningitis in humans.

Flavobacterium spp. and *Pseudomonas* spp. which were identified in this study are known to be fish pathogens. According to Cabral (2010), *Pseudomonas* are widespread and are usually associated with septicemias in aquatic animals including fish. They are considered opportunistic bacteria which cause diseases when the host is stressed (Huicab-Pech *et al.*

2016). *P. aeruginosa* was the species of *Pseudomonas* isolated from the fish skin. The results of the biochemical test of the isolated *P. aeruginosa* was in accordance with the results of Hasnson *et al.* (2006).

Loch *et al.* (2013) and Johansen *et al.* (2011) reported that *Flavobacterium* was a serious threat to both wild and cultured fish stock. In warm waters, *F. columnare* has been mentioned as the causative agent of the *columnaris* disease however, there is scanty information on the occurrence, pathogenicity and severity. And according to Hawke & Thune (1992), Durborow *et al*. (2000) and Wagner *et al.* (2002), it is the most prevalent bacterium that causes high mortality in fish. Its isolation from the rusty brown spots on the cultured Nile tilapia in Ghana is of grave concern.

Although many of the reports on *Flavobacterium* disease outbreaks in fish, along with researches aimed at mitigating them has focused mainly on three known *Flavobacterium* spp which include *F. psychrophilum*, *F. columnare*, and *F. branchiophilum* (Starliper & Schill, 2011, Loch & Faisal, 2017). However other previously unknown *Flavobacterium* spp. has been seen lately in fish disease outbreaks (Loch and Faisal, 2015). Based on works done by Decostere (1999), and Kumru *et al.* (2017), there are different strains of *F.columnare* and their virulence depends on the strain.

Some novel *Flavobacteria spp* are not known to cause systemic disease outbreaks in fish but are capable of generating symptoms that mimic their better-known fish-pathogenic counterparts according to Loch and Faisal (2016), it however complicates disease diagnosis and treatment. Unfortunately, very little is known about the ecology, how it is transmtted, and the pathogenesis of this emergent fish-associated *Flavobacterium* diseases (Chen *et al.*, 2017). *Flavobacterium spp* that have been associated with the rusty brown spots on the

skin of *O. niloticus* is not the same as that of known *Flavobacteria* infections in fish, (Shoemaker 2008) hence it is not possible to tell if it's the same species of *Flavobacteria* causing the rusty brown spots.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusions

The current study revealed the presence of a plethora of fungi and bacteria which are associated with the rusty brown spots on infected Nile tilapia. A total of eleven genera of fungi were identified in all the studied samples. These were *Achlya*, *Aspergillus*, *Cladosporium. Fusarium Mucor, Penicillium, Rhizopus, Saprolegnia, Syncephalastrum Trichoderma, and Yeast. Aspergillus* was found to be present in all the samples. *Aspergillus niger, Aspergillus flavus* and *Aspergillus glaucus* were found in over 50% of infected fish samples. The fungi found on the skin that were also found in the other samples suggest that they may have been transferred from the feed, water or sediment to the fish.

Bacteria identified in relation to the rusty brown spots, water and sediments included *Klebsiella pneumoniae*, *Flavobacterium* spp, *Pseudomonas aureginosa, Staphylococcus aureus* and *staphylococcus chonii*. The bacteria that were found on only the parts of the fish with the spots were *Pseudomonas aureginosa* and *Flavobacteria spp*. These two bacteria are known fish pathogens and are capable of causing severe damage to fish meat.

6.2 Recommendations

- Molecular screening using PCR should be conducted to identify the specific strain of *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus glaucus* and to determine its virulence factor.
- PCR should also be done on the *Flavobacterium* species identified to determine the strain of *Flavobacteria* and also determine the virulence factor of the pathogen.
- Water quality assessment in relation to the rusty brown spots should also be done to determine the preferred environmental factors for the growth of these associated pathogens.
- A challenge experiment should be conducted to determine which of the identified pathogens is responsible for the outbreak of the spots.
- Studies should be done to determine the acceptable levels of these pathogens in the fish to ascertain its safety for human consumption.

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APPENDIX

Appendix 1. Species of fungi belonging to the identified genera.

GENUS	SPECIES IDENTIFIED
Penicillium	*Penicillium cyclopium* *Penicillium corylophylum* *Penicillium digitatum*
Saprolegnis	*Saprolegnia Americana* *Saprolegnia parasitica*
Achlya	*Achlya Americana*
Cladosporum	*Cladosporum herbarum*
Fusarium	*Fusarium solani* *Fusarium oxysporum*

Rhizopus	*Rhizopus oxysporum* *Rhizopus stolonifera*
Paecilomyces	*Paecilomyces variotii*
Mucor	*Mucor racemosus*
Syncephalastrum	*Syncephalastrum racemosum*
Trichoderma	*Trichoderma harzianum*
Yeast	*Yeast spp.*
Aspergillus	*Aspergillus flavus* *Aspergillus niger* *Aspergillus terreus* *Aspergillus sulphereus* *Aspergillus versicolor* *Aspergillus alutaceus* *Aspergillus glaucus* *Aspergillus parasiticus* *Aspergillus tamarii* *Aspergillus wentii* *Aspergillus fumigatus* *Aspergillus nidulans*