ASSESSMENT OF MANAGEMENT PRACTICES AND OCCURRENCE OF *MYCOBACTERIUM MARINUM* INFECTION IN SELECTED MILKFISH (*CHANOS CHANOS*) FARMS IN ZANZIBAR, TANZANIA

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ABSTRACT

Milkfish farming in Zanzibar is at infancy stage, practiced at small-scale levels and faces a number of constraints including fish health problems. The purpose of this study was to assess the milkfish farming practices and possibilities for occurrence of *Mycobacterium marinum* infection in selected milkfish farms in Zanzibar, Tanzania. A questionnaire survey was administered to 24 milkfish farmers to acquire information on general management system, fish health and related problems. Pond physicochemical characteristics were assessed using standard procedures. Pond water (24), sediments (24) and fish (240) samples were collected for laboratory analysis ofw21 *M. marinum* using standard procedures. Most (92%) of farmers were smallholder with backyard ponds. About (91.7%) of the ponds were of earthen type adopted from salt pans and practiced polyculture (*Chanos chanos* + *Mugil cephalus*) technique. Fingerlings were obtained from the sea, some farmers did not feed their fish and there was no routine water exchange in ponds. Likewise, fish farmers were not aware about fish health related issues and fish mortalities were reported. Water temperature ranged between 29.3 ^{o}C to 37.1 ^{o}C varying significantly ($P<0.05$) between ponds. Dissolved oxygen ranged between 1.9 and 6.1 mg/l while the mean pH was 7.5 ± 0.5 and 8.2 ± 0.2. All sampled fish were apparently healthy. A total of 110 samples had bacterial growths on Lowenstein- Jensen media but only 12 (4.2%) were AFB positive. No any isolate was found to have DNA band size of 1030 bp for Mycobacteria which implied that they were not *Mycobacterium*. It is concluded that fish pond management practices was poor and farmers lack of knowledge on good management practices for optimal milkfish production. Health related problems exist in fish ponds but not mycobacteriosis. Education on fish diseases should be provided to farmers and researches on fish diseases including mycobacteriosis are recommended.

DECLARATION

I UBWA ABEID MASOUD, do hereby declare to the Senate of Sokoine University of Agriculture that, this dissertation is my own original work, it has neither been submitted nor being concurrently submitted in other institution.

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The above declaration is confirmed;

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DEDICATION

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LIST ABREVIATIONS AND SYMBOLS

AIDS	Aquired immuno- deficiency syndrome
bp	Base pair
CI	Confidence interval
DNA	Deoxyribonucleic acid
dNTP's	Deoxyribonucleoside phosphate
EDTA	Ethylenediaminetetra acetic acid
ELISA	Enzyme linked immuno – Sorbent assay
HCL	Hydrochloric acid
HIV	Human immunodeficiency virus
IU	International unit
KCl	Potassium chloride
P	Probability value (for statistical significance)
PCR	Polymerase chain reaction
pH	Logarism of hydrogen ions
pmoles	Picomoles
RNA	Ribonucleic acid
rpm	Revolution per minute
SUA	Sokoine University of Agriculture
TBE	Tris-HCL, boric acid and EDTA
UV	Ultra violet
V	Volts
v/v	Volume by volume

V/w	Volume by weight
WHO	World Health Organization
ZN	Ziehl- Neelsen stain

CHAPTER ONE

1.0 INTROUCTION

1.1 Background information

The aquaculture sector has been found to be an opportunity that can be utilized to contribute in to the effort of strengthening the Tanzanian economy and improve the livelihood of coastal communities (Sullivan *et al.*, 2007). Currently, both the Government of Tanzania and the Revolutionary Government of Zanzibar (RGZ) make remarkable effort to encourage aquaculture through involvement of more aquaculture candidates' species apart from the common species which are presently used in production (Rice *et al*., 2006). In recent years, there have been initiatives on establishment of demonstration farms for milkfish, crab fattening, shellfish farming and pearl production.

Milkfish farming has shown a great success by far than the other species because of their comparatively easy fingerlings availability from the wild, lower feeding costs and affordable raring technology used for milk fish production, an outcome which resulted on growing interest in milkfish farming throughout Tanzanian coastal regions (Msuya and Mmochi, 2007). According to Msuya and Mmochi (2007) milkfish farming activities in Tanzania was originated from the local community group in Zanzibar engaged into these activities since 1996. Then the idea and skills spread out to Mtwara, Lindi, Kilwa, Rufiji, Mkuranga, Bagamoyo and Tanga. Ling (1977) narrated that, the systems (commonly ponds), techniques and methods of milkfish farming in Tanzania were adopted from Asian countries namely Philippines,

Indonesia and Taiwan, that have been practicing these activities for over a hundred of years.

Milkfish (*Chanos chanos*) production is an income generating activity that is developing fast in the coast of Tanzania. Nearly 50% of the farms possess modified ponds that were previously used for salt production (Sullivan *et al.,* 2007). The milkfish aquaculture sector is facing a number of challenges including unreliable seed and feed supply, high initial investment cost and lack of basic information on health status of farms, the reared fish and the farmers (DMR-ZNZ, 2015). Recently, the RGZ developed a Memorandum of Understanding (MoU) with KOICA for construction of milkfish hatchery to solve the problem of seed supply (DFD-ZNZ, 2016). Among the other challenges that need close attention so as to boost production of milkfish and other mariculture products in Zanzibar was mentioned to be fish diseases in particular bacterial related diseases (DMR-ZNZ, 2015).

Fish mycobacteriosis is a chronic bacterial disease that may affect all freshwater, brackish and marine fish. The disease is caused by acid-fast *Mycobacterium* spp. and the bacteria involved are *Mycobacterium chelonae*, *M. fortuitum M. abscessus* and *M. marinum* (Chen *et al*., 1998; Chinabut, 1999). Mycobacterial infections led to systemic granulomas especially in kidney, spleen, skin and may cause serious mortality especially in farmed and aquarium fish (Belas *et al.,* 1995). *Mycobacterium marinum* infection in farmed fish is mostly experienced where there is high interaction between wild and reared fish. When the fish is infected; gills, skin and alimentary tract lesions are mentioned to be the sources of pathogen shedding in water potentiating the spread of infection between fish (Belas *et al.,* 1995).

The systemic infection of fish by *M. marinum* can produce severe contagious disease in commercial farmed fish operations, which may lead to widespread mortality and economic loss. During 1999, the annual Hybrid Striped Bass (HSB) production in USA was about 5 million (kg) with an approximate value of US $30 million (Carlberg *et al*., 2000). According to Cirillo (1999), the estimated loss due to *M. marinum* outbreak on HSB was more than US $125 million. Therefore, mycobacteriosis due to *M. marinum* is one of the remarkable constraints to the fish industry.

Mycobacteria that affect fish in particular *M. marinum* is zoonotic that usually causes skin lesions in humans (Huaman *et al*., 2015). A number of cases of cutaneous *M. marinum* infections associated with symptomatic skin ulceration in humans have been reported (Huaman *et al*., 2015). There are several risk factors for *M. marinum* infection in humans that include working and having direct contact with infected fish and contaminated water (Novotny *et al*., 2004).

Information about bacterial fish diseases in East Africa is limited. However, Haemorrhagic septicaemia, due to *Aeromonas hydrophila* and other Gram negative bacteria like *Edwardsiella tarda* in farmed tilapia in Mombasa Region of Kenya have been reported (Roberts and Sommerville, 1982). Mycobacterial skin lesions and gill rots also have been reported in cultured tilapia (*Oreochromis niloticus*) in Kenya (Roberts and Sommerville, 1982). Mycobacteriosis was reported from marine and freshwater fish species belonging to families *Cichlidae* and *Characidae* including *Sarotherodon andersonii* and *T. sparmanii* in the Okavango swamps in Botswana (Roberts and Sommerville, 1982). Other reports of mycobacteriosis in fish have been

published in Egypt on aquarium reared *Haplochromis multicolor, Hemichromis bimaculatus* (Nigrelli and Vogel, 1963) and *Oreochromis mossambicus* (Noga, 2010). *Leptospira* infection has been reported in catfish and tilapia in Mindu dam, Morogoro Tanzania (Mgode *et al*., 2014). Ulcerative *Aeromonas* infections have also been reported in tilapia from Mtera Dam, Tanzania (Shayo *et al*., 2012). However, there is no published information on mycobacteriosis in wild or farmed fish in Tanzania although some anecdotal information from fishermen have been reported to see fish with some nodular lesions on the skin. In addition to that, some fish farmers and fishers reported to suffer from skin rushes or itchiness when they had contact with pond or sea water. Therefore, the proposed study is aimed to assess the management practices and occurrence of *M. marinum* in selected milkfish farming areas in Zanzibar.

1.2 Problem statement and study justification

Fish diseases that sometimes lead to high morbidity and mortality rates in fish farms in the study area have been reported by fish farmers and fishermen. Reports from some fish farmers in Pemba island shown that, fish farmers suffered from skin rashes and irritation when working in milkfish ponds (Fish farmer. personal communication, 2015), a problem that may be caused by *M. marinum* infection in humans.

There are 70 milkfish farms scattered in different places in Unguja and Pemba (DMR-ZNZ, 2015). According to Chen *et al.* (1998) fish diseases particularly caused by bacteria are among the drawback to fish production under aquaculture that lead to reduced production performance in Zanzibar. Different studies have reported

existence of different bacterial diseases and infections in fish produced under aquaculture farming including mycobacteriosis in different countries in Africa (Nigrelli and Vogel, 1963; Roberts and Sommerville, 1982; Noga, 2010; Cirillo, 1999; Carlberg *et al*., 2000; Shayo *et al*., 2012; Mgode *et al*., 2014). However, there is limited information on fish diseases in aquaculture sector of Zanzibar. Possible existence of mycobacteriosis and other infectious fish diseases may be a threat to expansion and development of the aquaculture sector for some years (DMR-ZNZ, 2015).

The current study aimed to assess milkfish management practices and occurrence of *Mycobacterium marinum* infection in selected milkfish farms in Zanzibar, Tanzania. Findings of this study will increase the understanding on the status of mariculture and its challenges and provide useful information that can be used in advising farmers on better management practices of milkfish farms so as to improve the livelihood of people and contribute on poverty alleviation. The study has provided baseline data for further studies on fish diseases in particular mycobacteriosis.

1.3 OBJECTIVES

1.3.1 General objective

Assessment of the milkfish managerial practices and occurrences of *M. marinum* infection in selected milkfish farms in Zanzibar, Tanzania.

1.3.2 Specific objectives

i. To assess milkfish pond management practices in Unguja and Pemba, Zanzibar, Tanzania

ii. To evaluate the physicochemical water parameters in milkfish ponds water in Unguja and Pemba, Zanzibar, Tanzania

iii. To determine the occurrence of *M. marinum* in milkfish farms in Unguja and Pemba, Zanzibar, Tanzania

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Milkfish biology and ecology

Milkfish (*Chanos chanos*) is the sole living species in the family Chanidae. It is the marine finfish with morphology generally symmetrical and streamlined form, with a fork shaped caudal fin (Requintina *et al.,* 2008). Externally, milkfish is characterized by pale or yellowish colour with dark margins' dorsal, anal and caudal fins (Fig.1). The body colour of milkfish is silvery on belly and sides grading to olive-green or blue on back. Often, they can grow up to 1 m in length, in special cases male milk fish can grow up to 1.80 m (Bagarinao, 1991). Milkfish are usually found along the coasts of well-developed reefs and the most preferable environment for milkfish is warm waters above 20°C, clear shallow depth and moderate salinity. In Madagascar and Asian countries like Philippines and Indonesia, adult milkfish and Juveniles are reared in large coastal lagoons, atolls, and freshwater lakes (Bagarinao, 1999). They are abundantly found in all coastal regions of Tanzania. Milkfish life cycle include eggs, larvae, fry, juvenile and adults (Buri *et al.*, 1981).

Figure 1: Adult milkfish appearance. Source: Bagarinao (1991)

2.2 Milkfish farming in Zanzibar

Worldwide milkfish farming is well advanced in Asia and has been practiced for many years in particular like Philippines, Indonesia, and China. It is estimated that Asia produces 99.9% of the world milkfish consumed (FAO, 2007-2017). In these countries, the industry is well developed and farmers produce fish at a commercial level. Nevertheless, in African countries like Tanzania, milkfish farming is still in its infancy stage practiced by small scale farmers with backyard milkfish ponds and there have been a number of pilot (demonstration) projects (Msuya and Mmochi, 2007). Milkfish farming has a long history in Tanzania which started in 1996 in Zanzibar using experiences from Asia (Dubi *et al.*, 2004; Mmochi *et al*., 2005). It is now spread in many places along the coasts including Pemba, Mtwara, Lindi, Kilwa, Rufiji, Mkuranga, Bagamoyo and Tanga (Msuya and Mmochi, 2007). In 2007 it was estimated that there were more than 100 milkfish ponds in Tanzania. Milkfish farming if well practiced is a good enterprise that can contribute to poverty alleviation for many people living along the coastal regions (Rice *et al.*, 2006). The industry has not yet taken off on a commercial scale possibly because of poor knowledge on commercialization, lack of technical knowhow on production and the associated infrastructure especially the pond construction, information on the economic and marketing aspects and minimal government support on the industry. It is common that local people get fish harvested from natural water bodies. Another reason that makes people not to realize the importance of investing on milkfish production is because of good supply from the natural fisheries.

Milkfish production activities in Tanzania are conducted in mangrove areas where by traditional earthen ponds of about 1 ha are common (Sobo, 2013). Tanzania has over 50,000 hectares of saline areas with a potential for milkfish production (Sullivan *et*

al., 2007). The Milkfish production aquaculture as an income generating activity is developing fast along the coast of Tanzania due to the fact that, mariculture became alternative source of livelihood apart from capture fisheries (Msuya and Mmochi, 2007). In Zanzibar, milkfish farming activities are carried out along the coastal areas in modified ponds that were previously used for salt production (Sullivan *et al*, 2007). A total annual production of milkfish in Zanzibar for 2015-2016 was reached 6.66 tons from 4.48 tons (2010) (MANLF-ZNZ, 2016).

Before establishing milkfish ponds, there are a number of issues which need to be taken into considerations. Entrepreneurs interested to establish milkfish farms are advised to have a selection of good sites for pond constructions. Pond selection requires specific water levels, soil texture and supporting infrastructure (Requintina *et al.,* 2008; Sullivan *et al*, 2007). The pond layout and design is important for the better performance of the farm. Most farmers in Zanzibar possess backyard ponds because of limited capital to afford commercial ponds. The construction of a commercial milkfish ponds needs a number of specifications as detailed by Requintina *et al.* (2008). There are specific requirements on stocking and management of milkfish pond. Pond stocking need a reliable good source of fingerlings which under the Tanzania set up, the main source is from the sea (Dubi *et al.,* 2004; Requintina *et al.,* 2008).

Aquaculture management practices are important for optimal fish production. It referred to the effective practical methods of reducing environmental impact levels to those compatible with resource management goals (Hairston *et al.,* 1995) or structural, vegetative, or management activities needed to solve one aspect of a

resource management problem including not limited to, fish stocking, fertilization, feeding, rate of water exchange, water quality and liming (Baluyut, 1989) Maintaining proper water levels, monitoring salinity, dissolved oxygen, appropriate water temperature, pH, turbidity, pond fertilization and supplementary fish feed are among the important operational activities. Table 1, illustrates recommended optimal ranges of water quality parameters for milkfish farming. Precautions like pest control and biosecurity are important since diseases and predations can be the sources of fish mortalities (Requintina *et al.,* 2008; Sullivan *et al.*, 2007). Under good pond management system, the rearing time from fingering introduction in the pond to harvesting is up to 8 months and the fish size should be between 450 – 600 g (Requintina *et al.,* 2008).

Table 1: Optimal water quality parameters for milkfish farming

Parameter	Optimum ranges
Dissolve Oxygen	3 - 5 ppm
Temperature	22 – 35 ^{o}C
pH	6.8 – 8.7
Salinity	18 – 32 ppt
Turbidity	0.5 m

Source: Requintina (2006)

2.3 Causes of mycobacteriosis in fish (fish tuberculosis)

Piscine mycobacteriosis in fish is a systemic, chronic, progressive disease with granulomas scattered on skin and parenchymatous tissues, especially in the spleen, kidney and liver (Frerichs, 1993; Belas *et al.*, 1995). Mycobacteriosis has been reported to affect a wide range of freshwater and marine fish species and aquatic

mammals, suggesting a ubiquitous distribution in environment (Belas *et al.,* 1995). Most commonly reported species isolated in many cases of *Mycobacterium* infection in fish and other animals include *M. marinum*, *M. fortuitum* and *M. chelonae* (Falkinham, 2009). However, other species known to cause mycobacterial disease in fish are *M. chelonei, M. neoaurum*, *M. simiae*, *M. shottsii*, *M. peregrinum*, *M. scrofulaceum*, *M. szulgai*, *M. interjectum* and *M. scrofulaceum* (Falkinham, 2009).

Mycobacteria species belong to the Phylum; *Actinobacteria*, Order; *Actinomycetales*, Suborder; *Corynebacterineae*, Family; *Mycobacteriaceae*, Genus; *Mycobacterium* (Gauthier and Rhodes, 2008). *M. marinum* is a natural pathogen of ectotherms such as frogs and fish. It has an extensive habitat and can live saprophytically in a warm aquatic environment. It is also reported to be isolated from human as zoonotic pathogen (Bhatty *et al.,* 2000). Large outbreaks of infection due to this atypical mycobacterium in human have been described in association with swimming, ornamental fish aquarium and fish handling and processing.

2.4 Mycobacteriosis caused by *M. marinum* in human

Mycobacteriosis in human due to *M. marinum* accounts for 0.04 to 0.27 per 100 000 cases worldwide (Pang *et al.*, 2007). The age of the most patients infected is reported to range from 38 to 45 years and 2–5 years and the source is reported to be water or other environmental sources through superficial abrasions. In a Singaporean study of 38 patients, 34% kept fish at home, 11% had fish-related occupations, and 32% had a history of trauma (Ang *et al.,* 2000). In a French study of 63 patients, 84% had exposure to fish tanks. In a Hong Kong report on 24 patients, 67% were fishermen

and 67% had sustained a puncture wound prior to seawater contact (Chow, 1987). Furthermore, there are several cases where *M. marinum* was reported to be isolated from human with severely symptomatic skin ulceration. Novotny *et al.* (2004) reported that, most of these patients have had worked with fish or had direct contact with contaminated water bodies.

2.5 Biology of *Mycobacterium marinum*

Mycobacterium marinum are aerobic, motile bacteria and characterized as acid fast (Ryan and Ray, 2004). *M. marinum* have an outer membrane (Niederweis, 2010), they possess capsules and sporulate (Jaydip *et al.*, 2009). However, the above fact has been contested by further research conducted by Traag *et al.* (2010).The general characteristic of all Mycobacterium species is that the cell walls are thicker than in many other bacteria, being hydrophobic, waxy, and rich in mycolic acids. The cell wall of *M. marinum* consists of the hydrophobic mycolate layer and peptidoglycan layer held together by a polysaccharide, arabinogalactan. The cell wall makes a substantial contribution to the hardiness of this genus. They adapt readily to growth on very simple substrates, using ammonia or amino acids as nitrogen sources and glycerol as a carbon source in the presence of mineral salts. *M. marinum* is said to be fast grower among mycobacteria since they may form colonies clearly visible to the naked eye within seven days on subculture (Austin and Austin, 1999). The cells are straight rods between 0.2 and 0.6 µm wide and between 1.0 and 10 µm long. They were classified in photochromogens (group I) since, they produces non-pigmented colonies when grown in the dark and pigmented colonies on exposure to light and reincubation. Staining characteristic of *M. marinum* include Fite's, Ziehl-Neelsen,

and Kinyoun stains. Mycobacteria species appear phenotypically closely related to members of *Nocardia, Rhodococcus* and *Corynebacterium* (Rhodes *et al.*, 2004).

Sequencing and assembly of the *M. marinum* genome are complete. Its 6.6 Mb genome is about 1.5 times the size of the *M. tuberculosis* genome, reflecting its expanded host and environmental range relative to *M. tuberculosis*. The *M. marinum* genome is 85% identical to orthologous regions of the *M. tuberculosis* genome, and coding sequence amino acid identity averages 85% between orthologues (Stinear *et al.,* 2008). Analysis of 16S rRNA among 80 mycobacteria species both confirmed the intimate evolutionary relationship between *M. marinum* and *M. tuberculosis* and suggested that the two species are derived from a common ancestor with the capacity for both host and environmental niches (Tonjum *et al.,* 1998; Gey van Pittius *et al.*, 2006). *Mycobacterium marinum* and *M. tuberculosis* have adaptive primitive immune system which preceded a divergence between them. In terms of sheer bulk, the greatest difference in genome composition is the extra 2.2 Mb that *M. marinum* carries relative to *M. tuberculosis*. It has been hypothesized that much of this sequence resulted from *M. tuberculosis* genome loss as its ancestral species relinquished an environmental niche and specialized to survive exclusively within a host. For example, the light induced production of beta-carotene protects *M. marinum* from photo-oxidation damage (Matthews, 1963). Thus, genes specifically required for pigment production like crtB – a gene encoding a phytoene synthase – have disappeared from *M. tuberculosis* (Ramakrishnan *et al*., 1997). Other genes with dual roles in pigment synthesis and protection from host singlet oxygen species are present in both species (Gao *et al*., 2003). Finally, *M. marinum* has continued to

acquire new loci. There is evidence of lateral gene transfer and gene duplication to expand *M. marinum* genome after the divergence from *M. tuberculosis* (Stinear *et al.,* 2008). The evolutionary history of *M. tuberculosis*, though predominantly marked by genome reduction, also includes 600 kb that is not shared with *M. marinum*. Many of these tuberculosis-specific regions were acquired by lateral gene transfer and preserved, perhaps as part of evolutionary exigencies of niche specialization. Altogether, 14% of the *M. tuberculosis* genome has no counterpart in *M. marinum*, and 8% of the *M. tuberculosis* genome is thought to have arisen by lateral gene transfer (Stinear *et al.,* 2008).

2.6 Epidemiology of *Mycobacterium marinum*

Mycobacteriosis is frequently reported to affect both wild and cultured fish species around the world. So far, more than 150 fish species have been reported to be infected by the Mycobacteria (Nigrelli and Vogel, 1963). The infection was observed to affect fish from tropical to subarctic latitudes (Bruno *et al.,* 1998; Diamant *et al.,* 2000; Rhodes *et al.,* 2004). *Mycobacterium marinum* has also been isolated from Nile tilapia (*O. niloticus*) in Campeche, Mexico (Lara-Flores *et al.,* 2014) whereas Gauthier and Rhodes (2009) reported high outbreak that occurred in Taiwan affected more than 50% of *C. chanos* farms. According to Ostland *et al.* (2008), *M. marinum* has been the major challenge in strip bass production industry in USA since 1999. According to Cirillo (1999) estimated loss due to *M. marinum* outbreak on HSB aquaculture production in USA was more than US $125 million in 1999.

Mycobacteriosis transmission in fish occur vertically through transovarian in live bearing fishes (Conroy, 1966) and horizontally through ingestion of contaminated

food and water (Gauthier *et al.,* 2003; Nenoff and Uhlemann, 2006). In addition, observational study on zebra fish revealed that, embryonic transmission occurred when experimental fish challenged with *M. marinum* (Davis *et al.,* 2002), both bath exposure and gavage have been used to infect adult zebra fish during experiment with *M. marinum* and *M. peregrinum* (Hariff *et al.,* 2007). The latter study indicated that, gut is more susceptible and a primary site for infection.

The risk factor for disease transmission in fish and human are several. According to Noga (2000), closed aquatic systems with a high density of fish and warm waters appear to be favourable condition to *M. marinum*. Poor overall water quality and various nutritional deficiencies in fish rearing system have also been implicated as contributing factors to the outbreak of mycobacteriosis (Noga, 2000). The possible causes of *M. Marinum* infection in human including; poor handling practices, healthy hosts possesses aquatic trauma (injured) and a person with immune suppression condition e.g. HIV or AIDS etc. reported to be more susceptible to *M. marinum* infection (Sette *et al.,* 2015).

2.7 Clinical sign in fish and human

Clinical signs that can be observed in sick fish during diagnosis include; lethargy, anorexia, fin and scale loss, exopthalmia, emaciation, skin inflammation and ulceration, edema, peritonitis and nodules in muscles that may deform the fish (Bhatty, 2000). Post-mortem examinations usually reveal gray or white nodules in the liver, kidney, heart or spleen, there also may be skeletal deformities (Sette *et al.,* 2015; Bhatty, 2000). In humans, *M. marinum* infection is categorised into 3 types. Type I forms self-limiting verrucal lesions, type II forms single or multiple

subcutaneous granulomas with or without ulceration, and type III results in deep infections involving the tenosynovium, bursa, bones or joints causing tenosynovitis, septic arthritis or osteomyelitis (Bhatty, 2000). Deep infections of the latter type usually results from extension of cutaneous infections or direct inoculation, rather than through haematogenous spread (Sette *et al.*, 2015).

2.8 Diagnosis of fish mycobacteriosis

The diagnosis of *M. marinum* infection is based on clinical signs, post-mortem examination of the fish and the presence of acid fast bacteria in tissue sections. Bacteriological culture and identification of the organisms is among the conventional diagnosis method. Bacteriological examinations comprise the isolation of *M. marinum* on Lowenstein-Jensen (L-J media) with glycerol or pyruvate followed by demonstration of acid-fast bacilli through ZN staining. The use of appropriate media and biochemical tests are preferred as diagnostic tools on routine basis (Chinabut, 1999; Rhodes *et al.,* 2004). Recently, molecular techniques like diagnostic Polymerase Chain Reaction (PCR) are used in confirmation of the infection in fish. Real time PCR may be used for determination of the occurrence of *M. marinum* in fish and water samples, using ITS primers 16SCF and 23S-23R, consisting of 250 nucleotides (nt) of ITS sequence and 250 nt of flanking SSU rDNA sequence. For hsp65, reverse-primer TB12 was used with either forward primer TB11 or HS1F to generate a fragment approximately 400 or 550 bp, respectively as described by (Ucko *et al.,* 2002; Whipps *et al.,* 2003).

2.9 Treatment and control

Kanamycin with Vitamin B-6 for 30 days is the most effective treatment of mycobacteriosis in fish. For effective outcome and avoiding disease from spreading and infecting other population, fish should be quarantined during treatment time. Supplementation with vitamin B-6 is important during the course of treatment (Cheung, *et al.*, 2010). Optimum stocking is highly recommended to minimize outbreak of the disease in culture systems.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The field work that involved interview with fish farmers and sample collection was conducted in milkfish farming areas which are located in five districts of Unguja and Pemba (Figure1) in Zanzibar Island, the study involved 24 farming sites of which, 19 are located in Pemba and five in Unguja (Annex 3). The milkfish farming sites were purposively selected based on the criterion that, they were still producing fish during the sample collection period. Geographically, Zanzibar is positioned at latitude 06 º 08 'S and longitude 39 º19 'E with average annual precipitation of 84.63 cm. The peak rain is during April with a monthly average precipitation of 17.86 cm. Zanzibar Islands have binomial mode of rainfall distribution, the heavy rainfall occurs between March and May and the short rainfall between October and December. The annual temperature ranges between 21.3 °C and 30.7 °C.

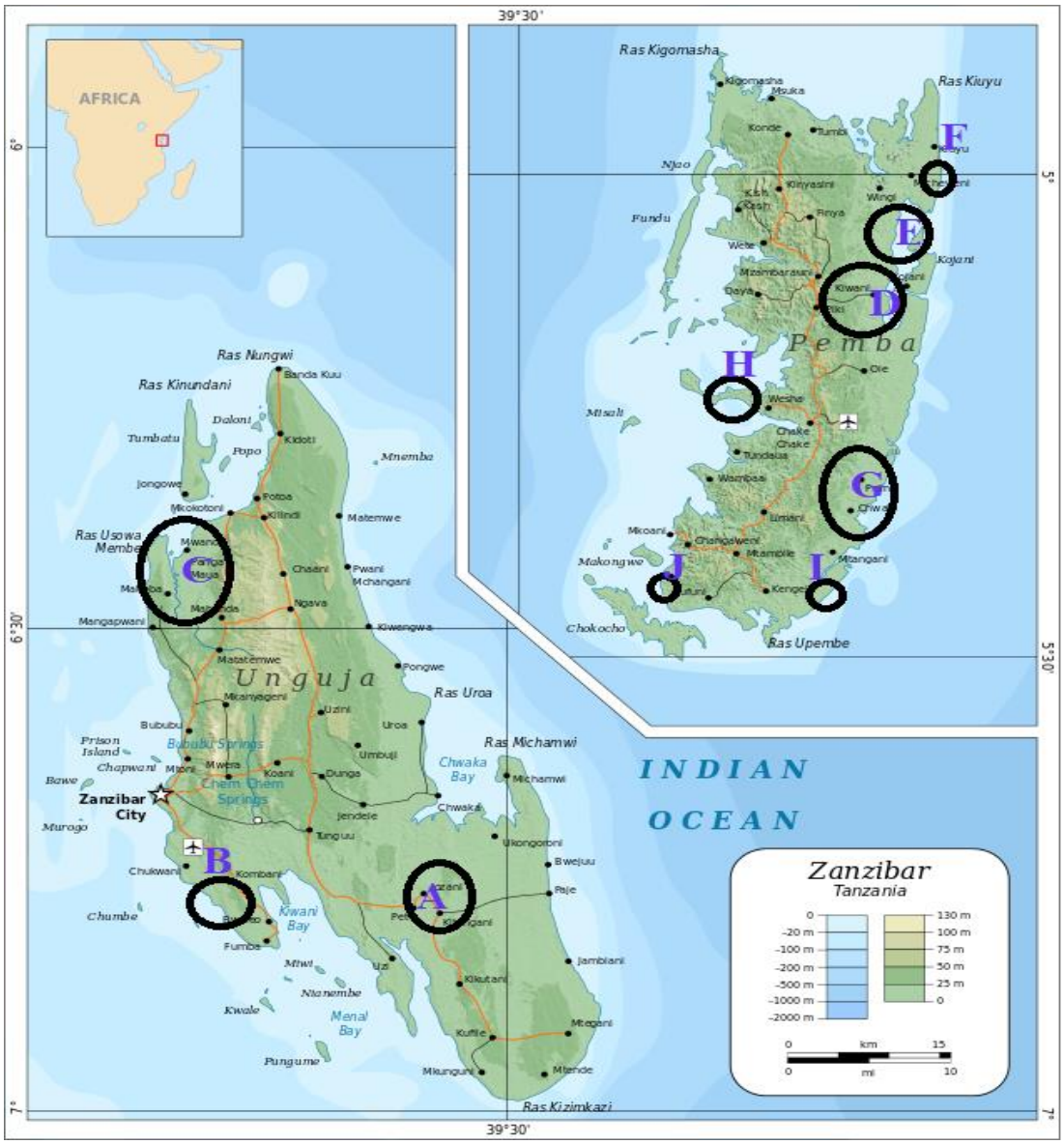

Figure 2: Milkfish farming areas of Zanzibar involved during the study. *Insert* Map of Africa shows the geographical location of Zanzibar.

Note that the black circled areas labeled: A = Jozani (South District); B = Shakani (West A District); C = Donge Muwanda and Bumbwini fish (North B District); D = Kangagani, Kiuyu, Shengejuu, Kiwani and Kambini fish (Wete Distric); E = Chwale and Kiungoni (Wete District); F = Mjananza ; G = Chambani, Pujini and Furaha (Chakechake District); H = Ndagoni (Chakechake District); I = Mwambe (Mkoani District); and J = Chokocho (Mkoani District).

3.2 Study design, inclusion criteria and sample size

The cross-sectional study design was used where interviews with fish farmers was administered and samples of fish, water and sediments were collected between November 2016 and May, 2017. During the study, both fish which showed clear clinical sign of the mycobacteriosis and the apparently healthy fish were analysed for *M. marinum*. Pond sediment and water samples were also collected from fish ponds and were analysed in the laboratory. The size of the fish sampled ranged between 10 cm and 45 cm. A total of 24 water and 24 sediment samples (one sample from each farm) were collected and analysed for *M. marinum*.

The sample size was determined by a formula by Thrusfield (2005): $N= Z^2 P (1-P) /\Sigma^2$. N is sample size, Z is constant (1.96), P is prevalence and Σ is error margin (0.05). Since there have been no study on *M. marinum* in milkfish farming in Zanzibar, an assumed prevalence of 50% was used in the calculation, which gave a sample size of 384 for each. However, due to financial, time constraint and uncertainties, the total number of the samples including, fish, water and sediment collected were 288.

3.3 Study Fish and management system

When selecting fish for sample collection, fish weight, size and sex were determined together with farm location, fish age and management practices. *Chanos chanos* was the only fish species used in this study. For the purpose of this study, fish which were 10 – 35 cm in length were considered for sampling because, they were easy to identify their anatomical feature and easy to handle during dissension.

3.4 Data collection

Two types of data were collected; managerial and laboratory based data.

3.4.1 Managerial data collection

Structured questionnaires were used which focused on all selected fish farmers to obtain information regarding the general fish farm management practices (Appendix 1). In addition to that, check list was used for physicochemical parameters of the pond's water (Appendix 2). The questionnaires were made of pre coded close ended questions with very few open ended questions.

3.4.1.1 Pretesting of questionnaires

Prior to starting of data collection, the questionnaires were tested for clarity and time management. After testing they were revised and corrected accordingly. The revised questionnaires were translated into Swahili language for easy understanding by respondents.

3.4.1.2 Administration of the questionnaire

Before sampling of water, fish and sediment a questionnaire was administered to the fish farm owners. The key information collected included demographic characteristic of the respondent, aquaculture management system and water utilization scheme, fertilizer application, fish disease management, feeding regime and fish stocking density. A total of 24 respondents were interviewed. In addition, group leaders (chairmen or secretaries) of fish farmers associations were intervened to get more information regarding milk fish farming in the study areas.

3.4.1.3 Physicochemical water quality assessment and fish sampling

After the questionnaire administration, pond location, pond type and fish raring system including feeding were assessed and recorded accordingly. Physicochemical water quality parameters like temperature, pH, salinity, conductivity and dissolved oxygen were measured *in situ* using a portable water quality checker (Horiba U-10, Japan) (Figure 2A). Then the farmers were requested to harvest fish from the ponds for sampling (Figure 2B). The commonly used gear was a 10 mL x 2 mD net with the mesh size of 2". A total of ten fishes were collected randomly from the net at each fish farm and used as sources of samples. The live fishes were placed in a plastic bucket and anaesthetized using clove oil and externally examined for lesions which may suggest mycobacteriosis infection. Attention for lesions suggestive of mycobacteriosis included lethargy, fin and scale loss, exopthalmia, emaciation, skin inflammation and ulceration. Thereafter, weight and total length of each fish determined and recorded. The cutoff point of the fish size selection used for sampling was between 10 cm and 35 cm.

Figure 3: ***In situ*** **data and samples collection**
Note that: Physicochemical water quality assessment (A); harvesting of fish (B); dissection of fish (C); water, sediments and swabs samples in universal bottle (D)

Each fish was placed in a dissection plate and dissected to display out internal visceral organs for examination and swab sample collections. The fish was laid on a dissection plate on its right side with the abdomen directed towards the dissector (Figure 2). Using a pair of scissors, the body cavity was opened in two slits. The first cut run from the rectal opening up to the branchial cavity, the second cut through an arch from the anus up to the spine and further to the upper side of the branchial cavity. Organs and tissues were first examined in situ for any gross pathological abnormalities. The lesions which were being observed included edema, peritonitis, nodules in muscles and skeletal deformities. Other expected lesions in the visceral organs included gray or white nodules in the liver, kidney, heart or spleen. Ethanol

(99.79%) was used for sterilization of the dissection surface, scissors, forcepts and other equipment after completion of sampling of one fish. Handgloves were also changed after every fish. All the internal organs were carefully examined for any lesions suggestive of mycobacteriosis. Swab sampling involved the kidneys as an organ which is very susceptible to mycobacteriosis infection . Moistened sterile cotton swab sticks were used to swab the kidney and immediately immersed in universal bottles containing Middle brook broth (BDH Chemicals Ltd, Poole, UK) which were properly capped before they were preserved in cool box with ice packs which was later taken to Laboratory of the Department of Livestock Production Zanzibar for further preservation. A total of 240 kidney swab samples were collected and subsequently transported under cold chain to Sokoine University of Agriculture (SUA) TB lab for *M. marinum* analysis.

3.4.1.4 Water sampling

Water samples were collected from ponds of each farm by using sterile plastic Pasteur pipette. A total of 50 to 60 ml of water sample was collected at 10 cm below the water surface and added into sterile universal bottles (Figure 2) which were caped tightly and preserved in cool box with ice packs. A total of 24 water samples were collected and subsequently transported under cold chain to SUA TB lab for *M. marinum* analysis.

3.4.1.5 Sediment sampling

The metal scooper was used to collect sediment samples from the pond bottoms. The sediment were scooped and filtered off water, then filled into sterile universal bottle carefully capped and immediately preserved into cool box with ice packs. Twenty

four sediment samples were collected and transported under cold chain to SUA TB lab for *M. marinum* analysis.

3.4.2 Laboratory sample analysis

3.4.2.1 Mycobacterial culture from fish kidney swab samples

In the laboratory, culture for isolation of *M. marinum* was done as described by OIE (2006). All the processes of sample preparations and inoculations into media were done under biosafety cabinet level 2 conditions. The kidney swab samples in Middle brook broth (BDH Chemicals Ltd, Poole, UK) were used in inoculation to the Löwenstein-Jensen media (BDH Chemicals Ltd, Poole, UK) for isolation of *M. marinum*. The Middle brook broth with the sample was transferred to a sterile 50 ml universal container and an equal volume of 3% Oxalic acid was added for decontamination purposes followed by intermittently shaking of the mixture for at least 30 minutes. This was followed by addition of 2% solution of Sodium Hydroxide to neutralize the oxalic acid. Phenol Red solution (1%) was added to the final mixture of the acid and base to check if the neutral point of pH 7 was attained. About 3 ml of the sample was added into two Löwenstein-Jensen (LJ) slants supplemented with either sodium pyruvate or glycerol and the cultures were incubated at 37 ℃ and observed for 2-3 consecutive days to assess if there are contaminations.

The cultures were observed weekly for growth of *Mycobacterium* colonies for 8-10 weeks. Positive cultures with colony morphological features as described by Vestal and Kubica (1966) were sub cultured onto blood agar and incubated for another two to four weeks. Macromorphology of colonies was used as the first stage of

Mycobacterium identification on L-J glycerol and L-J pyruvate media. *M. marinum* produces yellowish pigments (chromophogenic) when exposed to light.

The suspect colonies were subjected to Acid Fast Bacteria (AFB) analysis as a preliminary confirmatory test for *Mycobacteria* as described by Ziehl-Neelsen. Briefly, the bacterial smears were prepared and allowed to air dry. Then the smears were heat fixed and covered with carbol fuchsin stain. Heat was applied to the mixture until vapour began to rise at about 60 °C. The slide was allowed to stain for another 5 minutes and then washed with clean water. The smears were covered with 3% v/v acid alcohol solution for 5 minutes to allow for sufficient decolourization followed by washing with tap water. The smear was covered by malachite green stain for 1-2 minutes and then washed again with tap water. The slides were air dried on the draining rack and examined with light microscope at 100X magnification. Results were interpreted as positive when bright red, straight or slightly curved rods, singly or in small groups were seen.

3.4.2.2 Culture of Mycobacterium from sediments and water samples

The water and sediment samples were transferred to a sterile 50 ml universal container and centrifuged for 10 minutes at 30 revolutions per minute. An equal volume of 3% Oxalic acid was added for the purpose of decontamination and the mixture was shaken for 30 minutes. The supernatant was discarded followed by addition of 2% solution of Sodium Hydroxide into the sediment so as to neutralize the oxalic acid. Phenol Red solution (1%) was added to the final mixture to attain the pH of 7. Aliquots of the remaining sediments were inoculated onto Lowenstein-Jensen (L-J) media with Pyruvate and Glycerol (BDH Chemicals Ltd, Poole, UK)

and incubated as previously described for fish samples. Also preliminary identification of colonies macromorphologically and AFB analysis was done.

DNA extraction and* Mycobacterium *identification

Cultures that had colonies suggestive of *Mycobacterium* and AFB positive were subjected to the Mycogenus typing heat-killed method. Mycobacteria colonies were harvested with disposable sterile wire loop and added into a screw capped sterile 1.5 ml eppendorf tube (Eppendorf, AG, Hamburg, Germany) containing 200 ul of nuclease free water and mixed thoroughly. Thereafter, the sample was incubated at 80 °C for 1 hour to allow for inactivation of the Mycobacterial cells and also enable release of bacteria genome as extraction method for the DNA. The sample was transferred directly in the tray containing ice cubes ready for PCR. Alternatively, the heat-killed cells were stored at 4 °C until further laboratory analysis.

3.4.2.3 Molecular identification of Mycobacteria suspect samples

All the heat-killed cell samples stored at 4 °C were thawed for Polymerase Chain Reaction (PCR) laboratory analysis.

3.4.2.4 DNA quality and quantity determination

The DNA quality and quantity was determined through electrophoresis on 1.5% agarose gel. Through visual comparison, the intensity and conformation of bands between template DNA and the makers were determined. A single clean band of the DNA with high intensity indicated that the DNA extracted was of good quality.

3.4.2.5 Molecular identification by PCR

The technique was adopted from the PCR protocol described by Berg, (2007; 2008a and 2008b). It is specific for identification of species of *Mycobacterium* and used to differentiate species of the *M. tuberculosis* complex from *M. avium*, *M. intercellulare* and other *Mycobacterium* species. The PCR setup for genus typing incorporated equipment like, Laminar flow cabinet (DNA free), filter tips, Nuclease free water (qiagen), DNA polymerase, buffer, $MgCl_2$, dNTPs and DNA templates. It also involve a set of primers, forward 100 µM MYCGEN- F 5'- AGA GTT TGA TCC TGG CTC AG- 3' and 100 µM MYCGEN- R 5' –TGC ACA CAG GCC ACA AGG GA- 3' which are designed to target and amplify a sequence of highly conserved region within the 16S rRNA gene that is specific for the *Mycobacterium* genus. The primers produce PCR product of the size 1030 bp band (Berg *et al.,* 2008b).

3.4.2.6 Preparation of DNA template

The template of DNA from heat killed cells were immediately quenched from ice before adding 2 µl of it in the 18 µl of the master mix in 0.2 ml flat cap PCR tube to make a final volume of 20 µl of the reaction mixture.

3.4.2.7 Preparation of the master – mix for Mycogenus PCR

The PCR amplification was performed in a final volume of 20 µl containing 12 samples reaction of a Qiagen product protocol as shown in Table 2.The reaction contains mixture for 22 samples plus two controls.

Table 2: Mycogenus PCR 20 µl reaction mixture for 12 samples

Reagent	X1 reaction unit vol (µl)	X 12 reaction total vol. (µl)
RNase free water	7.4	88.8
100 µM Mycogen - Forward	0.3	3.6
100 µM Mycogen - Reverse	0.3	3.6
Qiagen Master mix ($MgCl_2$, DNTPs)	10	120
Total	18	216
Template DNA	2	24
Total reaction mixture	20	240

3.4.2.8 Setting of the mycogenus PCR

The total reaction mixture above was put into PCR reaction tubes and ran into the eppendorf thermocycler using the conventional PCR protocol. The samples were run in a PCR thermocycler machine (Bio-Rad, Hercules, USA). The reaction was subjected to 35 cycles as shown in Table 3.

Table 3: Reaction phases undertaken in Multiplex PCR

Reaction stage	Temperature (°C)	Time (minutes)
Initial denaturation	95	10
Actual denaturation	95	1
Annealing	61	30
Extension/elongation	72	2
Final elongation	72	10
Cooling/hold	4	4

3.4.2.9 Preparation of agarose gel

Agarose gel was prepared by mixing 1.65 of Agarose powder LE, Analytical Grade (PromegaMadson, U.S.A) with 1 x TBE buffer filling to 150 ml to obtain 1.5% concentration of the gel. Agarose powder was dissolved by heating the solution on a hot plate at 100 °C. A volume of 3.0 µl of ethidium bromide (0.06% v/v) solution was added to every 150 ml at 60 °C of molten agarose to obtain the final

concentration to 1.5 µg/ml and mixed thoroughly by a mixer. Molten agarose was then poured into the electrophoresis gel casting equipment and left for half an hour to set.

3.4.2.10 Loading of PCR products into agarose gel and electrophoresis

Before loading DNA samples, a 2 µl of loading blue dye 6X (Promega MADISON, WI USA) was added and mixed in every 8 µl of DNA to be analyzed. For each analysis, the first well of the gel was loaded with DNA molecular maker 100 bp DNA ladder (promega MADISON. WI USA) of 1 or 1.5 kb size. The molecular weight marker was run parallel with the DNA of sample, positive and negative control (Nuclease free water) in 1 x TBE buffer horizontal gel electrophoresis apparatus at a constant voltage of 100 V for 90 minutes. Samples were electrophoresed in 1.5% agarose gel stained with Ethidium Bromide (3 µl into 110 ml- 1 x TBE buffer trough) and observed under UV light.

3.4.2.11 Visualization of DNA

Visualization of bands was done by placing the electrophoresed agar rose gel to a medium wavelength ultra- violet (UV) light transluminator (STX- 20, Jencons Ltd, USA). A digital camera was used to document digital image of both DNA ladder and samples viewed within agar rose gel and then the bands were evaluated.

3.5 Data analysis

All data were entered into a Microsoft Excel spread sheet and transferred into Epi Info™ version 7 (Center for Disease Control and Prevention (CDC) (2014) software.

The number of fish with lesions suggestive of mycobacteriosis and the number of *M. marinum* detections in tissues, water and sediments were summarized using descriptive statistics. The prevalence of *M. marinum* fish, water and sediments were calculated using the formula:

$$\text{Prevalence} = \frac{\text{Number of fish or water or sediments testing positive}}{\text{Total number of fish or water or sediments tested}} \times 100$$

The mean and standard deviation values of physicochemical parameters were calculated using Microsoft Excel and presented in Tables. Differences in physicochemical parameter values between sampling stations were compared by one-way ANOVA ($Yij = \mu+Ti+ij$) whereby the, F- test was used to measure the mean variation among the fish farming sites (in Pemba or Unguja) and T-test to measure variation between two areas (Pemba and Unguja) at 95% significance level. Descriptive statistics like frequencies, mean and standard deviations was computed for proportions of infected fish, ponds and locations.

3.6 Ethical Consideration

Research permit was granted by Sokoine University of Agriculture and permission letters were obtained from the Principal Secretary of the Ministry of Agriculture, Natural resources, Livestock and Fisheries Development – Zanzibar. Verbal consent was obtained from each fish farmer after explaining the purpose and importance of the study prior to commencement of interviews and sampling. Participation in the study was on voluntary basis. All the information collected from the participants and laboratory results obtained will remain confidential.

CHAPTER FOUR

4.0 RESULTS

4.1 Questionnaire results

4.1.1 Demographic characteristics of the Respondents

The demographic characteristics of respondents are summarized in Table 4. A total of 24 respondents were interviewed from 24 representatives of fish farms in Zanzibar. Among the respondents (91.7%) were males and most of them aged between 36 and 45 years. Majority of the respondents (70%) had been doing fish farming for about 10 years. Out of 24 milkfish farms studied, 19 were based in Pemba and 5 in Unguja. It was found that farmers get training on milkfish farming twice in a year which is coordinated by the Fisheries Department of Zanzibar in collaboration with experts from China. Most of milkfish farmers (92%) use milkfish farming practices as a supplementary source of income.

Table 4: Demographic characteristics of respondents

Demographic information	Category	Number (%) of respondents
Sex	Male	22 (91.7)
	Female	2 (8.3)
Location	Unguja	5 (20.8)
	Pemba	19 (79.2)
Districts	West "A"	1 (4.2)
	South	1 (4.2)
	North "B"	3 (12.5)
	Mkoani	2 (8.3)
	Chakechake	7 (29.2)
	Wete	9 (37.5)
	Micheweni	1 (4.2)
Age	20 - 35	3 (12.5)
	36 - 45	10 (41.7)
	46 - 55	9 (37.5)
	56 - 65	2 (8.3)
Fish farming experience (years)	Up to 10	18 (75)
	11 – 20	5 (20.8)
	21 & above	1 (4.2)
Training on milkfish farming	Yes	24 (100)
	No	0 (0.0)
Is milkfish farming the sole source of income?	Yes	2 (8.3)
	No	22 (91.7)

4.1.2 Aquaculture systems and management techniques

The responses from respondents on milk fish farming system and management practices are shown in Table 5. Most (91.7%) pond type were earthen one which were mostly found in salt flats behind the mangroves and the numbers of raring unit per farm ranged from 1 to more than 10 units. Half of the milkfish farms (50%) studied use the ponds that were formerly used as salt pans. The rearing techniques differed from one farm to another and majority of farmers applied polyculture rearing technique. The main sources of fingerlings were from the wild environments (sea) which were sometimes not available in some seasons. Accordingly, most

farmers (92%) were overstocking ponds. In some cases, fish were fed with fish formulated feed from maize or rice bran mixed with fish meals but In the most cases fish sources their feed from the natural environment. Supplementary feed like leftovers from the farmer's households were introduced. Fish farms were fertilized with livestock and poultry manures. Water exchange in fish farms was reported to be done twice a month during the spring tides. Stocking seeds are collected from the wild and harvesting of the fish reaching the market size (fish weight ranging between 450 g to 600 g) normally is carried out between six and eight months from the first date of introduction to the pond. Although, to the other farms harvesting took long and reported to exceed one year. Mosquito nets were the common fishing gears for fingerling collection. All respondent (100%) interviewed confessed not to use protective gears when doing their fish farming activities. There were no strategies of fish diseases control like vaccination and or other veterinary services provided to the milkfish farms.

Table 5: Milk fish farming system and management (n=24)

Variables assessed	Category	Number of response (%)	P value
Farm location	Mangrove parchment areas	22 (91.7)	0.0021*
	Other areas	2 (8.3)	
Did you build the fish pond?	Yes	12 (50.0)	1.0000
	No, it was being used as salt pan	12 (50.0)	
Culturing system	Earthen pond system	22 (91.7)	0.0021*
	Other systems	2 (8.3)	
Number of system units per farm	1- 6	19 (79.2)	0.0155*
	Above 6	5 (20.8)	
Sources of fingerings	Wild environment (sea)	24 (100.0)	NA
	Hatcheries	0 (0.0)	
Knowledge on stocking density	Yes	0 (0.0)	NA
	No	24 (100.0)	
Farming techniques	Monoculture	15 (62.5)	0.2448
	Polyculture	9 (37.5)	
Feeding	Yes	18 (75)	0.0320*
	No	6 (25)	
Feeding frequency	Once / day	7 (29.2)	0.3451
	Twice - thrice / day	11 (45.8)	
	Never	6 (25.0)	
Feed composition	Carbohydrate + Fat sources	10 (41.7)	0.76891
	Carbohydrate + Protein + Fat + Vitamin sources	8 (33.3)	
	Neither	6 (25)	
Water exchange	Yes	11 (45.8)	0.6881
	No	13 (54.2)	
Manure application	Yes	9 (37.5)	0.2448
	No	15 (62.5)	
Manure type	Cow / goat dung	6 (25.0)	NA
	Poultry drops	1 (4.1)	
	Fresh leaves	0 (0.0)	
	Composites	0 (0.0)	
	Cow /goat dung + poultry	2 (8.3)	
	NA	15 (62.5)	
Fishing gears used	Mosquito nets	18 (75.0)	0.0320*
	Seine net	6 (25.0)	
Period from introduction of fish in a pond to harvesting	More than one year	22 (91.7)	0.0021*
	Six months to one year	2 (8.3)	
Incidences of harvested fish not to get customers then spoils	Yes	0 (0.0)	NA
	No	24 (100.0)	

** = statistically significant*
NA = not applicable

4.1.3 Health and related problems in milkfish ponds

Almost all fish farmers did not know issues related to fish health, diseases prevention and control. The biosecurity measures were not in place which makes the fish ponds more prone to infections. In case of fish health problems and mortalities, few farmers (17%) reported to fisheries officers for a help. Problems of incidences of pond drying, fish diseases and mortalities were also being experienced in some farms. Majority of the fish ponds (92%) had no system of pest control (Table 6). Fish farmers reported to experience problems of birds and predator fishes feeding on the milkfish.

Table 6: Summary of health and related problems in milkfish ponds in Zanzibar

Fish health issues assessed	Response	Number (%) of response	P value
Knowledge on fish health, diseases prevention and control	Yes	0 (0.0)	NA
	No	24 (100.0)	
Disease incidences	Often	4 (16.7)	0.2385
	Sometime	7 (29.2)	
	None	13 (54.1)	
In case of disease occurrence in the fish ponds, what do you do?	Report to fisheries officer	4 (16.7)	0.0077*
	Do nothing	20 (83.3)	
Are you aware that tuberculosis can affect milkfish?	Yes	0 (0.0)	NA
	No	24 (100.0)	
Ever encountered fish with skin lesions which may be suggestive of *Mycobacterium* infection?	Yes	0 (0.0)	NA
	No	24 (100.0)	
Did you experienced fish mortality?	High	9 (37.5)	0.2448
	Normal	15 (62.5)	
Are there pest control systems in the ponds?	Yes	22 (91.7)	0.0021*
	No	2 (8.3)	
Use of protective gears during fishing activities	Yes	0 (0.0)	NA
	No	24 (100)	
Problems of pond drying?	Often	8 (33.3)	0.06738
	Sometime	7 (29.2)	
	Do not	9 (37.5)	

** = statistically significant*
NA = not applicable

4.1.4 The physicochemical characteristic of water in the milkfish farms

The Table 7 summarizes the physicochemical characteristic of the water assessed in the 24 fish farms. The water temperature of all fish farm ranged from 29.3 ^{o}C to 37.1 ^{o}C. The mean water temperature recorded was highest (37.3 ± 1.4 ^{o}C) in Micheweni district and lowest (29.7 ± 0.3 ^{o}C) in ponds of West "A" District. Ponds found in the four districts had the temperatures exceeding the upper range of the recommended level of 32 ℃ for milkfish ponds (Requintina *et al.*, 2006; Pickering, *et al.*, 2012). The variations of water temperature in milkfish farms among the districts in Pemba varied significantly ($P < 0.05$). Comparisons of temperature variation between Unguja and Pemba, indicated statistical significance differences ($P < 0.05$).

The DO (mg/L) ranged between 1.9 and 6.1 mg/l. The concentration of dissolved oxygen was highest (6.1 ± 0.9 mg/l) at West A district and lowest (3.2 ± 1.5 mg/L) at North. Almost all farms (except one in South) had concentrations of dissolved oxygen exceeding the maximum of 5 mg/l recommended for milkfish farming. Three farms had at certain period a DO below the recommended level of 3 mg/L.

The pH reading indicated a range of 7.2 to 7.8. The mean pH range was from 6.9 to 8.4 and; only one pond in Chakechake had pH level beyond the recommended upper limit of 8.5 (Requintina *et al.*, 2006; Pickering *et al.*, 2012). The fish ponds of South district in Unguja were recorded to be alkaline with mean pH (8.2) higher by 1.2 pH value against West A district.

Salinity was high in all the assessed ponds ranging between 32.3 g/l to 67.7 g/l. The mean salinity and TDS were observed to be higher in both Wete and Chakechake compared to other districts in Pemba (Table. 6). The statistic $F (4.09) > F (3.29)$

indicated that there was a significant differences in mean salinity of the farms among the districts in Pemba at 95% confidence level and the tested statistic for TDS was F (2.00) < f (3.29). All the ponds except one in West “A” district had salinity exceeding the upper recommended optimum range of 35 (Pickering *et al.*, 2012).

Table 7: Physicochemical characteristic of water in milkfish farms

Districts where ponds are located	Physicochemical parameter	Mean ± SD	Range
	Temperature (°C)	31.5 ± 02	31.3 - 3.7
	Dissolved oxygen (mg/l)	4.6 ± 1.6	2.9 – .0[b]
South	pH	8.2 ± 0.2	8.0 – 8.4
	Salinity (g/l)	35.8 ± 3.0	32.3 - 37.5[c]
	TDS (g/l)	35.2 ± 2.6	32.3 – 36.9
	Temperature (°C)	32.1 ± 1.4	29.9 – 34.6 [c]
	Dissolved oxygen (mg/l)	3.2 ± 1.5	1.7 – 6.3 [b]
North B	pH	7.5 ± 0.5	6.9 – 8.6 [b]
	Salinity (g/l)	38.7 ± 5.0	30.5 – 46.7 [c]
	TDS (g/l)	37.9 ± 4.4	30.7 – 44.6
	Temperature (°C)	29.7 ± 0.3	29.3 – 29.9 [c]
	Dissolved oxygen (mg/l)	6.1 ± 0.9	5.1 – 6.7 [c]
West A	pH	7.6 ± 0.2	7.5 – 7.8
	Salinity (g/l)	7.1 ± 0.6	6.7 – 7.9
	TDS (g/l)	8.2 ± 0.7	7.8 – 9.0
	Temperature (°C)	33.4 ± 1.6	30.6 – 36.5 [c]
	Dissolved oxygen (mg/l)	5.1 ± 1.1	2.3 – 6.9 [b]
Wete	pH	7.9 ± 0.3	7.2 – 8.6 [b]
	Salinity (g/l)	48.3 ± 9.7	33.1 – 67.7 [c]
	TDS (g/l)	42.7 ± 13.4	10.6 – 62.4
	Temperature (°C)	37.3 ± 1.4	36.2 – 39.4 [cc]
	Dissolved oxygen (mg/l)	4.6 ± 0.5	4.2 – 5.1
Micheweni	pH	7.8 ± 0.1	7.7 – 7.9
	Salinity (g/l)	40.4 ± 5.4	37.6 – 48.5 [cc]
	TDS (g/l)	39.4 ± 4.7	36.7 – 46.5
	Temperature (°C)	35.0 ± 1.9	31.4 – 38.5 [c]
	Dissolved oxygen (mg/l)	4.7 ± 0.9	3.4 – 7.1 [c]
Chakechake	pH	7.9 ± 0.3	7.5 – 8.9 [c]
	Salinity (g/l)	47.5 ± 8.6	39.3 – 68.2 [cc]
	TDS (g/l)	45.5 ±7.1	38.2 – 62.2
	Temperature (°C)	30.5 ± 1.4	28.5 – 31.9
	Dissolved oxygen (mg/l)	5.6 ± 2.3	3.2 – 9.3 [c]
Mkowani	pH	7.9 ± 0.3	7.3 – 8.2
	Salinity (g/l)	21.8 ± 17.8	5.1- 38.3 [c]
	TDS (g/l)	21.9 ± 16.9	5.9 – 37.5

Note that the optimal range of water parameter for milk fish ponds are:
Temperature = 26 – 32; dissolve oxygen = 3 – 5; pH = 7.5 – 8.5 and salinity = 0 – 35 (Requintina *et al.*, 2006; Pickering, *et al.*, 2012).
Key:
cc = Both lower and upper values of reported range exceeded the optimum range;
a = Only lower value was below the optimum range;
b = Lower value below the optimum range and the upper value was above the optimum range;
c = Only upper value was above the optimum range;
TDS = Total Dissolve Solids

4.1.5 Results on fish measurements and pathological lesions

Fish measurements showed the total length ranged from 10 cm to 35 cm (mean 20.63 cm) while the weight ranged from 14.1 g to 521.5 g (mean = 95.8 ± 2.3 g). Most of the fish were female 62%. On detailed examination of the external skin surface and internal visceral organs, none of the fish was found to have lesions which could suggest presence of mycobacteriosis. All of the fish appeared normal, with their elongated body and had small, smooth scales, olive green and silvery flanks.

4.1.6 Bacteriology and acid fast testing in fish, water and sediments

A total of 240 fish kidney swab samples were collected from 240 fish and cultured on L-J media. Table 8 summarizes the results on bacterial growth and AFB positive isolates. It was found that 34.2% of the fish kidney samples had bacteria colonies on LJM but only 7 (2.9%) were found to be AFB positive isolates (Fig. 4) suggestive of *Mycobacterium*. In total, 12 (4.2%) of all 110 isolates from fish, water and sediments were AFB positive.

Table 8: Bacterial growth and test results for acid fast bacteria

Samples type	Bacteria isolates on Lowenstein-Jensen media (LJM)		Total number (%) of growth	Number (%) of AFB positive
	Isolates on LJM with pyruvate	Isolates on LJM with glycerol		
Fish (n=240)	49 (20.4)	33 (13.8)	82 (34.2)	7 (2.9)
Water (n=24)	5 (20.8)	6 (25.0)	11 (45.8)	2 (8.3)
Sediments (n=24)	8 (33.3)	9 (37.5)	17 (70.8)	3 (12.5)
Total (n=288)	62 (21.5)	48 (16.7)	110 (38.2)	12 (4.2)

Key:

AFB = Acid Fast Bacteria

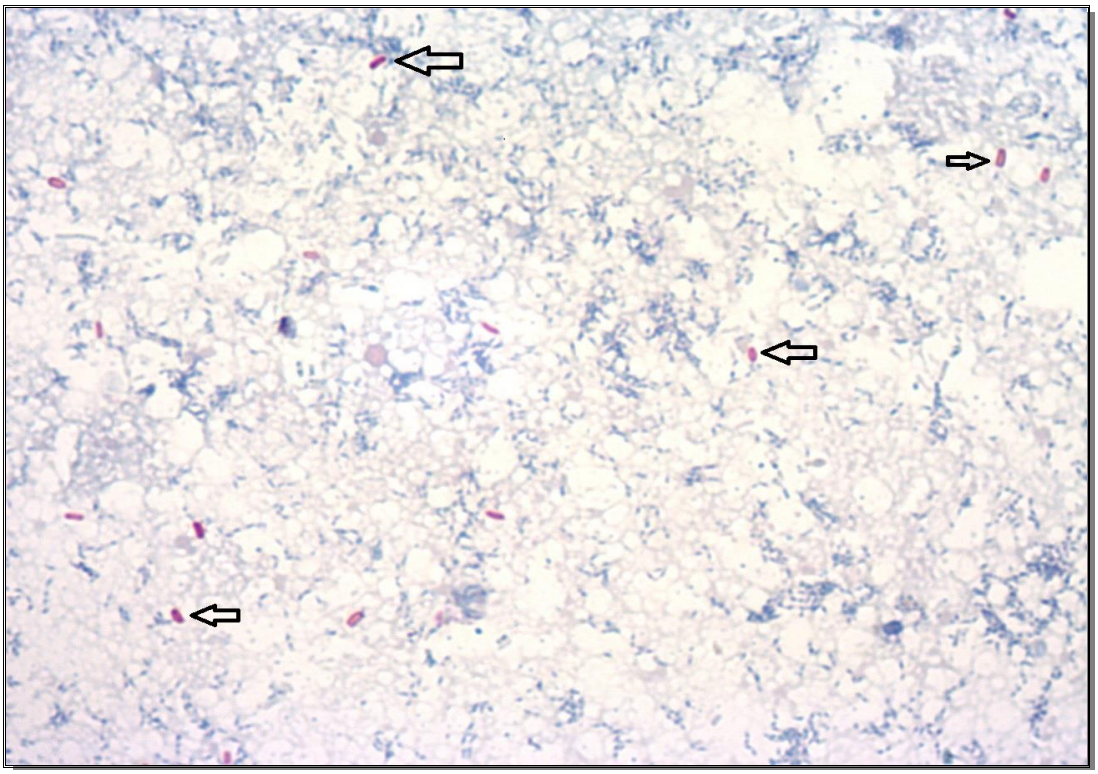

Figure 4: AFB positive smear. Note the cells that appear bright red, straight or slightly curved rods are Mycobacteria.

4.1.7 Results of *Mycobacterium* genus typing

All the 12 isolates from fish, water and sediments which were AFB positive were subjected to molecular identification by using a multiplex PCR protocol. It was expected that the members of Mycobacteria would exhibit band size of 1030 bp but all the 12 AFB positive isolates did not exhibit the expected band size of *Mycobacterium* (Figure 5). This implies that they were not *Mycobacterium*.

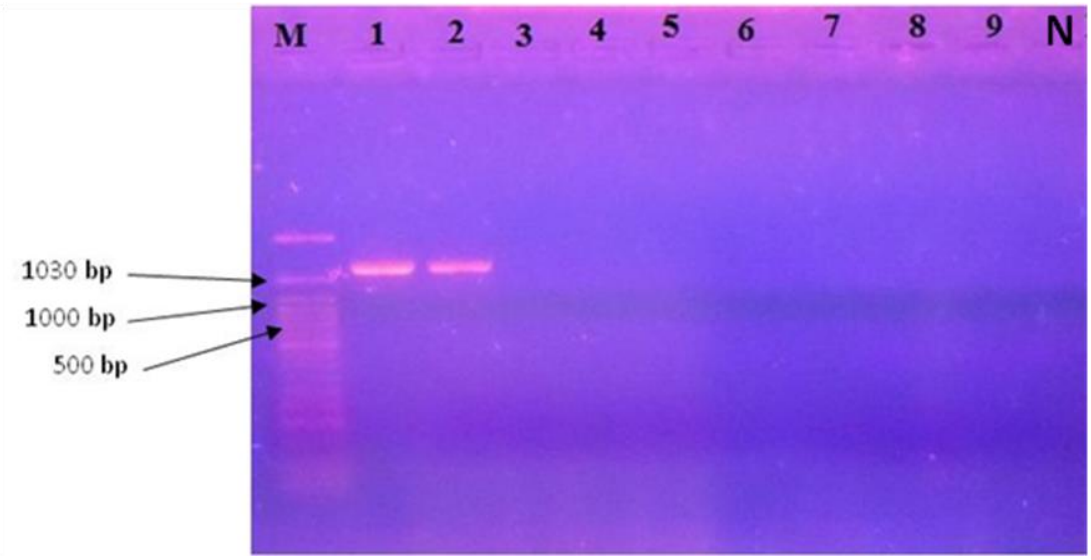

Figure 5: PCR products by Mycogenus typing of mycobacteria isolates from fish kidney, water and sediment samples Unguja and Pemba in Zanzibar. Lanes marked M is a molecular weight marker (100 bp), lanes marked 1 and 2 are positive control; lanes 3, 4, 5, 6, 7, 8 and 9 were AFB positive isolates; lane N is negative control (water). Note that all the isolates in lanes 3, 4, 5, 6, 7, 8 and 9 that were AFB positive did not show the expected band size of 1030 bp and were regarded as negative.

CHAPTER FIVE

5.0 DISCUSSION

The purpose of the current study was to assess milkfish farms management, evaluate the physicochemical parameters of water in milkfish ponds and determine the occurrences of *M. marinum* in selected milkfish farms in Pemba and Unguja Islands, Zanzibar. Generally, it was established that most fish farmers were males (91.7%) with backyard earthen ponds practicing polyculture production system. Knowledge on stocking density, pond management and fish health was low regardless of training on milkfish production which possibly leads to low production. Farmers experience several problems including fish diseases and mortalities. Some of the physicochemical water parameters in fish ponds like temperature, dissolved oxygen, pH, salinity and TDS were high beyond the normal recommended levels such that could be the sources of stress to the fish.

The average milkfish size (95.8 g) at harvesting time was below the recommended one of up to 400 g within rearing period of 6 to 8 months. Examination of the external skin surface and internal visceral organs of all milkfish found no lesion suggestive of mycobacteriosis. Although bacteriology and acid fast testing of some bacteria isolates of fish, water and sediments revealed to be AFB positive, they were all negative on molecular confirmation implying that they were not *Mycobacterium*. Therefore, although the fish pond management was not good and some fish diseases were reported as among the drawbacks to milkfish farming in Zanzibar, *M. marinum* is not prevalent in the Island.

Mariculture is one of the important enterprises to the wellbeing of the people living along the coastal regions. In Zanzibar, milkfish farming is commonly practiced by the local people as alternative sources of income. The current study established that most milkfish farmers were males (91.7%) with the age category ranging between 36 and 45 years and majority of whom were farmers (70%) that doing fish farming for 10 years but at a small scale level (Table 4). Comparing between districts, Wete in Pemba Island had high number of milkfish farms in Zanzibar as previously reported by Msuya *et al.* (2016). In general, milkfish farming is only picking up recently with 14 farmer units in Unguja and 71 in Pemba (Msuya *et al.,* 2016). A total annual production of milkfish is 6.7 metric tons (MANLF-ZNZ, 2016) compared to countries like Philippine with annual production of 76 metric tons which is 55% of the total world milkfish produced (Philippine Statistics Authority, 2015). More education is still needed to the people living along the coastal regions so that they can utilize the opportunity of milkfish farming since it is an avenue which can be used in poverty alleviation.

It was also established that aquaculture systems and farming techniques mostly involved backyard earthen ponds type (91.7%) in mangrove catchment area. Half of the milkfish farms (50%) used ponds that were formerly used as salt pans of which salinity levels of the water is always high, the set up and design was not meant for milkfish farming. Different categories of fish ponds like fries/fingering pond, rearing pond and feed ponds were not considered during constructions. According to Sullivan *et al*. (2007) explained that, farmers were not able to afford the commercial ponds which are expensive to construct and maintain. Financial constraint and lack

of knowledge on how to run milkfish as a business are also reported to be obstacles hindering milkfish production (Sobo, 2013). The use of poorly constructed and poorly managed ponds is among the constraints to production of milkfish (Sullivan *et al.,* 2007; Requintina *et al.,* 2008).

During the current study, it was also observed that majority of farmers practiced polyculture rearing technique whereby more than one species of fish are reared in the same culturing unit. Most common pair of fish species often reared in the same unit was, *Chanos chanos* + *Mugil cephalus*. Lacking of knowledge to differentiate fingerlings of these two species of fish during fingerling collection, resulting on stocking both species unintentionally and grown togather. It was also realized that majority of the farmers were feeding the fish with different kinds of feed formulations that included carbohydrate, fat, protein and vitamin sources. However, others were just depending on animal manure added as fertilizers to the ponds to boost algae bloom development which served as fish feed. For optimal growth and development of milkfish to reach the harvesting size (~400 g) within 6 – 8 months of rearing it is important to feed the fish properly (Sullivan *et al.,* 2007; Requintina *et al.,* 2008; Sobo, 2013). Interviews with fish farmers disclosed that, from fingering introduction to harvesting period (rearing time) was more than one year but still the fish sizes were below the recommended size. This shows that, the fish were stunted probably because of poor pond management, feeding, water quality and diseases (Sullivan *et al.,* 2007; Requintina *et al.,* 2008; Sobo, 2013).

In all the fish farms, the sources of fingerings were from the wild environment (sea) such that the quality is questionable especially in terms of species, health and performance. In some cases, some of the fish are reported to be predators which feed on milkfish. It was realized that sometimes, the availability of fingerings was a problem since it is seasonal and farmers had to stay for a longer time before restocking their ponds. Currently, there are no hatcheries for milkfish fingerlings production in Zanzibar which could be the good source of a quality fingerlings supply but the facilities are under establishment and soon will be operating and serving milkfish fingerlings to the fish farmers (DFD-ZNZ, 2016). In areas where milkfish culture has been practiced extensively, there are individuals or communities that are specialized in fingerling collection, as a separate business from the actual fish farming (Requintina *et al.*, 2006). This would have also been a good source of fingerlings for the farmers. Milkfish fingerlings collected from the wild have been found to be relatively more resistant against fish disease and they have lower probability of being a product of interbreeding compare to hatchery produces. Although, unreliable supply and high biosecurity risk that might be imposed to the fish farms, has discouraged the dependence of fingerling collections from the wild (Bagarinao, 1999). The stocking density was also observed to be poor since 100% of the fish farms were over stock. Fish farmers did not follow a proper stocking procedure per fish pond as explained during the trainings. This can partly lead to poor performance for milkfish production. The recommended stocking density for optimal production, should be one milkfish per meter square (Requintina *et al.*, 2006).

Fish farmers have reported a number of drawbacks which included fish pond drying, incidences of fish diseases, predators and mortalities (Table 6). These problems compromised the fish farming and unfortunately, there were no reliable supports from the fisheries extension officer to provide some advices on how to overcome the problems because most of the fish farms are located in remote areas. In addition, the knowledge on fish health, diseases prevention and control was poor. Pest control practices (parasites like copepods and predators like birds, carnivorous fish species and crustaceans) were not in place to most (92%) of the milkfish ponds. In absence of biosecurity measures, farms can predispose fish ponds to outbreak of diseases (Sadler and Goodwin, 2007). Incidences of diseases were reported by farmers but not at the high rate probably due to the high salinity above the tolerance level of many micro-organisms.

Physicochemical water parameters in the milkfish ponds showed some variations between districts and between ponds (Table 7). The water temperature of all visited milkfish ponds ranged between 29.3 ^{o}C and 37.1 ^{o}C which reflected the ambient temperature in Zanzibar. In some ponds found in some districts like Wete, the water temperature was high exceeding the upper range of recommended level of 32 ℃ for milkfish production. The milkfish ponds found in Pemba ($P < 0.05$) had significantly higher temperature than Unguja ($P > 0.05$). Temperature of surface waters may be influenced by latitude, altitude, seasons of the year, time of day, air circulation, cloud cover, flow rate and depth of the water body. In turn, temperature affects many physicochemical and biological processes in water bodies.

The amount of dissolved solutes is directly related to the water temperature of the water body. Due to the above fact, the mean salinity level in Pemba was reported to

be 39.3 ppt which is relatively higher compared to the reported mean salinity of Unguja; 27.5 ppt (Table 7). Probably, this might be a reason behind which led to the higher pond water temperature in Pemba than Unguja. Water temperature in fish ponds when is high may cause stress conditions to fish. Temperature affects the growth of fish, as it slows metabolism, reduced feed intake and growth rates. High temperature (35 °C) beyond the optimum of 32 °C may lead to disastrous high mortalities and therefore, water exchange every two weeks may help to overcome the problem (Requintina *et al.,* 2008). Temperature may accelerate diseases development leading to mortalities of fish (Sadler and Goodwin, 2007). Nevertheless, optimum temperature in brackish water ponds is also necessary for better growth of natural foods of fish like benthic algae ("lab-lab"), or good plankton growth in freshwater ponds (Requintina *et al.,* 2008).

Salinity as an outcome of dissolved solutes was also high in all the assessed ponds which ranged between 35.8 ± 3 g/l and 48 ± 9.7 g/l with the highest levels observed in Wete and Chakechake compared to other districts in Pemba. The optimal recommended salinity in milkfish ponds is 18 – 32 g/l (Requintina *et al.,* 2008). Salinity in fish ponds as other water bodies can be influenced by degree to which solutes dissociate into ions, amount of electrical charge on each ion, ion mobility and environmental temperature (Chapman, 1996). The observed high salinity in most of the assessed ponds may be influenced by lack of regular exchange of water. Salinity is important in brackish water ponds in maintaining good growth of benthic algae without affecting the milkfish, because they can survive and grow in wide range of salinities (Requintina *et al.,* 2008). Although marine fish like milkfish need saline environment, when the salinity is high may have influences on fish performance. At

high salinity levels, milkfish can survive but start to become stressed and growth rates can be reduced or stopped. For example, salinity of 60 g/l can cause fish mortalities (Requintina *et al.,* 2008). The reported mortalities in this study may partly be contributed by hypersaline conditions of the ponds.

Oxygen content of water bodies vary with temperature, salinity, turbulence, photosynthetic activity of algae and, plants and atmospheric pressure. Solubility of oxygen decreases as temperature and salinity increases (Chapman, 1996). Less oxygen can be held in fully air-saturated sea water than fully air-saturated freshwater. The current study established that the dissolved oxygen in milkfish ponds ranged between 1.9 which is hypoxic state and 6.1 mg/l which is hyperoxic state (Svobodova *et al.,* 1993; Chapman, 1996; Mallya *et al.,* 2007; Requintina *et al.,* 2008). A few fish ponds (3/24) had dissolved oxygen below the recommended level of 3 mg/l. For the fish ponds with low dissolved oxygen, fish may be seen floating on the water surface while gasping for air. The fish become lethargic, stop feeding and swimming which may lead to mortalities. If the levels of dissolved oxygen are not at anoxic state but are persistently low, an assortment of stress related diseases such as fin rot and white spot may occur (Svobodova *et al.,* 1993; Mallya *et al.,* 2007).

Aeration through showering or peddling on the fish ponds is recommended together with encouraging algae development (Svobodova *et al.,* 1993; Mallya *et al.,* 2007). Most of the milkfish ponds had dissolved oxygen concentration of dissolved oxygen exceeding the upper limit of 5 mg/l recommended for milkfish ponds (supersaturated) and therefore they were in the hyperoxic state (Svobodova *et al.,* 1993; Mallya *et al.,* 2007). Too much dissolved oxygen in fish ponds may cause

undesirable effects where by bubbles form in the blood (gas bubble diseases) and these can block the capillaries which can causes death to occur due to blockage of the major arteries. It is recommended that under such situations, either to remove the fish to normally equilibrated water or to provide vigorous aeration to strip out the excess gas (Svobodova *et al.,* 1993; Mallya *et al.,* 2007).

The mortalities and general poor performance of the milkfish ponds in most of the studied ponds could partly be contributed by the hypoxic or hyperoxic states of the water. This further underpins the importance of routine monitoring of water quality in the fish ponds.

Interestingly, examination of the external skin surface and internal visceral organs of fish found that all had no any lesion suggestive of mycobacteriosis. All of the milkfish appeared normal, with their elongated body and small, smooth scales with olive green and silvery flanks. This was a good finding since *M. marinum* apart from causing fish granuloma, it also affects human being.

Laboratory analysis of fish kidney swabs, water and sediment samples isolated 110 bacteria isolates but only 12 (4.2%) were AFB positive (Table 8). Confirmation of AFB positive isolates by multiplex PCR was done and expected the isolates were tested at band size of 1030 bp but all the 12 AFB positive isolates did not exhibit the expected band size which implied that they were not *Mycobacterium*. It is possible that the ones displayed as AFB positive on ZN staining were other microorganisms such as Nocardia or Coriobacteriia rather than *Mycobacterium*.

The negative results to all fish, water and sediment samples may indicate that, the bacterium is not present in the milkfish ponds in Zanzibar or coincidentally, the ponds which were sampled were not contaminated with *Mycobacterium*. Indeed, all the fish which were sampled were apparently health as had no any observable lesions suggestive of *M. marinum* infection. Other studies elsewhere have reported occurrences of mycobacteriosis in milkfish and other finfish (Bragg *et al.*, 1990; Chang *et al.*, 2006; Gauthier and Rhodes, 2009; Puk *et al.*, 2017). Further research is recommended before concluding with certainty that there is no *Mycobacterium* infection in milkfish farms.

CHAPTER SIX

6.1 CONCLUSIONS

From the findings of this study, it is concluded that:

(i) Milkfish farming in Zanzibar is practiced under subsistence level and is used as alternative livelihood and source of income generation for the coastal communities. Fish farmers usually get training on milkfish farming once or twice a year but still the management practices and production were reported to be poor. The fact was based on the low annual production yield per capital as reported by Department of Fisheries Development, Zanzibar (2016).

(ii) Most of the fish farming structures were earthen pond which was formerly used as salt pans for salt production. The higher salinity levels than recommended for milkfish farming was observed during this study in some milkfish farms, was possibly due to the impacts of salt residuals imposed in the areas during salt production period.

(iii) Sources of fingerlings collection were from the wild and there were neither pre-conditioned before were introduced in ponds nor proper stocking density practices were implemented by fish farmers. This behavior imposes high risk of infectious diseases to fish and fish farmers.

(iv) Milkfish rearing from fingering to harvesting period was too long. It was explained to be more than one year and still was rare to get the fish catches obtained the market size of 450 g to 1 kg. While, the mean Total length and weight of the fish catch were 20.63 cm and 95.8 g, respectively.

(v) According the findings of this study, fish farmers had low knowledge on fish health, diseases prevention and control as a result milkfish disease incidences were likely to occur which could be associated with high mortalities. In addition, the fish diseases and mortality were reported to reduce the overall revenue of the fish farmers.

(vi) Most of the physicochemical water quality parameters namely temperature, dissolved oxygen, pH, salinity and total dissolved solutes had exceeded the recommended levels for milkfish farming. Therefore, the reported abnormal physicochemical water parameters' might be among the factors that contribute to negative effect on fish growth and production performance of fish farmers.

(vii) External skin surface and internal visceral organs of all milkfish examined were found to have no lesions suggestive of mycobacteriosis. Although, seven (2.9%) fish samples had AFB positive isolates, it was later confirmed by PCR that they were not Mycobacterium.

6.2 RECOMMENDATIONS

Based on the findings from this study, the following are recommended:

(i) More education should be given to milkfish farmers on better management practices for optimal production

(ii) Government support including financial assistance should be given to groups of milkfish farmers so as to expand their farming activities which include establishing commercial milkfish ponds and managing them as a business

(iii) Hatcheries for fingerlings have to be established in Zanzibar so as to ensure reliable supply of good quality fingerlings to farmers

(iv) To minimize losses, milkfish farmers have to be advised on the importance of routine monitoring of water quality in the milkfish ponds

(v) More research on fish diseases including Mycobacteriosis so as to ascertain the health and wellbeing of the fish produced in Zanzibar as well as health of the consumers.

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APPENDICES

Appendix 1: Questionnaire for risk factors assessment of *Mycobacterium marinum* infection in milkfish farming areas in Zanzibar

I. General information

a. Fish farmer information

Name of Respondent _____
Date of Interview_____/_____/_____
Region & Village
_____
Name of farmer group/enterprise_____
Age: _____ years. Sex: male ☐ female ☐

b. Farm information

i. Size of the farm? _____ m^2
ii. Type of rearing system:_____Tank/ Pond/ Cage
iii. Age of the system: ____ years;
iv. Year operation started: _____;

II. <u>General pond description and use of water</u>

1. How many ponds do you own? _____ ponds
2. Is milkfish farming your sole source of income?
3. How big are the respective ponds (m^2)? A: _____ B: _____ C: _____
4. Please describe the location of your pond. A: _____ B: _____ C: _____
(1 = residential area, 2 = paddy field area, 3 = upland field area, 4 = other(s).
5. What are the respective ponds used for? A: _____ B: _____ C: _____
(1 = grow-out, 2 = nursery, 3 = other(s); other(s): _____)
6. Was the fish pond dug and constructed for the purpose of milk fish production?
Yes….No….
7. If no, what was the original purpose of the pond?.....
8. When did you build the respective pond (year)? A: _____ B: _____ C: _____
9. Do you get trainings on milkfish production?
10. Do you dry-out the pond before stocking? A: _____ B: _____ C: _____
(1 = yes, always, 2 = yes, often, 3 = yes, sometimes, 4 = never, 5 = other(s); other(s): _____
11. Do you remove the pond mud? A: _____ B: _____ C: _____
(1 = yes, always, 2 = yes, often, 3 = yes, sometimes, 4 = never, 5 = other(s); other(s): _____)
12 If yes, what do you do with the pond mud
?_____

III. Fish stock

1. When did you stock the respective pond? A: ____/____ B: ____/____ C: ____/____
(Month and year)
2. Where do you get fingerings for restocking your ponds?..... kg-1) Source of fish
3. How were the fish transported from the place of purchase to your farm? _____
4. Do you also stock non-fish aquatic products (e.g. shrimps, snails etc.) in your pond? Yes ☐ No ☐ If yes, what?_____
5. Do you know the stocking density of your ponds?_____
6. How long do you harvest the fish?-_____
7. What are the harvesting gears do you use?_____
8. Is there any incidences of harvested fish not to get customers?

IV. Feed management

1. Which ingredients do you use to make your fish feed?
_____
_____
2. How often do you feed per day?
(1 = once, 2 = twice, 3 = seldom, 4 = never)

3. Are there differences in feeding practices between the different ponds? Yes ☐ No ☐ If yes, which?
_____
_____
_____

V. <u>Manure management</u>

1. Do you use manure in your ponds? Yes ☐ No ☐ If yes, which ponds? _____
2. What kinds of manure do you usually use? ____/____/____
(1= cattle, 2 = pig, 3 = chicken, 4 = green manure, 5 = humus, 6 = other(s) _____)
3. Please estimate the amount and frequency of manure application. A: ____/____ B: ____/____ C: ____/____
(e.g. 3 kg / twice a week)
4. In which form do you usually supply manure? _____
(1 = fresh, 2 = dried, 3 = processed, 4 = other(s);_____)

VI. Fish diseases

1. Have you ever had to cope with fish diseases in the respective pond? A: _____ B: _____ C: _____ (1 = often, 2 = sometimes, 3 = seldom, 4 = never)
2. Do you know anything fish diseases? Yes------No------
3. Do you know the name of the disease?
_____ please describe the symptoms:
_____

4. Since which year have you faced problems with this fish disease? _____
Do you know the disease prevention and control?
5. How many fish have died? _____ .What was the approximate weight of dead fish (g)?
_____
6. Do you practice pest control in fish ponds?

Appendix 2: Parameters record sheet

1. Water quality parameters

Name of the owner ……. Date……. Pond number…… District……

Pond Number	Water quality parameters					Time
	pH	T^0c	DO	Salinity	Transparency	
1						
2						
3						
4						
5						

2. Sampled fish bio-data sheet

S/N	Weight(g)	Length (cm)	Sex

Appendix 3: List of fish farms and their geographical location

FARM SITE	FARM NAME	CORDINATES
Jozani	UWEMAJO	S 06 °16' 34" E 039 °25' 35.3"
B. Mafufuni	Rabi tuokowe	S 05 °56' 54.5" E 039 °12' 06.5"
D. Muanda	Fanya utunzwe	S 05 °54' 28.2" E 039 °13' 23.1"
B. Kiongwe	Magereza	S 05 °56' 48.6" E 039 °12' 06.8"
Kangagani	Tunaweza	S 05 °08' 56.0" E 039 °49' 59.4"
K. Minungwini	Kichakaasishangi	S 05 °09' 02.3" E 039 °49' 24.9"
Shengejuu	Mwisho mgumu	S 06 °11' 11.9" E 039 °15' 19.5"
Ambasha	Kinema	S 05 °06' 13.6" E 039 °49' 23.2"
Chwale	Twende pamoja	S 05 °06' 13.6" E 039 °49' 23.2"
Kiungoni	Batawi	S 05 °04' 26.8" E 039 °49' 36.3"
Kiungoni	Kidunda	S 05 °04' 23.1" E 039 °49' 47.7"
W. Mjananza	U. Ni nguvu	S 05 °02' 41.8" E 039 °49' 36.3"
Ndagoni	Tujipange sote	S 05 °12' 42.0" E 039 °41' 16.0"
Pujini kijili	Popular Salt	S 05 °18' 08.2" E 039 °48' 58.3"
Pujini kibaridi		S 05 °18' 24.5" E 039 °48' 42.9"
Chambani	Mwanzo mgumu	S 05 °20' 22.5" E 039 °47' 35.0"
Chambani	K. Kichimba	S 05 °20' 32.4" E 039 °47' 41.8"
Chambani	Kilakichwa	S 05 °20' 39.3" E 039 °47' 40.7"
Kiwani	Niasafi	S 05 °06' 47.3" E 039 °49' 34.8"
Kambini	Chabwi	S 05 °08' 02.9" E 039 °49' 18.8"
Furaha	Mwagiwa	S 05 °16' 00.7" E 039 °49' 24.2"
Mwambe	Hakiliki	S 05 °25' 48.9" E 039 °38' 20.8"
Chokocho	Muelekeo	S 05 °26' 01.6" E 039 °38' 20.7"
Shakani	Z. African F.Farm	S 06 °15' 40.7" E 039 °14' 33.1"

Appendix 4: Media composition of the Löwenstein–Jensen

i) LJ glycerol medium (LJM-G) was made up of 61.7 % whole egg, 36.9 % (v/v) IUT buffer salt solution (50mM K_2PO_4 ; 25mM $Na_2HPO_4.2H_2O$; 1.6mM $MgSO_4.7H_2O$; 14mM citric acid; 67 mM L-Asparagine; 0.2 % glycerol) and 2.4 % (v/v) of 1.2 % (w/v) malachite green. Add egg to buffer salt and then the Malachite green to complete the solution.

ii) LJ Pyruvate medium (LJM-P)was made of 61.6 % whole egg, 36.9 % (v/v) Pyruvate medium buffer salt solution (50mM K_2PO_4; 25mM $Na_2HPO_4.2H_2O$; 114mM sodium Pyruvate; 14mM citric acid), 1.2 % v/v of 1 % (w/v) malachite green and 0.25% (v/v) of 1 % (w/v) trypan blue. Add Egg into buffer salt followed by Malachite green and lastly with Trypan blue solution. After mixing all the components, 4ml of the medium was dispensed into 30 ml glass universals and then insipissated at 85 ° C for one hour to solidify the media. L-J glycerol medium had a pale green colour, while L-J pyruvate medium had pale blue. About 0.1 ml of the sediments from each sample was spread on the surface of each media using a sterile disposable pipette and, in order to avoid sedimentation of inoculums at the bottom of the slope, at incubation chamber all the slopes were laid horizontally overnight before being placed vertical for continued incubation at 37 ° C until culture growth or at least ten weeks.

Appendix 5: Summary of the procedure for PCR typing of *Mycobacterium* species

1. Initial denaturation at 95°C for 10 mins

2. Followed by 35 cycles of: Denaturation at 95°C; 1 min → annealing primer at 61°C; 30 seconds

→ Extension at 72°C; 2 mins.

3. Final elongation at 72°C; 10 mins

4. Hold at 4°C

Electrophoresis performed at 100 volts on Agarose 1.5% gel of 1x TAE buffer and ethidium bromide at 0.3 μg/ml.

RESULT S

1. 1030 base pairs – all members of the genus of Mycobacteria (i.e. primers Mycgen-F/R)
2. 180 base pairs – M. Avium subspecies including M. Paratuberculosis (primers Mycgen – F/M YcAv – R) plus 1030 base pairs
3. 850 base pairs – M. Intracellulare (primers Myc int-F/ Mycgen-R) in addition to 1030 base pairs genus product
4. 372 base pairs – M. Tuberculosis complex (with TB-1-F/ TB-1-R) in addition to 1030 base pair genus product.

Two bands indicate a specific PCR product and respective species-specific PCR product