UNIVERSITY OF CAPE COAST

OCCURRENCE OF NONTUBERCULOUS MYCOBACTERIA IN SOME SELECTED WATER SOURCES IN THE CENTRAL REGION OF GHANA

BY

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Thesis submitted to the Department of Molecular Biology and Biotechnology, School of Biological Sciences of the College of Agriculture and Natural Sciences, University of Cape Coast, in partial fulfilment of the requirements for the award of Master of Philosophy degree in Molecular Biology and Biotechnology

MARCH 2020

DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original research and that no part of it has been presented for another degree in this University or elsewhere.

Candidate's Signature ……. Date …….

Name: Yvonne Edem Quist

Supervisor's Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

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ABSTRACT

Nontuberculous mycobacteria (NTM) are common saprophytes found in the environment, specifically in soil and water. These mycobacteria have recently been reported to be implicated in opportunistic infections with increasing frequency, especially in immunocompromised individuals. This study aimed to find the occurrence of NTM in some selected water sources in the Central Region of Ghana using treated water from, and water sources employed by the Ghana Water Company Limited (GWCL) as the main medium for the study. Water samples were decontaminated with 4% NaOH solution and cultured on Löwenstein-Jensen (LJ) slants at 30^{o}C and 37^{o}C for the recovery of Acid-fast bacilli (AFB). Post-culture Ziehl-Neelsen microscopy was done to identify AFB from isolates. This was followed by categorization of isolates as NTM using the Capilia TB-Neo assay. The 360 bp fragment of the *rpoB* gene was amplified for 17 (30.9%) isolates categorized as NTM and amplified products were separately subjected to digestion with the restriction endonucleases *Msp*I and *Hae*III. Resulting fragments were compared with those in a standard algorithm and the NTM species identified were *M. celatum*, *M. gordonae* type II, *M. simae*. *M. genavense* and *M. kansasii* V strain. The results of this study suggest that the water sources investigated harboured NTM, which can pose a potential public health hazard especially to immunodeficient individuals.

KEY WORDS

Acid-fast Bacilli (AFB)

Ghana Water Company Limited (GWCL)

Mycobacteria

Nontuberculous mycobacteria (NTM)

Treatment plants

Water

ACKNOWLEDGEMENTS

I would like to express my profound gratitude to my supervisors, Dr. Daniel Asante of University of Cape Coast and Dr. Kwasi Oti Gyamfi of Ghana Atomic Energy Commission, RAMSRI-CCRC for their encouragement, advice, guidance, support and patience with which they guided this work. I am most grateful.

I am also grateful to Mr. Kofi D. Bedzra of Ghana Atomic Energy Commission, RAMSRI-CCRC for his unflinching support and dedication towards the successful completion of this work.

I thank the management of Ghana Water Company Limited (GWCL) Central Region for giving me the permission to use their facility for the research. I specially thank Mr. Amihere, Mr. Adams, Mr. Felix Appiah, Mr. Kwaku Doh and all staff at the Central Region treatment plants who assisted me in this work.

Finally, I would also want to thank my family for their support especially, my husband, Joseph Obiri-Yeboah, my mother, Mrs Evelyn Effie Quist and my siblings; Lord, Evelyn and Frances.

DEDICATION

To my husband, Joseph Obiri-Yeboah and our lovely Children, Jeanelle and Jedidiah.

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LIST OF ACRONYMS

AFB	Acid-fast bacilli
BCG	Bacillus Calmette-Guerin
Bp	Base pair
BLAST	Basic Local Alignment Search Tool
BU	Buruli Ulcer
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
GWCL	Ghana Water Company Limited
LJ	Lowenstein Jensen
MAC	*Mycobacterium avium* complex
MAP	*Mycobacterium avium subspecies paratuberculosis*
MAIS	*Mycobacterium-avium-intracellulare-scrofulaceum*
mg/L	Milligram per litre
MGIT	Mycobacteria Growth Indicator Tubes
MOTTS	Mycobacteria Other Than Tuberculosis Strains
MTBC	*Mycobacterium tuberculosis* Complex
NTM	Nontuberculous Mycobacteria
PCR	Polymerase Chain Reaction
PRA	Polymerase chain reaction Restriction enzyme Analysis
RFLP	Restriction Fragment Length Polymorphism
TB	Tuberculosis
WHO	World Health Organisation
ZN	Ziehl Neelsen
%	Percentage

CHAPTER ONE

INTRODUCTION

Background to the Study

Nontuberculous Mycobacteria (NTM) are opportunistic pathogens which are found in the environment, specifically in water and soil. Nontuberculous Mycobacteria (NTM) are also referred to as 'Environmental Mycobacteria' or 'Atypical Mycobacteria' and they can cause infections in humans and animals (Griffith *et al*., 2007; van Ingen, 2013). They have assumed public health importance because of the different infections they cause in humans with chronic, weakening pulmonary disease among immunocompromised persons being the most common clinical manifestation (Winthrop *et al*., 2010). The American Thoracic Society has indicated that NTM infections of organs such as skin and lung are becoming increasingly important public health considerations (ATS and IDSA, 2007). However, in contrast to developed countries where the prevalence of NTM disease is well documented (McCarthy *et al*., 2012), little data is available on the occurrence of NTM and its clinical importance in developing countries (Chanda-Kapata *et al*., 2015). Apart from the infections NTM cause, these organisms also have the ability to reduce the efficacy of vaccines like the Bacillus Calmette-Guerin (BCG) vaccine which protects people against pulmonary tuberculosis (TB) (Falkinham, 2013). These organisms proliferate in the environment and daily contact with them pose a high risk of acquiring infections from them especially for immunocompromised individuals.

Nontuberculous mycobacteria belong to the genus *Mycobacterium* which is made up of gram positive aerobic, non-motile, non-spore forming acid

fast organisms. This genus is known for its two major obligate pathogens; *Mycobacterium tuberculosis* and *Mycobacterium leprae.* The genus *Mycobacterium* also includes the *Mycobacterium tuberculosis*-Complex (MTBC), a group of closely related mycobacterial species known to cause TB disease in humans and animals (Gopinath and Singh, 2010; Sharma, 2015). As a group, NTM includes *Mycobacterium ulcerans* which causes Buruli or Bairnsdale ulcer and *Mycobacterium marinum* which also causes fish handlers' disease. Advances in Mycobacteriology have led to the identification of different species of NTM. In 1997, the American Thoracic Society (ATS) published that there were approximately 50 NTM species that had been identified (ATS, 1997). Currently, the genus *Mycobacterium* contains more than 170 different species (Forbes, 2017).

A major NTM species of public health significance in Ghana and West Africa is *Mycobacterium ulcerans*, the causative agent of Buruli ulcer (BU). Like other NTM species, controlling *M. ulcerans* is difficult because of a lack of understanding of the pathogen ecology and mode of transmission (Aboagye *et al*, 2016). *Mycobacterium avium* complex (MAC), consisting of *Mycobacterium avium, M. intracellulare, M. kansasii, M. chelonae, M. interjectum, M. septicum and M. fortuitum* are the most common NTMs responsible for diseases in humans (Kazda *et al*., 2009; Katoch, 2004). In non-HIV patients, different NTM may cause localized pulmonary diseases, adenitis, soft tissue infections, infections of joints/bones, bursae, skin ulcers and generalized disease in individuals with leukaemia or transplant patients. Nontuberculous mycobacteria are also involved in nosocomial (hospital acquired) infections and pseudo-outbreaks. Nosocomial infections result in

increase in positive results through microscopy to detect acid-fast bacilli but without evidence of disease in patients (Wallace, 1998; Fraser and Wallace, 1996). Treating NTM infections is complicated because NTM are resistant to some anti-mycobacterial drugs (van Ingen *et al*., 2012). Currently no vaccine is available for the prevention of NTM infections (van Ingen, 2017) making NTM infections a serious health problem.

Transmission of NTM from human to human is rare but rather human diseases are thought to be acquired from environmental sources like soil, dust and water. Ingestion, inhalation and inoculation of NTM from the environment have been hypothesized as causes of NTM infections (Griffith *et al*., 2007). These organisms can survive in many different water sources like treated water, ground water, surface water, waste water and recreational water. (Lee *et al*., 2008). The species *M. avium, M. chelonae, M. fortuitum, M. gordonae, M. kansasii*, *M*. abscessus, *M*. marinum and *M. xenopi* are the most frequently reported mycobacteria occurring in drinking water (Beran *et al*., 2006; Gauthier & Rhodes, 2009). They have been isolated from public water distribution systems, home distribution systems, hot and cold water taps, ice machines, heated nebulizers, and showerhead sprays (Schulze-Robbecke *et al*., 1995; Falkinham, 1996; Dailloux *et al*., 1999). Nontuberculous mycobacteria can survive in a wide range of temperatures and pH and because of this, they can live in tap water for a long period of time and common disinfectants cannot kill them (Le Dantec *et al*., 2002). This makes water a very important medium for the transmission of NTM. However, the routine bacteriological examinations that are carried out by providers of treated water, such as the Ghana Water

Company Limited, do not include a search for NTM by employing microscopic examination for acid-fast bacilli.

The availability of water in terms of quantity and quality is essential to human existence and every effort should be made to achieve water quality as much as practicable. The first line of action in achieving good water quality is by protecting water supplies from contamination (WHO, 1996). Protecting the sources of water which are treated for human consumption from contamination is the best method of ensuring the delivery of safe drinking water and it is better than treating contaminated water for consumption. Failure to provide adequate protection and effective treatment of water will expose the communities to the risk of outbreaks of intestinal and other infectious diseases (WHO, 1996). Those at greatest risk of waterborne diseases in our communities are children, people who are debilitated or living under insanitary conditions, the immunocompromised individuals, the sick and elderly. It is very important to control microbial contaminations like NTM because the potential consequences from mycobacterioses are very alarming and sometimes cannot be managed. The greatest risks are associated with ingestion of water that is contaminated (WHO, 2011).

As with the other regions, people in the Central Region of Ghana use water for a lot of activities including drinking, cooking, bathing, washing, and for agricultural purposes. Sources of water for the people in the Central Region of Ghana include rivers, streams, lakes, sea, boreholes, hand dug wells and treated water from Ghana Water Company Limited.

Ghana Water Company Limited (GWCL) is a utility company, fully owned by the state. Ghana Water Company Limited was established on 1st July,

1999 following the conversion of Ghana Water and Sewerage Corporation into a state-owned limited liability company under the Statutory Corporations (Conversion to Companies) Act 461 of 1993 as amended by LI 1648. The First public water supply system in Ghana, then Gold Coast, was established in Accra just before World War I. Other systems were built exclusively for other urban areas among them the colonial capital of Cape Coast, Winneba and Kumasi in the 1920s. The company is responsible for potable water supply to all urban communities in Ghana. It currently operates eighty-eight (88) urban water supply systems throughout the country. Average production is about eight hundred and seventy-one thousand, four hundred and ninety-six cubic meters (871,496 m^3) per day (192 million gallons per day). Present potable water demand is estimated at one million, one hundred and thirty-one thousand, eight hundred and eighteen point eighteen cubic meters (1,131,818.18m^3) per day (249 million per day). Urban water supply coverage is therefore about seventy-seven percent (77%). With a staff strength of three thousand, four hundred and seventy-six (3,476), GWCL serves five hundred and fifty (GWCL, 2016) districts. Portable water from GWCL is the main source of water used by the inhabitants of the Central Region for their activities and it feeds about 80% of the inhabitants of the region (GWCL, 2016).

Statement of the Problem

The discovery that NTM can cause opportunistic infections in humans especially those with predisposing conditions like immune dysfunctions has made NTM a great public health threat worldwide. It is therefore necessary for the initiation of public health strategies for the prevention, management and control of infections caused by NTM.

Control of NTM is challenging because the current understanding of how the organisms are transmitted and the actual place they live in the environment is limited. In the developed countries, knowledge about NTM forming amoeba-associations and biofilms in water distribution systems has been used to design effective control measures. Unfortunately, in developing countries like Ghana, there is little knowledge on the occurrence of these mycobacterial infections and practically no awareness of these organisms as opportunistic pathogens. There is therefore the need for a better understanding of the ecology of NTM and the habitats they successfully proliferate in. This will enhance knowledge regarding disease epidemiology and also guide the design of public health strategies which will help control and prevent NTM transmissions and infections. Thus, it would be beneficial in terms of good public health practice if a study is carried out to determine the occurrence of different NTM in selected water sources.

Purpose of the Study

The purpose of this study was to recover and identify NTM from some selected water sources in the Central Region of Ghana. Isolation and characterization of NTM is important to improve the understanding of their epidemiology and also to help in the design and implementation of control measures for NTM.

Main Objective

This main aim of the study was to study the occurrence of NTM in the main sources of drinking water in parts of the Central Region of Ghana.

Specific Objectives

The specific objectives of this study were:

1. To detect acid-fast bacteria from water sources.
2. To recover NTM from water sources.
3. To identify the species recovered using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analyses.

Research Questions

The study was guided by the following research questions;

1. Are acid-fast bacteria present in the selected water sources in the Central Region of Ghana?
2. Are the acid-fast bacteria in the selected water sources NTM?
3. What are the species of NTM in the selected water sources?

Significance of the Study

Nontuberculous mycobacteria are considered an important group of bacteria because of their increasing pathogenic importance. The knowledge of the occurrence of NTM will lead to the awareness by all stakeholders the possible health risk from contamination of these microorganisms in water sources. This will lead to the design of measures and implementation of appropriate interventions to manage and control NTM contamination. Also, providers of portable drinking water will be aware of the possible NTM contaminations and this will guide them to design control strategies on proper disinfection protocols which will minimize NTM contamination in water distribution systems. This study will also yield information on the sources of NTM contamination in the Central Region.

Delimitations

The main focus of the study was to isolate NTM from treated water which is produced and supplied by Ghana Water Company Limited (GWCL) in the Central Region. Ghana Water Company Limited has nine treatment plants in the region but six were used for the study because two of the nine GWCL treatment plants: Twifo Praso and Dunkwa treatment plants were not easily accessible and also production in Breman-Asikuma treatment plant was at a halt at the time of the research. Only rivers which supply GWCL treatment plants with raw water for treatment were included in the study so that the river sources could be checked for their suitability for treatment and the level of treatment they requires. These rivers were Kakum, Ochi Amissah**,** Ochi Nakwa, Ayensu and Pra. Some of the GWCL reservoirs were not in use as at the time of sampling and others did not have outlets where water could be sampled. As a result, only two GWCL reservoirs: Mbroboto and Mawukpor reservoirs were included in the study. The community taps from which water samples were taken are the taps strategically positioned in the communities by GWCL which are accessible by all members of a community and not personal taps of individuals in their homes.

Limitations

The research plan for sample collection was altered because of rains. Samples were to be taken weekly for three weeks but because of the rains, sample collection lasted for five weeks. Decontamination was done to eliminate the associated flora like other bacteria and fungi with 4% NaOH treatment. Mycobacteria are more resistant to this treatment than the background flora because of the nature of their cell walls but they are not fully resistant.

Definition of Terms

1. Immunocompromised person- a person with an impaired or weak immune system.
2. Immunocompetent individuals-a person with a normal immune system which is able to develop an immune response.
3. Hydrophobic- tending to repel or fail to mix with water.
4. Hydrophilic- tendency to mix with, dissolve in, or be wetted by water.

Organisation of the study

The overall experimental design for this study is summarized in Figure 1

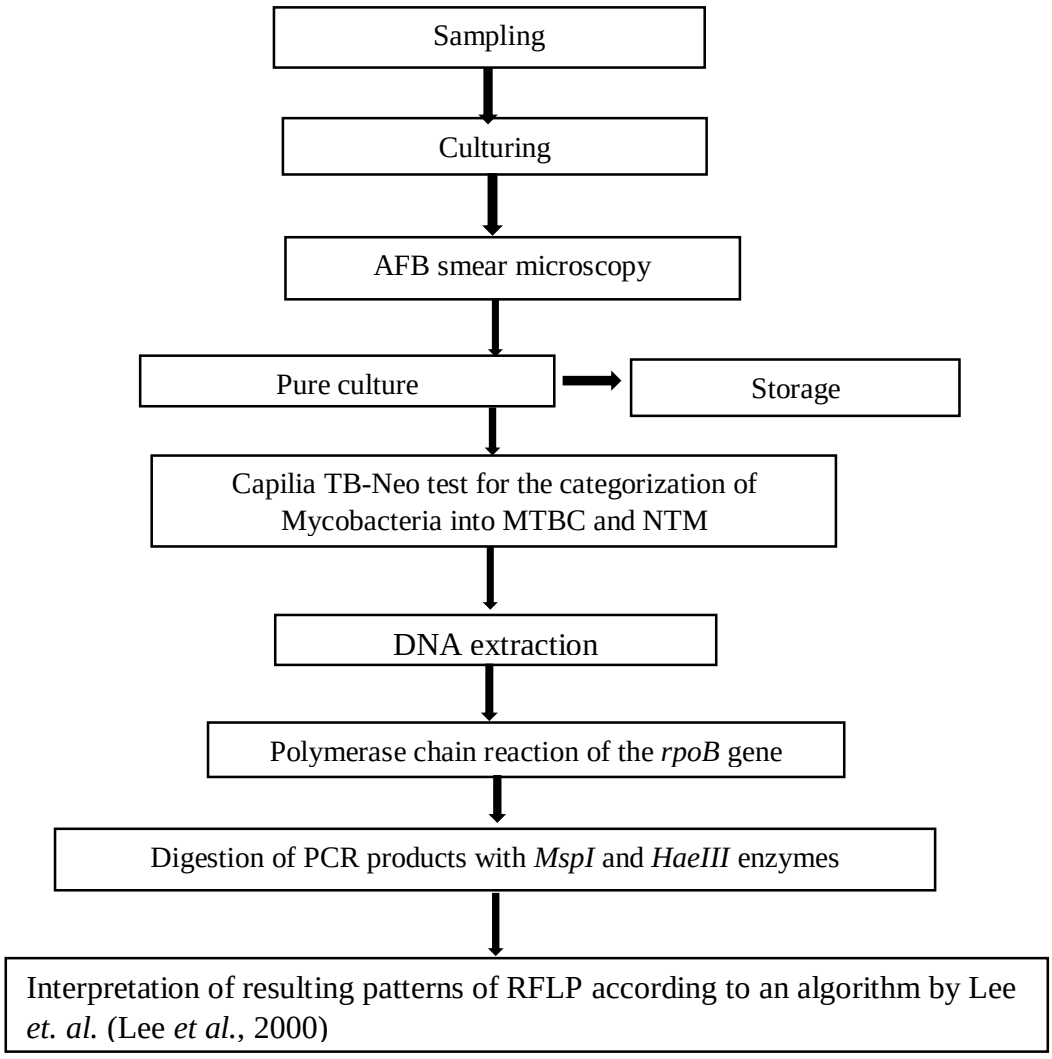

Figure 1: Stages of the study from sampling to identification of Nontuberculous mycobacteria recovered from water sources

CHAPTER TWO

LITERATURE REVIEW

Taxonomy and Microbiological Characteristics of Mycobacteria

The name *Mycobacterium* which means fungus-bacterium was introduced in 1896 by Lehmann and Neumann (Van Ingen, 2017). The name Mycobacteria was given because the bacteria grow as mould-like pellicles on the surface of liquid media (Gangadharam & Jenkins, 1998). Mycobacteria belong to the Order *Actinomycetales*, Family *Mycobacteriaceae* and Genus *Mycobacterium*. The early techniques used to classify mycobacteria included the Adansonian and Runyon taxonomy systems. The Adansonian system of taxonomy even though outdated, helped to identify mycobacteria using biochemical and culture properties (EPA-US, 2002). Dr. Ernest Runyon, a botanist brought about the Runyon system of classification in the mid-1950s (Grange, 1996). In Runyon's classification, Mycobacteria, excluding those in the *Mycobacterium tuberculosis* complex (MTBC) and non-cultivable taxa (e.g., *Mycobacterium leprae*), were divided into four groups based on growth rates and pigmentation properties (Butler and Guthertz, 2001; Jarzembowski and Young, 2008). Mycobacteria that form colonies clearly visible to the naked eye within seven days on solid media were termed as rapid growers, while those requiring longer periods were termed as slow growers. The Runyon classification system was not able to define species within the Mycobacterium genus but it provided information for identifying NTM, resulting in better characterization of diseases. Biomarkers are now used to identify mycobacteria at the species level but that notwithstanding, the Adansonian and Runyon classification systems are still used to classify some mycobacteria species that

are not familiar (WHO, 2004). According to the growth rate and production of pigments, mycobacteria are classified into four groups: Photochromogens, Scotochromogens, Non-chromogens and Rapid growers. The characteristics and examples of these four groups, as classified by Runyon, are shown in Table 1.

Table 1: Runyon's Classification of Mycobacteria

Runyon group	Description	Growth rate	Pigment formation	Examples
I	Photochromogen	Slow	Produce a yellow-orange pigment when exposed to light	M. kansasii M. marinum M. asiaticum M. simiae M. szulgai
II	Scotochromogens	Slow	Produce a yellow-orange pigment regardless of whether they are grown in the dark or the light	M. gordonae M. scrofula-ceum M. szulgai
III	Non-chromogens	Slow	Never produce pigment, regardless of culture conditions	M. avium M. intracel-lulare M. ulcerans
IV	Rapid growers	Rapid growth (produces mature colonies in agar ≤ 7 days)	Some do not produce pigment and others produce late pigmentation	*M. fortuitum* *M. peregrinum* *M. abscessus* *M. chelonae*

Source: Jarzembowski and Young, (2008)

Biology of Nontuberculous Mycobacteria (NTM)

Mycobacteria are generally aerobic, non-motile rods, non-spore forming and able to enter into dormant states. Mycobacterial cells are straight or slightly curved rods between 0.2 and 0.6 µm wide and between 1.0 and 10 µm long (Ryan and Ray, 2004). Mycobacterial cell walls have unique characteristics which make them hydrophobic and acid fast as they retain dyes after acid or alcohol decolourisation. Mycobacterial cell walls are lipoid in nature, consisting of a mycolate layer and a peptidoglycan layer held together by arabinogalactan, a polysaccharide (Bhamidi, 2009). The hydrophobic, waxy and mycolic acid rich cell wall makes them resistant to many hydrophilic compounds, disinfectants and common antibiotics (Hoffmann *et al.*, 2008). These characteristics of the cell wall allow the Mycobacteria to survive in different environments, for example in biofilms in water habitats. Many Mycobacteria grow well on very simple substrates which contain ammonia or amino acids as nitrogen sources and glycerol as a carbon source in the presence of mineral salts. Optimum growth temperatures vary widely according to the species. They have unusual life cycles and this may explain the phenomenon of mycobacterial dormancy and persistence in some habitats (Grange, 1996). These organisms are capable of adapting to prolonged periods of dormancy in tissues, and this dormancy may be responsible for the latency of disease. Wayne (1994) indicated that there may be two or more stages involved in the process leading from active replication to dormancy. The first step involved is a shift from rapid to slow replication and the second is a complete shutdown of replication which does not result in the death of the cell. This may be the reasons why

mycobacteria can inhabit a host for long periods of time before signs of the disease are recognized.

Nontuberculous mycobacteria like other mycobacteria have certain characteristic structures and processes which allow them to survive and persist in the environment. Discussed below are some aspects of the biology of NTM which particularly help in their survival and proliferation in the environment.

Growth and Ecology of Nontuberculous Mycobacteria

The composition and structure of the mycobacterial outer membrane determines the growth, physiology, ecology and virulence of these opportunistic pathogens. The impermeable, hydrophobic, lipid outer membrane limit growth while providing some level of impermeability to anti-microbial agents. Hydrophobicity of the outer membrane drives the attachment of mycobacterial cells to liquid surfaces (Falkinham, 2009), thus preventing the washing off and dilution of cells in flowing systems (rivers and drinking water distribution systems). Hydrophobicity of the cell wall is also responsible for the concentration of NTM at air–water interfaces, that is transfer of cells from water to air (aerosolization) where organic compounds are also concentrated, providing nutrients (Harvey and Young, 1980 as cited by Falkinham, 2009).

The surface charge of NTM is different from that of other bacteria. For example, *M. avium, M. intracellulare* and *M. scrofulaceum* cells are electronegative above a pH of between 3.5– 4.5 (Falkinham, 2002). Thus, at near-neutral pH values, cells of these mycobacteria are rather strongly negatively charged and would be expected to attract positively charged ions (e.g., cations) and repel like-charged ions (e.g. anions). This characteristic is related to the ability of these mycobacterial cells to grow at pH 6 under

conditions of Mg^{2+} limitation. Thus, only under acidic conditions (e.g. pH 4) would these cells lack a charge. This may be the reason why *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum* exhibit optimal growth at acidic pH values (Piddington *et al*., 2000).

Some NTM have been shown to also grow within amoebae (Miltner & Bermudez, 2000) and it is suggested that the amoebae provide a safe haven for them when environmental conditions deteriorate. For example, protozoa and amoebae have been shown to protect intracellular *Mycobacterium avium* cells from antibiotics (Miltner and Bermudez, 2000). These amoebae form cysts during harsh conditions so NTM growing in amoebae are able to survive in the cyst. Nontuberculous mycobacteria in a cyst of protozoa or amoebae are therefore protected from disinfection (Falkinham, 2009). Falkinham again observed that mycobacteria lose their acid fastness and cultivability when inside another cell. During cultivation of amoebae or protozoa infected with mycobacteria, the detection of mycobacteria as acid fast cells decreases to the point where no acid fast cells are observed, yet colonies can be isolated and species-specific DNA sequences (e.g. IS900) can be amplified by polymerase chain reaction. This suggests that perhaps, intracellular mycobacteria change their cell structure particularly the outer membrane when they find themselves in a host cell and this can give false negative results during an investigation. Strahl *et al.* (2001) have also shown that NTM can be phagocytized and thus grow within the ciliate Tetrahymena (genus of free-living ciliates found in freshwater ponds that can switch from commensalistic to pathogenic modes of survival). This association with amoebae and ciliates may enhance the entry, growth and virulence of NTM (Cirillo *et al*., 1997). The ecology of NTM may

be more complicated than it is being thought of currently because, for example, *M. ulcerans* has been found in the salivary glands of aquatic insects (Portaels *et al*., 1999; Marsollier *et al*., 2002) and the demonstration of *Mycobacterium avium subspecies paratuberculosis* (MAP) in trichostrongylid nematode larvae (Lloyd *et al*., 2001; Whittington *et al*., 2001) and earthworms (Fischer *et al*., 2003) suggest that NTM can live in more diverse environments that it is currently thought of thus, there is a need for further research into the ecology of NTM.

Nontuberculous mycobacteria grow over a wide range of pH values and temperatures. Growth optima for almost all mycobacteria are at acidic pH values and there is little growth at alkaline pH values above 7.5 (Falkinham, 2002). The optimal pH for growth of *Mycobacterium kansasii, M. marinum, M. avium, M. intracellulare,* and *M. xenopi* is between 5.0 and 6.5 (Piddington *et al*., 2000). The pH optima for growth of the rapidly growing mycobacteria, *Mycobacterium fortuitum, M. chelonae,* and *M. abscessus* is also between 5.0 and 6.5 (Piddington *et al*., 2000). The optimal temperature for most cultures for NTM is between 28°C and 37°C. Most clinically significant slow-growing mycobacteria grow well on primary isolation at 35° to 37°C with the exception of the following: the newly described *M. conspicuum,* which requires temperatures from 22° to 30°C for several weeks and only grows at 37°C in liquid media, *M. haemophilum* which prefers temperatures from 28° to 30°C, *M. ulcerans,* which grows slowly at 25° to 33°C, and some strains of *M. chelonae,* which require temperatures between 28° and 33°C (Griffith *et al*., 2007). Cultures for rapid growing mycobacteria, skin, joint fluid and bone specimens and *M. marinum* are incubated at 28° to 30°C for optimal recovery

of NTM. *M. avium and M. xenopi* can grow at 45°C, which explains their recovery from hot water systems (Slosarek *et al*., 1993). The ability of *Mycobacterium avium*, *M. intracellulare* and *M. xenopi* to grow over a temperature range of 10° to 45°C suggests that these species must be able to modify the composition of their membrane lipids to maintain a fluid, yet intact, permeation barrier (Liu *et al*., 1995; Liu *et al*., 1996). It is best to culture mycobacteria at two different incubation temperatures for optimum recovery of all species.

Biofilm formation by Nontuberculous Mycobacteria

Nontuberculous mycobacteria cells readily adhere to surfaces and form biofilms (Carter *et al*., 2003) because of their high surface hydrophobicity. Biofilms are described as colonies of microorganisms irreversibly attached to each other and to a surface (Kumar *et al*., 2015). During the development of a bacterial biofilm, the bacteria undergo several changes in their phenotypic state to form a heterogeneous, dynamic, and differentiated community and this is a strategy for bacterial survival in severe environments, since biofilm provides protection against environmental stressors, for example, antimicrobial agents and disinfectants (Johnson *et al*., 2008; Usui *et al*., 2013).

Mycobacterial cells grown in biofilms are resistant to disinfectants (Steed and Falkinham, 2006) and antibiotics (Falkinham, 2007) for a short period of time. Biofilm-grown cells can be released from surfaces by scraping or vortexing and the cell suspensions exposed to antimicrobial agents without the protection of layers of cells as in biofilms. Such biofilm-grown, but suspended cells are more resistant to antimicrobial agents than cells grown in suspension, but of reduced resistance compared with cells grown and exposed to

antimicrobial agents in biofilms (Steed and Falkinham, 2006; Falkinham, 2007). However, the resistance of biofilm-grown but suspended cells to antimicrobial agents is also transient because after 24 hours growth in medium as suspended cells, their susceptibility is equal to that of cells grown in suspension (Steed and Falkinham, 2006; Falkinham, 2007). In the absence of attachment and growth in a biofilm, mycobacterial cells eventually revert back to the susceptibility of suspension-grown cells. The adaptation to antimicrobial resistance as a consequence of biofilm growth has important ramifications. In pipes, cells growing on surfaces in drinking water distribution systems and in households are more resistant to disinfection. Furthermore, cells released from the biofilm would transiently be of intermediate disinfectant resistance. If methods to identify the contact time for killing 99.9% of cells employ suspension-grown cells, the resulting estimates would direct the use of too low a concentration of disinfectant. Likewise, selection of concentrations of antibiotics for killing or inhibiting the growth of mycobacterial cells should not be based on those of suspension-grown cells, but rather upon cells grown in biofilms (Falkinham, 2007).

Mycobacterial Adaptations and Environmental Survival

One advantage of the slow growth of mycobacteria is that their cells are capable of adapting to changing conditions (Falkinham, 2009). Mycobacterial adaptations are geared towards survival as a consequence of exposure to anaerobiosis (Dick *et al*., 1998), starvation (Archuleta *et al*., 2005), acid (Bodmer *et al*., 2000) and temperature (Scammon *et al*., 1964), and elevated antibiotic and disinfectant resistance of biofilm-grown cells (Steed and Falkinham, 2006; Falkinham, 2007). Temperature, pH, organic matter, salinity

and humidity are factors which may influence the survival of NTM in habitats that are potential reservoirs or sources of infection. Unbalanced growth as coined by Mall and Kjeldgaard in 1966 results in death of NTM by antimicrobial agents or environmental stresses (e.g. oxygen deprivation) and a microbial cell may die if a cellular process like cell wall synthesis is inhibited while DNA, RNA and protein synthesis continue unabated. Also, rapidly growing cells are more susceptible to antimicrobial agents or environmental stressors compared with slowly growing cells. Cells of *M. avium* have been shown to enter a dormant stage upon starvation (Archuleta *et al*., 2005).

Humic and fulvic acids are known to affect the survival of NTM either directly (Brooks *et al.*, 1984) or indirectly in combination with other physiochemical variables such as temperature, oxygen content, pH and inorganic substances (Kirschner *et al*., 1992). The combination of higher temperatures, low oxygenated waters, low soil pH, and waters high in zinc, humic and fulvic acids from swamp waters most likely favour the growth and survival of *Mycobacterium-avium-intracellulare-scrofulaceum* (MAIS) organisms (Flaig *et al*., 1975; Schnitzer, 1982). A number of physiological traits support the survival, growth and persistence of mycobacteria in the environment. Furthermore, it is a determinant of the concentration of mycobacteria in air bubbles and their ejection into the air in droplets (Parker *et al*., 1983). The hydrophobic surface layers reduce transport (Brennan & Nikaido, 1995) but also protect against antimicrobial agents. The resistance of nontuberculous mycobacteria to the disinfectants used in water treatment (e.g. chlorine) promotes mycobacterial growth and persistence in drinking water distribution systems and household plumbing (Falkinham, 2010).

Nontuberculous mycobacteria are also oligotrophs, able to grow in water with very low levels of organic carbon (i.e. over 50 mg assimilable organic carbon per litre). This collection of traits supports the notion that engineered habitats such as drinking water distribution systems and household plumbing support mycobacterial persistence and proliferation.

Colony variations in Nontuberculous Mycobacteria (NTM)

Culturing of NTM show colony variations in the different species. There is a long history of reports of colony variation among the NTM, particularly those of *Mycobacterium avium* and *Mycobacterium intracellulare* (*M. avium* complex or MAC). *Mycobacterium avium* colonies switch between two types, smooth (opaque) and smooth (transparent) (Cangelosi *et al.*, 2001). Similar variation has also been reported for *Mycobacterium abscessus* (Howard *et al.*, 2006). These differences are significant because the transparent types are relatively more virulent, hydrophobic, and antibiotic resistant compared with the hydrophilic, less virulent, and more antibiotic sensitive opaque types (McCarthy 1974; Schaefer *et al*., 1970; Kajioka & Hui 1978). The rate of switching (i.e. opaque to transparent and transparent to opaque) occur at frequencies of 1 per 1000 colonies as the opaque colonies are easy to see and count against the background of small transparent colonies. Transparent types are usually isolated from patients, rather than opaque types (Schaefer *et al*., 1970). NTM colonies on agar medium, cells in biofilms, and cells in patients will contain cells of both colony types.

Occurrence of Nontuberculous Mycobacteria in the Environment

Nontuberculous mycobacteria reside in a variety of natural and artificial environments. Generally they can be found in water, soil, air, on equipment,

food and even in surgical implants such as catheters (Edun *et al*., 2016). Nontuberculous mycobacteria have been isolated from drinking water pipelines (Torvinen *et al*., 2010; Thompson *et al*., 2013), water tanks (Tuffley & Holbeche, 1980), hot tubs (Mangione *et al*., 2001), residential faucets (Slosarek, Kubin & Jaresova, 1993; Thompson *et al.*, 2013), hospital faucets and ice machines (Galassi et al., 2003; Cooksey et al., 2008), diagnostic laboratories (Chang *et al*., 2002), bottled and municipal water, commercial and hospital ice (Covert *et al*., 1999), potting soil (De Garoote *et al*., 2006), house dust (Dawson, 1971; Torvinen *et al*., 2006), water damaged building materials (Torvinen *et al*., 2010), showerheads (Falkinham *et al*., 2008), shower aerosols (Thompson *et al*., 2013), hot-tub aerosols (Mangione *et al*., 2001), coniferous forest soils and brook waters (Iivanainen *et al*., 1993), cigarettes (Eaton, Falkinham & von Reyn, 1995), livestock (Leite *et al*., 2003), coastal mosses (Schroder *et al*., 1992) and seawater (Gruft *et al*., 1979). Nontuberculous mycobacteria species which have been isolated from animals, birds and fish include: MAC, *M. marinum, M. ulcerans, M. paratuberculosis, M. simiae, M. fortuitum* and *M. smegmatis.* The isolates from sewage samples include *M. gordonae, M. scrofulaceum, M. flavescens, M. phlei, M.* terrae, *M. fortuitum, M. chelonei* and *M. smegmatis* (Won Jin *et al.,* 1984). Mycobacterial species including, *M. marinum, M. chelonei, M. scrofulaceum* and *M. gordonae* have been isolated from samples taken from swimming pools (Falcao *et al*., 1993) and species in the *M. avium* complex have also been recovered from hot tubs (Embil *et al*., 1997; Kahana *et al*., 1997).

NTM in Soil

Nontuberculous mycobacteria such as *Mycobacterium kansasii*, MAC, *M. malmoense*, and *M. fortuitum* have been recovered from the soil, as well as from house dust (Paull, 1973). Mycobacteria, including *M. avium* and *M. intracellulare*, have also been recovered from anaerobic river sediments (Iivanainen *et al.*, 1999). Potting soil samples collected from the homes of HIV patients were found to contain serotypes of MAC that were similar to the isolates from the HIV patients of the study group (Yajko *et al.*, 1995).

Evidence of the presence of rapidly growing NTM in polluted soils (Wang *et al.*, 2006) suggests that rapidly growing NTM may be important agents of mineralization of pollutants. Polluted sites may be ideal habitats for rapidly growing NTM because they can metabolize a variety of major groundwater hydrocarbon pollutants (Burback & Perry 1993; Heitkamp *et al.*, 1988) and also attach to particulates where pollutants are concentrated (Stelmack *et al.*, 1999). Nontuberculous mycobacteria hydrophobicity-driven adherence to particulates would prevent flushing from polluted sites (Bendinger *et al.*, 1993), and limited oxygen levels are unlikely to restrict mycobacterial metabolism (Dick *et al.*, 1998). Numbers of rapidly and slowly growing NTM are relatively high in soils, particularly acidic, boreal forest soils and peats (Iivanainen *et al.*, 1997), water draining from peat-rich soils (Iivanainen *et al.*, 1999), and acidic, brown water swamps (Kirschner *et al.*, 1992). High NTM numbers, particularly *M. avium* and *M. intracellulare* are found in commercially available, peat-rich potting soil (Yajko *et al.*, 1995; De Groote *et al.*, 2006).

NTM in Dust/and Aerosols

Slowly growing NTM can readily be transmitted between habitats. Cells of *M. avium* and *M. intracellulare* are readily aerosolized from water to air, through droplet formation (Parker *et al*., 1983). Hydrophobic NTM cells adhere to air bubbles rising in a water column and, at the water surface, the bubble bursts ejecting droplets to heights of 10 cm (Parker *et al*., 1983). The densities of *M. avium* and *M. intracellulare* are 1000 to 10,000 fold higher in the ejected jet droplets than in the water (Parker *et al*., 1983). A substantial proportion of the droplets are small enough to enter human alveoli (Wendt et al. 1980; Parker *et al*. 1983). Evidence of *M. avium* pulmonary infection and disease associated with exposure to aerosols generated in showers (Falkinham et al., 2008) and hot tubs and spas (Embil *et al*., 1997; Kahana *et al*., 1997; Mangione *et al*., 2001) is indirect evidence of aerosol-borne mycobacterial infection. Shelton *et al*., (1999) investigated aerosolized NTM as a possible cause of hypersensitivity pneumonitis in three machine workers. They recovered *M. chelonae* and mycobacteria which were identical to *M. immunogen.*

NTM on Instruments

There have been a variety of reports documenting the presence of mycobacteria in bronchoscopes. Biofilms in water lines in dental drilling and cleaning devices contain a variety of mycobacteria. Bronchoscopes and catheters have been shown to be contaminated with *M. avium* (Gubler *et al*., 1992), *M. intracellulare* (Dawson *et al*., 1982), *M. xenopi* (Benett *et al*., 1994) and *M. chelonae* (Gubler *et al*., 1992; Takigawa *et al*., 1995). In most cases, contamination was suspected because of unusual increases in the isolation of a particular mycobacterial species. The presence of Mycobacterium species on

bronchoscopes has been attributed to inadequate decontamination of the bronchoscope following its use with an infected patient (Benett *et al*., 1994; Takigawa *et al*., 1995).

NTM in Animals

Infection in animals has been traced to the presence of mycobacteria in water. Simian virus-infected macaques were shown to have acquired *M. avium* infection from potable water (Mansfield *et al*., 1997). *Mycobacterium avium* has been recovered from tuberculous cervical and mesenteric lymph nodes of pigs and domestic fowl. The mycobacteria, *M. avium, M. fortuitum*, and *M. genavense* have been recovered from captive exotic birds (Hoop *et al*., 1993) and from wild birds. In contrast to *M. avium* infection in wild and domestic birds, *M. avium* infection in mammals occurs only sporadically and is rarely transmissible (Thorel *et al*., 2001). Infection is usually chronic and generalized disease is uncommon, but disseminated disease has been reported in captive hoofed animals and immunosuppressed dogs and cats. The zoonotic potential of *Mycobacterium avium* complex infections is poorly understood. *Mycobacterium avium* complex, MAC, causes infections in a wide range of animals including water buffalo (Freitas *et al*., 2001), cattle (BolIo *et al*., 1998), pigs (Ramasoota *et al*., 2001), deer (O'Grady *et al*., 2000) and horses (Leifsson *et al*., 1997). *Mycobacterium avium* complex (MAC) causes infections in cats (Kaufman *et al*., 1995) and dogs (Hom *et al*., 2000), armadillos (Dhople *et al*., 1992) and primates such as cynomolgus and rhesus macaques (Bellinger & Bullock 1988; Goodwin *et al*., 1988). *Mycobacterium avium* complex, MAC disease is more common in farmers (Falkinham 1996) possibly as a result of contact with animals or their products and MAC also cause infections in

chickens (Odiawo & Mukurira 1988), white cameaux pigeons (*Columbia livia*) (Pond & Rush 1981), commercial emus (Dromaius novaehollandiae) (Shane *et al*., 1993) and farmed rheas (*Rhea americana*) (Sanford *et al*., 1994). *Mycobacterium avium* complex infections in birds appear not to be the source of most human infections (Martin & Schimmel 2000; Pavlik *et al*., 2000), although MAC lymphadenitis was reported in two children who lived in close proximity to a pigeon loft (Cumberworth & Robinson 1995).

Nontuberculous Mycobacteria in Water

As stated earlier, NTM have been repeatedly isolated from natural and treated water. Piped water supplies are readily colonized by mycobacteria which can thereby lead to more frequent exposure to humans. Mycobacterial characteristics such as surface hydrophobicity and charge, as well as certain physiochemical factors like salinity, temperature, humidity and wind currents can influence the distribution of mycobacteria in water systems (Falkinham, 1996).

In Sri Lanka, the frequency of isolation of NTM from different water sources for aquarium water, surface water, ground water and chlorinated water were 29% (20/70), 26% (20/76), 5% (4/76) and 1% (1/68), respectively (Edirisinghe *et al*., 2014). Forty-five isolates belonging to the genus Mycobacterium were isolated from the collected water samples. Twenty of the isolates were identified as M. fortuitum, *M. phlei*, *M. gordonae*, *M. scrofulaceum*, M. chelonae, *M. marinum*, and *M. parafortuitum*. Furthermore, 25 *Mycobacterium* isolates remained unidentified since their phenotypic characteristics could not be matched with the existing schemes.

Early studies in the 1990s report of a variety of NTM isolations from different water sources (Hilborn *et al.*, 2002). *Mycobacterium avium, Mycobacterium intracellulare*, or the *Mycobacterium avium* complex have been isolated from drinking water (Falkinham *et al*., 2001), public bath waters, hospital water systems, and water supplies of hemodialysis centres. In addition, other NTM that have been recovered from drinking water include *Mycobacterium kansasii, Mycobacterium marinum, Mycobacterium malmoense, Mycobacterium scrofulaceum, Mycobacterium xenopi, Mycobacterium fortuitum, Mycobacterium abscessis*, and *Mycobacterium chelonae*.

Mycobacterium-avium-intracellulare-scrofulaceum (MAIS) has been recovered from acid-brown swamps and lake water samples. The high rate of recovery of MAIS was attributed to the combination of higher temperatures, low oxygenated waters, low pH soils, higher zinc, and fulvic was detected in 40% of surface water samples collected from streams. *Mycobacterium malmoense* was detected in two stream waters at concentrations of 320 and 750 CFU/L. In Valencia, Spain, 15 strains of *M. gordonae* and 10 strains of MAC were identified in a variety of surface water samples (Sabater & Zaragoza, 1993). Mycobacterial species including, *M. marinum, M. chelonei, M. scrofulaceum* and *M. gordonae* were isolated from swimming pool samples collected in Araraquara, Sao Paulo, Brazil (Falcao *et al.*, 1993). The number of isolates ranged from 1-3 per site.

Water reservoirs constructed of concrete or steel tanks, located below and above ground in a small town in Texas were reported to contain *M. kansasii and M. gordonae* (Steadham, 1980). The investigators postulated that the NTM

species could have entered the reservoirs and passed into the water distribution system from deep water wells or through seepage of water and soil which accumulate in both concrete and steel reservoirs. False report of NTM outbreaks have been traced to the use of tap water on medical devices and equipment during certain surgical and lab procedures, which is contaminated with *M. xenopi* (Sniadack *et al*., 1993), *M. gordonae* (Stine *et al*., 1987) and *M. fortuitum* (Jacobsen *et al*., 1996).

Drinking water and Nontuberculous Mycobacteria

Management of the microbial quality of drinking water is aimed primarily at minimizing illness caused by waterborne pathogens. The increasing prevalence of infections due to nontuberculous mycobacteria (Morimoto et al., 2014; Bar-On *et al*., 2015), combined with growing evidence linking such infections to drinking water (Nishiuchi *et al*., 2007; Falkinham *et al*., 2008; Falkinham, 2011), has highlighted NTM exposure through drinking water as an emerging public health challenge. Two trends that could influence the occurrence of nontuberculous mycobacteria in drinking water may be global warming and increase in the number of immunocompromised individuals. Global warming due to climate change, results in higher surface and drinking water temperatures and it is expected that growth of most opportunistic pathogens in drinking water is enhanced when water temperatures rise above 20 to 25°C (van der Wielen & van der Kooij, 2013). Also, the number of immunocompromised humans, who are prone to infection with opportunistic pathogens are increasing in the First World because of ageing of the population and the longer life spans of patients who suffer from serious diseases like AIDS, cancer, cystic fibrosis and others.

Potable water is not routinely monitored for the presence of mycobacteria, and there is limited information on the occurrence or concentrations of these bacteria in drinking water systems. For microbial water quality, verification is based on the analysis of faecal indicator microorganisms, with the organism of choice being *Escherichia coli* or, alternatively, thermotolerant coliforms. Monitoring of specific pathogens may be included on very limited occasions to verify that an outbreak was waterborne. *Escherichia coli* provides conclusive evidence of faecal pollution and should not be present in drinking water. Under certain circumstances, additional indicators, such as bacteriophages or bacterial spores, may be used. However, water quality can vary rapidly, and all systems are at risk of occasional failure. For example, rainfall can greatly increase the levels of microbial contamination in source waters, and waterborne outbreaks often occur following rainfall. According to the Water Services Association of Australia (WSAA, 2017), Mycobacterial species are present in natural water sources from which drinking water is drawn, and in contrast to most other bacteria, these microorganisms are relatively resistant to drinking water disinfection processes. This resistance is attributable to the thick cell wall, with its high lipid content, which is common to all mycobacteria. Mycobacteria are believed to grow mainly as a component of biofilms attached to pipe surfaces within water supply systems, although they can also be found in the water phase.

The chlorine in treated water is regarded as an effective means to reduce risks from a range of microbial pathogens, but this measure appears to be ineffective against mycobacteria. Research has shown that levels of chlorine-based disinfectants that kill other microorganisms in pipe biofilms may have

little effect on mycobacterial numbers (WHO, 2011). One United States study in a system that switched from chlorine to chloramine disinfection showed a reduction in the rate of Legionella colonization, but an increase in mycobacterial colonization (WSAA, 2017). This raises the possibility that efforts to reduce risk from one pathogen in water systems may have the unintended consequence of increasing exposure to another. It is not clear whether good management practices such as flushing and disinfection after repairs, and regular cleaning of sediments from pipes and storage tanks have any significant effect on mycobacterial growth in water distribution systems. A study of eight Dutch water supplies found NTM in all systems, and with abundance increasing through the distribution systems, compared to levels at the associated water treatment plants. Reduction of nutrient levels in water has also been advocated as a possible means of controlling mycobacteria, but in the Dutch study the nutrient levels in water appeared to have no effect on mycobacterial numbers, while higher water temperatures were associated with increased abundance (WSAA, 2017). Current understanding of mycobacterial ecology in water supply systems and their interactions with other potential pathogens is limited, and there is insufficient knowledge to formulate control strategies to reduce the prevalence of NTM in water distribution systems.

Reducing Nontuberculous Mycobacteria in Potable Water

Water filtration has been shown to reduce NTM numbers, but without changing the filter regularly (<3 weeks), the filter can become a source (Rodgers *et al*., 1999). Filters provide an ideal habitat for NTM; they attach and can grow on the filter material on the organic compounds collected and concentrated on the filters, even if the filter is impregnated with an antimicrobial agent e.g. silver

(Rodgers *et al.*, 1999). Nontuberculous mycobacteria numbers in drinking water distribution systems are higher in systems with higher turbidity (Falkinham *et al.*, 2001), likely because of the hydrophobicity-driven adherence of NTM to soil particulates (Stelmack *et al.*, 1999). Thus, reduction of water turbidity would be expected to reduce NTM numbers in both water treatment systems and households.

Water Treatment Methods

Clean, safe water is vital for everyday life. Water is essential for health, hygiene and the productivity of our communities. The water treatment process may vary slightly at different locations, depending on the technology employed at the water treatment plant and the water it needs to process, but the basic principles are largely the same. In general, drinking water treatment methods can be separated into two modes of action: removal and disinfection. Methods used to remove physical contaminants typically are physical rather than chemical techniques. Removal methods such as filtration, sedimentation, coagulation, flocculation and adsorption are primarily physical operations that remove bacteria from the water. Disinfection is defined as the destruction or inactivation of pathogenic microorganisms including bacteria, amoebic cysts, algae, spores and viruses (Montgomery, 1985). Disinfection treatment methods may kill bacteria using chemicals such as chlorine and ozone which are added to the water, or may inactivate microbes by UV radiation (EPA-USA, 2002). Nontuberculous mycobacteria are relatively resistant to standard water disinfection procedures and, therefore, can occur in potable water (EPA-USA, 2002). The quality of the raw or source water and the measures that are taken to improve water quality prior to disinfection will have a significant effect on the

efficacy of any treatment method. Water quality factors that influence disinfection efficiency include particulates or aggregates (suspended solids or turbidity), dissolved organic matter, inorganic constituents, pH and temperature. Physicochemical treatment methods are generally used prior to disinfection to 'clarify' the source or raw waters for improvement of water quality. These treatment methods are also used to improve the efficiency of disinfection (called disinfection demand) by reducing the inorganic and organic loads present in source water prior to drinking water treatment. The key steps in water treatment processes are discussed below.

Steps involved in water purification

The steps shown in Figure 2 are the basic steps used by most companies which produce portable water for human consumption. Based on the source of water to be treated, sometimes some steps are added to these major steps for water treatment. These steps are discussed below.

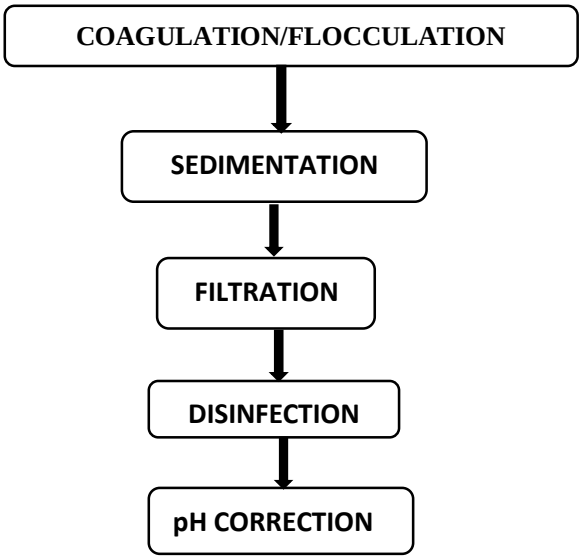

Figure 2: Major steps involved in water purification for human consumption
Coagulation / Flocculation

During coagulation, liquid Aluminium Sulfate (alum) is added to untreated (raw) water. When mixed with the water, this causes the tiny particles of dirt in the water to stick together or coagulate. Next, groups of dirt particles stick together to form larger, heavier particles called flocs which are easier to remove by settling or filtration.

Sedimentation

As the water and the floc particles progress through the treatment process, they move into sedimentation basins where the water moves slowly, causing the heavy floc particles to settle to the bottom. Floc which collects on the bottom of the basin is called sludge, and is piped to drying lagoons. In Direct Filtration, the sedimentation step is not included, and the floc is removed by filtration only.

Filtration

Water flows through a filter designed to remove particles in the water. The filters are made of layers of sand and gravel, and in some cases, crushed anthracite. Filtration collects the suspended impurities in water and enhances the effectiveness of disinfection. The filters are routinely cleaned by backwashing.

Disinfection

Water is disinfected before it enters the distribution system to ensure that any disease-causing bacteria, viruses, and parasites are destroyed. Chlorine is used because it is a very effective disinfectant, and residual concentrations can be maintained to guard against possible biological contamination in the water distribution system.

pH Correction

Lime is added to the filtered water to adjust the pH and stabilize the naturally soft water in order to minimize corrosion in the distribution system, and within customers' plumbing.

Clinical Significance of Nontuberculous Mycobacteria

A number of species of NTM are epidemiologically important. For example, *Mycobacterium marinum* causes infections in fish and skin granulomas in humans where infection is associated with occupation in the fishing industry or exposure to aquaria (Aubry *et al*., 2002). The prevalence of *Mycobacterium malmoense* infections has steadily risen since its first description in 1977 and infections are commonly associated with damaged lungs due to infection or inhalation of particles (Zaugg *et al*., 1993). In adults, pulmonary infection is the most commonly recognized form of NTM infections. These infections often present clinically as chronic cough, sputum production and fatigue. Members of the MAC or *Mycobacterium kansasii* are the NTM species most commonly associated with pulmonary infections. However, other species known to occasionally cause pulmonary disease include: *Mycobacterium xenopi, M. fortuitum, M. abscessus, M. szulgai, M. malmoense* and *M. simiae*.

Tuberculous lymphadenitis (inflammation of the lymph nodes) which is caused by *Mycobacterium tuberculosis* or other related mycobacteria occurs predominantly in young children, between 1 and 5 years old, and typically affects the cervical, submaxillary, submandibular and preauricular lymph nodes (ATS, 1997; Jenkins, 1991). In the absence of HIV infection, this disease rarely affects adults. Historically, the classical cause of cervical lymphadenitis was

Mycobacterium scrofulaceum whereas today, the species most commonly involved is the MAC. *Mycobacterium abscessus* has recently been implicated in causing sporadic ear infections after placement of tympanotomy tubes (Correa & Starke, 1996).

Multiple species of mycobacteria have been identified as causative agents of skin and soft tissue infections. These include; *Mycobacterium marinum, M. ulcerans, M. haemophilum, M. fortuitum, M. abscessus, M. chelonae* and species within the MAC. These infections can be either community acquired or nosocomial infections. *Mycobacterium marinum* is a species of Mycobacterium most commonly associated with skin infections. Most often, infection from this species occurs following an exposure of cut or abraded skin to organisms present in aquaria, pools, natural water supplies and salt water (ATS, 1997). The typical outcome of infection is the development of a localized skin lesion on the arms or legs. *Mycobacterium haemophilum* has also been observed to cause joint and skin infections in immunocompromised patients resulting in lymphadenitis and skin lesions in healthy children. The rapidly growing mycobacterial species *Mycobacterium abscessus, M. fortuitum* and *M. chelonae* are also a common causes of skin and soft tissue infections following local trauma.

Although infrequent, catheter-associated mycobacterial infections have most often been associated with long-term central venous catheters and are linked to rapidly growing mycobacterial species. The disease may include exit site infections, tunnel infections or catheter-related bacteraemia (Correa and Starke, 1996). The most common NTM species associated with catheter-associated infections are *Mycobacterium fortuitum, M. chelonae, M. abscessus*

and *M. mucogenicum* (Wallace *et al*., 1993). Rarely, other species may be seen, including *Mycobacterium avium* complex.

Disseminated NTM infection in HIV patients appears to originate from a primary infection of either the respiratory or gastrointestinal tracts (Correa & Starke, 1996). These infections may involve any organ, but most commonly occur in the lungs, liver, spleen, lymph nodes or bone marrow (Correa & Starke, 1996). Common symptoms include prolonged fevers (often accompanied by night sweats), weight loss and occasional abdominal pain or diarrhoea. This disease is most commonly seen in patients with less than 50 CD4 cells (ATS, 1997). The primary Mycobacterium species associated with disseminated infections in HIV infected patients is *Mycobacterium avium*. However, *Mycobacterium kansasii, M. haemophilum* and *M. genavense* have also been implicated. Prior to the HIV epidemic, disseminated infection caused by MAC was rare, occurring primarily in patients with underlying immunodeficiency. The mycobacteria, MAC, *M. kansasii, M. chelonae, M. scrofulaceum, M. abscessus* and *M. haemophilum* have all been observed to cause disease in individuals without HIV infection. The typical symptom of disseminated infection with MAC is a fever of unknown origin, whereas symptoms caused by the other species consist of multiple subcutaneous nodules or abscesses that drain spontaneously (ATS, 1997). In immunosuppressed individuals other than those with HIV, particularly in patients undergoing chemotherapy for cancer, dissemination of disease from a cutaneous infection is the most common form of NTM disease. These infections are usually due to *Mycobacterium chelonae, M. abscessus, M. haemophilum* and rarely other species such as *M. kansasii*.

Mycobacterium ulcerans

Mycobacterium ulcerans is the Mycobacterium species that causes Buruli ulcer (BU), the third most common mycobacterial disease after tuberculosis and leprosy (Johnson *et al.*, 2005). BU was first described by Sir Albert Cook, a British physician, in patients from Buruli district in Uganda (now known as Nakasongola District) and the causative organism was first isolated by MacCallum and others in the Bairnsdale district of Victoria, Australia in 1948, hence the name the Bainsdale ulcer. The mode of transmission of *Mycobacterium ulcerans* is not known and unlike *Mycobacterium leperae* and *Mycobacterium tuberculosis* which are transmitted by person-to-person (Merritt *et al*., 2010). It is thought that infection with *Mycobacterium ulcerans* occurs through contact with the environment. *Mycobacterium ulcerans* causes distinctive, often severe, skin lesions. It is thought that the primary mode of infection with this species is through cuts from vegetation (e.g., grass) in wet swampy locations which allow the organisms to enter the skin. Lesions develop as small, palpable, painless, subcutaneous swellings approximately 4 to 10 weeks after infection. The growing nodule, which is firm and attached to the skin, remains superficial and extends laterally involving fat and fascia around muscle bundles or the muscles themselves. The skin overlying the lesion loses pigmentation, becomes filled with fluid and necrotic debris, and often ulcerates. The ulceration typically has undermined edges and enlarges over many months (Feldman, 1974). The most well recognized NTM in Africa and specifically Ghana is *M. ulcerans*.

Buruli ulcer cases are usually found in communities near wetlands such as swamps, marshes and slow moving rivers in areas that are prone to flooding.

Also, areas where the environment has been disturbed example as in areas which have suffered from deforestation, eutrophication, construction of dams and irrigation systems, mining and others have recently had an increase in the number of reported BU cases (Asiedu *et al*., 2000; Merritt *et al*., 2005; Duker *et al*., 2006;). Buruli ulcer has been reported from more than 33 countries worldwide, mainly in tropical and subtropical regions (WHO, 2010). The worst affected areas are countries lying along the Gulf of Guinea in West Africa, where Buruli Ulcer prevalence exceeds that of leprosy, making it the second most important mycobacterioses. In West and Central Africa, the disease typically affects impoverished communities primarily children of remote areas where medical services are unavailable or too expensive. It is estimated that more than 7000 people develop BU annually, with the West African countries like Benin, Côte d'Ivoire and Ghana having the highest incidence rates (WHO, 2008).

The first case of Buruli ulcer in Ghana was first reported in the Greater Accra Region in 1971 by Bayley from patients living along the tributaries of the Densu River which passes through the Central, Eastern and Greater Accra regions. In 1989, 96 cases were reported in Asante Akim North District of the Ashanti Region (Van der Werf., 2014). This was followed by the discovery of a major endemic focus in Amansie West in the same region (Amofah *et al*., 1993). Cases of the disease have been reported in all the ten regions of the country with the Ashanti Region reporting the highest number of cases of about 60% of all cases. The overall national prevalence is 20.7 cases per every 100,000 population making BU the second most important mycobacterial

disease after tuberculosis (Amofah *et al*., 2002). Globally, Ghana is the second most endemic country for Buruli ulcer after Cote d'ivoire (WHO, 2012).

Analytical Methods for the Detection of Acid-fast bacilli (AFB)

The isolation of AFB from environmental samples is achieved by culturing on a selective medium. Molecular methods and microscopy are also employed in the identification of mycobacteria. Modern trends have provided fast and easier ways of isolating and identifying mycobacteria. For the isolation of mycobacteria from water, many studies have employed minor modifications of methods developed for the diagnosis of Tuberculosis. Discussed below are some analytical methods employed for the isolation and identification of acid-fast mycobacteria.

Decontamination Methods

Most samples for environmental mycobacteria culture contain various amounts of other bacteria which usually grow faster. Therefore, recovery of mycobacteria is aided by a chemical decontamination process that effectively kills other microorganisms leaving behind mycobacteria to grow for isolation to be done. Sodium hydroxide, oxalic acid, benzalkolnium chloride and cetylpyridinium chloride (CPC) are the most commonly used decontaminants. Decontamination process is a balance between maximizing recovery of viable mycobacteria, and minimizing contamination by other bacteria and fungi. A wide range of decontamination methods and culture conditions have been used by a variety of researchers to selectively isolate mycobacteria from the environment. However, these decontamination methods kill some of the mycobacteria (Iivanainen *et al*., 1997). Their isolation from environmental samples requires both selective decontamination of samples and cultivation on

selective media due to the threat of overgrowth by more rapidly growing microbes (Falkinham, 1996). The stronger the decontamination agent, the higher its temperature during the time it acts on the specimen, and the longer it is allowed to act, the greater will be the killing action on both contaminants and Mycobacterium. Strict adherence to the timed killing period is necessary to maximize recovery (Pfyffer *et al*., 2003). Culturing can also not be done without decontamination because other bacteria may overgrow and overshadow the mycobacteria of interest so researchers most of the time use the mildest form of a decontamination agent so that both aims of killing and recovery are achieved.

Acid-fast microscopy

Acid-fast microscopy is the fastest, easiest, and least expensive tool for the rapid identification of patients with mycobacterial infections and also for water samples contaminated with nontuberculous mycobacteria. Although the specificity of acid-fast microscopy is excellent (all mycobacterial species are acid-fast), the sensitivity is not optimal, and this method is unable to distinguish within the Mycobacterium genus (Somoskovi *et al*., 2002) and may also detect other acid-fast species such as Nocardia and Corynebacterium. The sensitivity of microscopy is influenced by numerous factors such as the prevalence and severity of tuberculosis or NTM disease, the type of specimen, the quality of specimen collection, the number of mycobacteria present in the specimen, the method of processing (direct or concentrated), the method of centrifugation, and most importantly, by the staining technique and the proficiency of the examination (Somoskovi *et al*., 2002). Presently, two types of acid-fast staining techniques are used in clinical mycobacteriology laboratories. One is carbol

fuchsin (Ziehl-Neelsen (ZN) or Kinyoun staining methods), and the other is a fluorochrome (usually auramine or auramine-rhodamine and potassium permanganate).

Isolation of Acid-fast Bacilli (AFB)

Isolation of AFB involves cultivation on media after decontamination to kill other bacteria present in the sample being analyzed. Cultivation of mycobacteria is considered to be the gold standard for detection of mycobacteria in a sample and it is done on solid or in liquid media. However, solid media allow for the observation of colony morphology. The recommended solid media include either egg based media such as Löwenstein-Jensen medium (LJ) and Ogawa medium and agar-based media such as Middlebrook 7H10 and 7H11 agar. Löwenstein-Jensen (LJ) medium containing malachite green dye to inhibit growth of contaminating organisms, is the traditional solid media for culture of mycobacteria. However, with liquid media and modern culture systems such as the BACTEC AFB or Mycobacteria Growth Indicator Tubes (MGIT), growth can typically be seen in approximately 2 weeks. Isolation of AFB from environmental samples is an important step in the detection of mycobacteria in the environment.

The major problem encountered when isolating mycobacteria from the environment is the presence of high numbers of other fast growing microorganisms and fungi in the samples that overgrow on the media before the growth of mycobacteria. This leads to contamination of the medium by other fast growing bacteria which hinder the isolation of mycobacteria. Enrichment of culture media is needed to allow growth of mycobacteria. For example, *M. haemophilum* needs an iron source (ferric ammonium citrate or hemin) in the

medium and is best incubated at 30°C (Saubolle *et al*., 1996) while for *M. genavense* it has been reported that media composed of blood, charcoal, caseine, yeast extracts and acidified to pH 6.0, is successful in its isolation (Realini *et al*., 1999). Most mycobacteria grow optimally between 28°C and 37°C.

DNA extraction.

Generally, DNA extraction involves lysing or breaking up of cell walls, separating DNA from other cell components and isolating DNA. A variety of methods can be used to isolate DNA from cells of mycobacteria. Some of the methods for DNA isolation are boiling the sample in distilled water, autoclaving, disruption by glass beads or sonication, use of different enzymes and surfactants etc. (Hosek *et al*., 2006). The initial step in the extraction is breaking the cell so that its components can be released. Cells are lyzed by using chemical reagents such as lysozyme, EDTA, lysozyme and EDTA, detergents. Cells can also be lyzed by heating samples on a thermostat/heat block, boiling the sample in distilled water, autoclaving or disruption by glass beads or sonication. The simplest way of DNA release from mycobacterial suspension is boiling at 100°C for 10 to 15 min in distilled water (Tortoli *et al*., 1999; Aldous *et al*., 2005). After cells are lyzed, the next step is the removal of cellular components using organic extraction method, or silica-based technology. The last step involves DNA precipitation to obtain pure DNA at a high concentration. Selection of an adequate isolation method of mycobacterial DNA is essential because the quality of the extracted DNA may determine the success or failure of the amplification processes.

Categorizing mycobacteria culture isolates as NTM or MTBC

Advances in molecular biology have provided fast and easier ways to categorize or differentiate culture isolates as NTM or MTBC. Technologies such as the use of molecular probes, gas-liquid chromatography, and high-performance liquid chromatography are available to aid in the differentiation or categorization process but these tests are technically complex, cumbersome, and expensive, especially the ones that are based on molecular analyses (Ichiyama *et al*., 1997). Recently, the immunogenic protein MPB64 which has been identified as a *Mycobacterium tuberculosis* complex-specific secretory protein has been used to differentiate MTBC from NTM. *Mycobacterium tuberculosis* complex are known to produce 200 or more kinds of protein including MPB64, ESAT6, CFP10, Ag85B, Hsp16.3. Among these secreted proteins, MPB64, which was first described as MPT64, has been found in unheated culture media of *M. tuberculosis*, *M. bovis*, and some but not all sub strains of *M. bovis* BCG (Hasegawa *et al*., 2002). This is a highly specific protein for the *M. tuberculosis* complex and has been well characterized, and the antigen has been widely studied. The molecular weight of MPB64 is 24,000 (Harboe *et al*., 1986) and a number of rapid MPB64 antigen assays have been developed to categorize mycobacteria isolates as MTBC or NTM.

The Capilia TB-Neo assay (TAUNS Laboratories Inc. Japan) is a lateral flow imunochromatographic test which employs the presence or absence of MPB64 in categorizing AFB as NTM or MTBC. The Capilia TB assay is a rapid, simple, and inexpensive identification test and it has been evaluated extensively. The total assay time is 15 min, with reactivity determined by visual colour development of test and control lines.

Molecular Characterization of *Nontuberculous mycobacteria*

Molecular characterization targeting short regions of the bacterial 16S rRNA gene are widely used to profile bacterial communities in water; however, this target does not provide sufficient resolution to distinguish NTM species from each other (Halstrom *et al*., 2015). Sequencing based methods are becoming more widespread and the most common target genes include 16SrRNA (Cloud *et al*., 2002), heat shock protein (*hsp* gene) and *rpoB* (Adekambi *et al*., 2006; Adekambi *et al*., 2008). Polymerase chain reaction is done first of all to amplify fragment of the DNA, coding for these genes. The first step in a PCR-RFLP analysis is amplification of a fragment containing the variation. This is followed by digestion of the amplified fragment with an appropriate restriction enzyme or endonuclease. The restriction enzymes cut the amplicons at the restriction enzyme recognition site resulting in the formation of restriction fragments of different sizes. The resulting fragments are analyzed using gel electrophoresis after which the bands are visualized using UV light.

Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism Analysis

Currently, identification of clinical isolates of mycobacteria to the species level is primarily based on cultural characteristics and biochemical tests which take several weeks and sometimes even fail to provide precise identification (Lee *et al*., 2000). The procedures for these tests are complex and laborious, and they are usually impeded by the slow growth of mycobacteria in clinical laboratories. In addition, currently available DNA strip assay kits are limited to the identification of a few species. In contrast to the above-mentioned techniques, PCR-restriction fragment length polymorphism analysis of the *rpoB*

gene using restriction enzymes, *Msp*I and *Hae*III offer an easy, rapid, and inexpensive way to identify several mycobacterial species in a single experiment.

Polymerase chain reaction-restriction fragment length polymorphism analysis (PRA) using the novel region of the *rpoB* gene was developed for rapid and precise identification of mycobacteria to the species level (Lee *et al*., 2000). Lee *et. al.* (2000) developed a PRA method that is precise for mycobacterial species identification. From the PRA they performed, the sizes of the restricted fragments of each species were determined, and an algorithm was constructed based on this information. In the study, a total of 50 mycobacterial reference strains and 3 related bacterial strains were used to amplify the 360-bp region of *rpoB* gene, and the amplified DNAs were subsequently digested with restriction enzymes *Msp*I, *Hae*III, *Sau* 3A, *Kpn* 1 *and Bst* EII. The primer pair used for the amplification were 5′-TCAAGGAGAAGCGCTACGA-3′ (RPO5′) and 5′-GGATGTTGATCAGG GTCTGC-3′ (RPO3′). The primer sequences were selected from the region of the *rpoB* gene which have been previously identified from *M. tuberculosis*, *M. leprae,* and *M. smegmatis* (Lee *et al*., 2000). The primers amplified the region between the first variable region (V1) and second conserved region (C2) based on the genetic information for the *rpoB* gene of *Escherichia coli*. This resulted in PCR products of 360 bp, 171 bp and 189 bp of the variable and conserved regions respectively. The results from this study clearly showed that most of the mycobacterial species were easily differentiated to the species level by this method. In addition, species with several subtypes, such as *Mycobacterium gordonae, M. kansasii, M. celatum,* and *M. fortuitum*, were also differentiated by this method. Subsequently, an algorithm was

constructed based on the results. The algorithm developed from this research is shown in Figure 2. The lines in the algorithm represent the fragment/ bands as seen on a gel after digestion with the restriction enzymes *Msp*I and *Hae*III (and other enzymes *Sau*3AI, *Kpn*I and *Bst*EII) and the numbers indicate sizes (in base pairs) of resulting fragments/ bands.

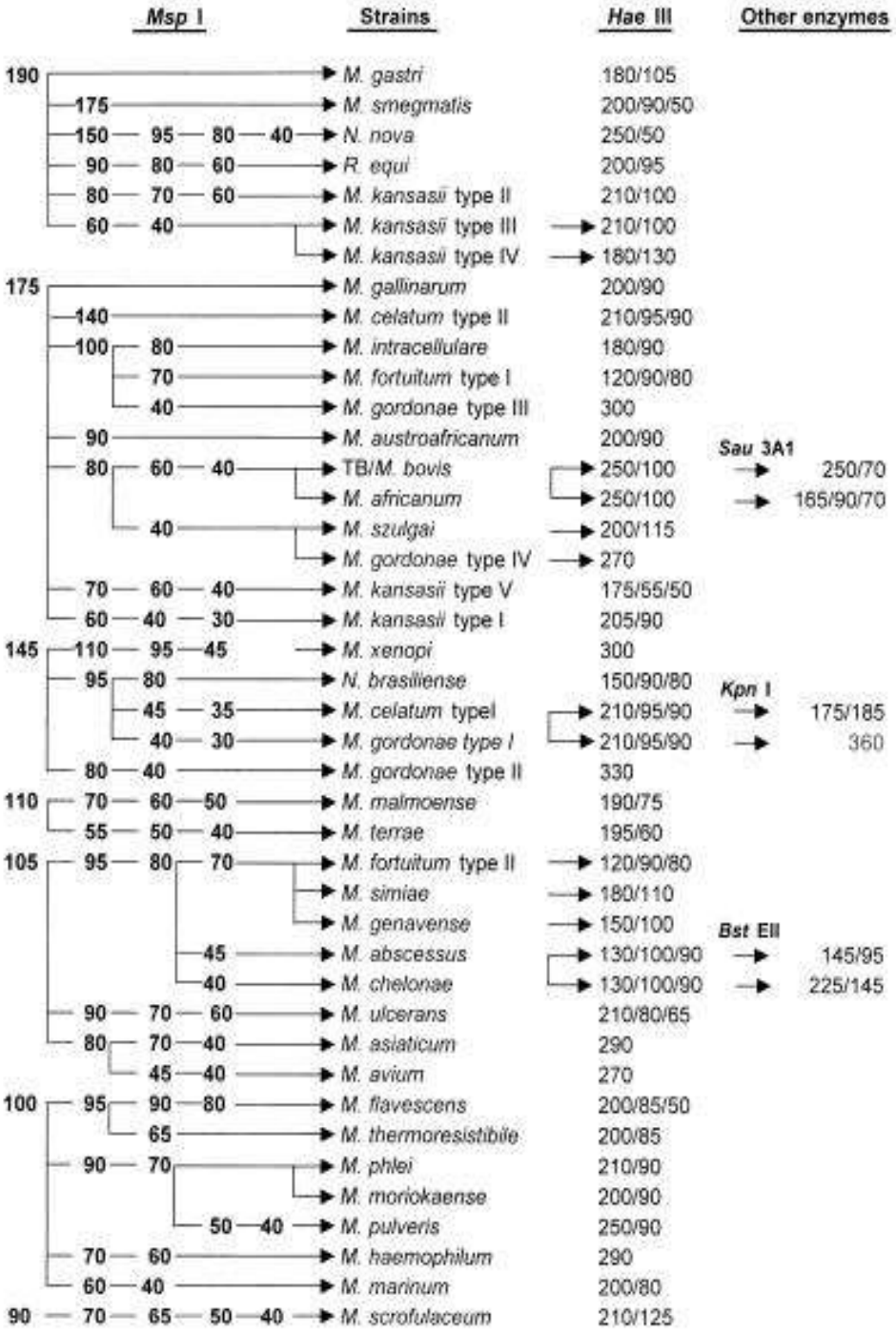

Figure 3: An algorithm constructed based on the results of PCR restriction enzyme analysis (PRA) with 40 mycobacterial reference strains and 3 other related bacterial strains (Lee et al., 2000).

CHAPTER THREE

MATERIALS AND METHODS

The main aim of the study was to find the occurrence of nontuberculous mycobacteria in the main sources of drinking water in parts of the Central Region of Ghana. These sources of water are exploited by the Ghana Water Company Limited (GWCL) in order to treat water for distribution to the general public. This chapter gives a detailed description of all the experiments performed in this study. The chapter is presented under the following headings: Study Design, Study Area, Sampling, and the Microbiological and Molecular aspects of the study.

Study Design

The main source of drinking water for the people of the Central Region is treated water from GWCL (GWCL, 2016). Few communities rely on water from other sources like boreholes and wells for drinking. As a baseline study, the main focus was on finding the occurrence of NTM in treated water from GWCL.

In finding the presence or absence of NTM in the water sources, the water samples were decontaminated and cultured on Lowenstein-Jensen (LJ) media and harvested colonies were confirmed as acid-fast by Ziehl-Neelsen (ZN) microscopy. Isolates which were found to be positive for AFB were categorized as NTM using the Capilia TB-Neo assay (TAUNS Laboratories, Inc. Japan). The particular species of NTM isolated from the water samples were determined by polymerase chain reaction-restriction fragment length polymorphism analysis. Primers for the amplification of the genus-specific *rpoB* gene and two restriction enzymes, *Msp*I and *Hae*III were used for PCR

and PCR products digestion respectively. These two enzymes were selected because of the sequence information they reveal about the *rpoB* genes of *M. tuberculosis, M. leprae,* and *M. smegmatis.* The resulting bands were interpreted using an algorithm designed by Lee et al. (2000) to identify the particular species of NTM.

Study Area

The Study Area for this research was the Central Region, specifically the towns where the treatment plants (TP) of Ghana Water Company Limited are located. Ghana Water Company Limited has nine headworks/treatment plants in the region which are located in the following towns: Sekyere Hemang, Brimsu, Baifikrom, Breman Asikuma, Essakyir, Winneba, Kwanyako, Twifo Praso and Dunkwa. Brimsu and Kwanyako have two separate systems; old and new serving different towns.

Water Treatment systems Employed by Ghana Water Company Limited

Seven treatment plants out of the nine use surface water and the conventional method of water purification which follows the steps: coagulation/flocculation, sedimentation, filtration, disinfection and pH correction. Twifo Praso and Dunkwa TPs use mechanized borehole systems and not the conventional water purification method. Twifo Praso, Dunkwa and Breman Asikuma TPs were not included in the study because accessibility to the headworks was very difficult. Breman Asikuma TP was also not running a continuous system as at the time of the research. Sekyere Hemang, Brimsu Old, Brimsu New, Baifikrom, Essakyir, Winneba, Kwanyako Old and Kwanyako New were the TPs used in this study and they also served as the key sampling points for the study. Towns supplied with potable water by these treatment

plants formed parts of the study area. Table 2 is a summary of the towns and sampling points considered in the study.

Table 2: Areas/Towns in the Central Region included in Study

GWCL Treatment Plant	River source	Location of Reservoir	Location of Community tap	Other source of drinking water
Sekyere Hemang	Pra	Mawukpor	Atabaadze Kissi	Abrodziwuram Kissi
Brimsu	Kakum	None	Brimsu Asanaadze	Asanaadze
Baifikrom	Ochi Amissah	None	Baifikrom Saltpond Mankessim	Saltpond Baifikrom
Essakyir	Ochi Nakwa	Mbroboto	Gomoa Adam Kotukwa Antseadze	None
Winneba	Ayensu	None	Winneba Bududuram	Winneba
Kwanyako	Ayensu	None	Kwanyako Potsin, Salem	Eduafo

The laboratory analyses were done at the Cellular and Clinical Research Centre (CCRC) laboratory of the Radiological and Medical Sciences Research Institute (RAMSRI) of the Ghana Atomic Energy Commission (GAEC).

Collection of Water Samples

Permission was sought from the GWCL, Central Region (Head Office) for the collection of water samples from GWCL treatment plant. A copy of the permission letter can be found in the Appendix. Water from the following points were collected:

a. Raw water source (river).
b. Treated water at the GWCL treatment plant before distribution to towns for consumption or storage in reservoir.
c. Water from GWCL reservoir.
d. Treated water from taps in the community (point of use).
e. Water from any other source used for drinking by the people in the community (boreholes or wells).

Sample bottles

Plastic bottles (500 ml volume) were used for sampling. The sample bottles were irradiated with gamma rays at 25 kGy and dose rate of 0.595 kGy/hr for two days at the Gamma Irradiation Facility at Ghana Atomic Energy Commission (GAEC) to sterilize them. All bottles were appropriately labelled with dates and sources of water samples.

Sampling procedure for treated water, reservoir water and river

Sampling was done for all the six headworks in a day and this was repeated after two weeks. The taps (metal) from which samples were taken were sterilized with heat flame for about two minutes then opened to let water run to waste for a minute. Sterilized sample bottles were then filled with water and covered immediately. Due to the aerobic nature of Mycobacteria, the bottles were not filled to the brim to allow air circulation, thus keeping the organisms alive until analysis and also to facilitate mixing by shaking before analysis. Samples were kept in an ice chest and transported to the lab, stored at room temperature and analyzed within forty eight hours of collection.

Twelve samples were from rivers, 16 from treated water from GWCL treatment plants before distribution to the communities, 4 from GWCL

reservoirs, 16 from community taps and 7 samples from boreholes and wells. The number of samples and the various sources are shown in Table 3.

Table 3: Number of water samples collected for each sampling point/ location

Location	Sources sampled	Number of samples
Sekyere Hemang	River	
	Treatment plant	10
	Reservoir	
	Community tap	
	Borehole	
Brimsu	River	
	Treatment plant	9
	Community Tap	
	Borehole	
Baifikrom	River	
	Treatment plant	9
	Community Tap	
	Borehole	
Essakyir	River	
	Treatment plant	
	Reservoir	9
	Community Tap	
Winneba	River	
	Treatment plant	
	Community Tap	7
	Borehole	
Kwanyako	River	
	Treatment plant	
	Community Tap	11
	Borehole	

Measuring Chemical and Physical Characteristics of Water Samples

Water from these sources had different characteristics. Some physical and chemical characteristics of the water samples were taken on the field during sampling. For this study, the physical characteristics considered were appearance, colour and odour and the chemical characteristics considered were

pH, temperature and residual chlorine for treated water. These measurements were taken on the field concurrently with sampling based on the procedure employed by GWCL for their routine quality assessment.

Measuring pH and Temperature of water samples

The temperature and pH were measured using the Multi-Parameter PCS Tester 35 (Oakton Instruments, Vernon Hills, IL 60061-USA). The instrument was calibrated for these two parameters at the GWCL office before taking it to the field. On the field, these parameters were measured by placing tester into each collected water sample and the tester allowed to measure. Measurements were then recorded.

Measuring residual or free chlorine levels in water samples

A Hach portable digital colorimeter (DR/820 Hach Company-Loveland, Colorado, U.S.A.) was used in measuring the residual chlorine level of the water samples following the manufacturer's instruction. Measurements were made and recorded for each collected sample.

Microbiological Analysis of Samples

The microbiological analysis was done by culturing the water samples on Löwenstein-Jensen media. Before culturing samples, they were decontaminated with 4% NaOH solution. After culturing, isolates were identified as AFB by Ziehl-Neelsen staining microscopy and categorized as NTM using the Capilia TB-Neo assay.

Decontamination of water with 4% NaOH solution

Equal volumes of sample and 4% NaOH solution were added in sterile 50 ml Polypropylene conical tubes. Mixture was shaken with a shaker (Heidolph Vibramax 100 Shaker) for twenty minutes at 400 rpm and then

centrifuged with IEC Centra Centrifuge (GP8R-USA) for another twenty minutes at 3000 rpm. Supernatant was poured off leaving pellets in tube.

Culturing on Löwenstein-Jensen media

Pellets were re-suspended in 2 ml of sterile double distilled water. For each sample, a 200 µL aliquot of the resulting suspension was inoculated onto Löwenstein-Jensen slants in replicates per each incubation temperature. The replicate slants (or tubes) were incubated (Memmert and Hereaus incubators) at two different temperatures, 30˚C and 37˚C for the growth of Mycobacteria.

Microscopy for the detection of Acid-Fast Bacteria

Growths or colonies on media were picked with loops and gently spread in the centre of the slides in a uniform manner to get circular shapes. Smears were allowed to air-dry and then stained using the Ziehl-Neelsen staining procedure as follows: The slides with smears were flooded with Carbol fuchsin and heat applied gently under the slides until vapour appeared. Slides were then decolorized with 20% H_2SO_4 for five minutes before counter staining with methylene blue for one minute. Slides were then rinsed with running water and left to air-dry. Smears were then examined first at 10X for rough focussing and then at 100X with a drop of immersion oil using a Cole Parmer digital microscope (Model DC5-163) for the presence or absence of Acid-fast bacteria which appear as pink rods in a blue background. All isolates whether positive or negative for AFB microscopy were used for the second stage of the work which involved the categorization of isolates into MTBC and NTM using the Capilia TB-Neo assay.

Capilia TB-Neo assay for the categorization of Nontuberculous Mycobacteria

Samples that were positive for AFB were selected for the Capilia TB-Neo assay for the categorization of isolates as MTBC or NTM. Capilia TB-Neo assay was performed following the manufacturer's instructions. A loop full of bacterial colonies was suspended in 200 µl of extraction buffer in 1.5 ml eppendorf tubes and vortexed for one minute using a Heidolph Reax Top vortexer machine. One hundred microlitres (100 µl) of the suspension was used as the specimen and placed in the test area of the Capilia TB-Neo assay. This was allowed to stand for fifteen minutes before the results were interpreted as positive or negative. NTM samples gave negative results for the Capilia TB-Neo assay. Samples which were identified as NTM were selected for DNA analysis.

Molecular Biology Analysis of Samples

DNA extraction

Crude DNA was obtained as follows: samples were put in a heating block (Block Thermostat, Model BT 100) at 95^{o}C for twenty minutes to lyse the bacterial cells. Samples were centrifuged at 14000 g for 10 minutes. The supernatant was discarded and the crude DNA (as pellets**)** stored at -20^{o}C until retrieved for further analysis.

Polymerase Chain Reaction (PCR) analysis

Polymerase chain reaction of the *rpoB* gene using primers RPO5' (5'-TCAAGGAGAAGCGCTACGA-3') and RPO3' (5'-GGATGTTGATCAGGG TCTGC-3') was done to amplify a 360 bp fragment, 171 bp of variable region and 189 bp of conserved region on the mycobacterial genome. Each PCR reaction mixture contained sterilized distilled, 2.5 µl of 10x reaction buffer, 0.4

µl of $MgCl_2$, 0.8 µl of dNTPs, 0.2 µl of 1 unit *Taq* polymerase, 0.5 µl each of forward and reverse primers and DNA template to make up a 25 µl reaction volume. The PCR amplifications were performed in BIO RAD Peltier Thermal Cycler as follows: denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, extension at 72°C for 1 minute and ended with final extension at the 72°C for 7 min. The PCR products were resolved for 1 hour at 80 mV 70 mA on 1.5 % (w/v) SeaKem LE Agarose gel (Lonza, Rockland ME, USA) in 3 µl Orange G 1X TBE buffer (Tris base, Boric acid, EDTA: pH 8.3) using a horizontal gel electrophoresis apparatus (Minicell Primo EC135). The gels were stained with ethidium bromide and PCR fragments were visualised under UV light. The PCR fragments were analysed with a software, Alpha Ease FC software on a gel analysis system with an Olympus digital camera (Model AT126 D). Molecular weight of bands on gel were mapped with a 100 base pair DNA standard ladder (Metabion, GmbH, Germany).

Restriction fragment length polymorphism analysis

After successful amplification of the 360 bp PCR products was confirmed and then subjected to restriction enzyme digestion with *Msp*I (R0106S, New England Biolabs Inc., USA) and *Hae*III (R0108S, New England Biolabs Inc.) following standard procedure and the manufacturer's instructions with slight modifications. The reaction mixture was prepared by adding 12 µl of sterile distilled water, 2.5 µl of 10X NE buffer, 0.5 µl restriction enzyme and 10 µl *rpoB* amplicons. For the digestion, 15 µl of the reaction mixture was aliquoted into each labelled PCR tube. 10 µl of PCR amplicons were then added. Samples were incubated at 37°C for 2 hours. For *Msp*I digested samples, 5 µl

of (6X) loading buffer was added to stop the reaction. For the *Hae*III digested samples, it was inactivated at 80^{o}C for 20 mins and the 5 µl of (6X) loading buffer was added. Digested samples were run on a 4% agarose gel at 100 V for 1 hour. Gel was then visualised on a UV transilluminator. Resulting fragment bands were interpreted using 50 bp and 100 bp ladders (Metabion, GmbH, Germany). Using these markers, the sizes of the restricted fragments of each species were determined, and compared with an algorithm constructed by Lee *et al*. (2000) for species identification.

CHAPTER FOUR

RESULTS

In determining the occurrence of nontuberculous mycobacteria (NTM) in the main sources of drinking water in parts of the Central Region of Ghana, microbiology and molecular biology techniques were employed. Treated water from, and water sources employed by the Ghana Water Company Limited (GWCL) were the main media for the study. Results of all the stages from sampling to identification of the organisms are presented in this chapter.

Physical and Chemical Characteristics of Water Samples

In this study, the physical and chemical characteristics of water samples considered were appearance, odour, colour, temperature, pH and residual chlorine value for treated water from GWCL. Measurements for the chemical characteristics and observations made for the physical characteristics of samples were done concurrently with collection of water samples on the field. The values obtained represent the characteristics of samples at the time of collection from the various sources before transporting them to the laboratory for analysis. Results of the physical and chemical characteristics of samples are displayed in Tables 4 and 5 respectively. From the thermometer measurements of samples, the temperature range was 35 ^{o}C-24.8 ^{o}C with sample from Brimsu treatment plant (TP) recording the highest temperature of 35 ^{o}C and sample from Essakyir reservoir recording the lowest temperature of 24.8 ^{o}C. The pH range was 8.13-4.9 with sample from Brimsu TP recording the highest pH of 8.13 and sample from Essakyir TP recording the least pH of 4.9. The Digital Colorimeter used to measure the residual chlorine values of samples gave a range of values of 0.1 mg/L-2.1 mg/L. Samples from Brimsu community tap, Essakyir reservoir,

Baifikrom community tap and Essakyir community tap all recorded the least value for residual chlorine levels of 0.1 mg/L and sample from Essakyir TP recorded the highest value for residual chlorine level of 2.1 mg/L. The physical characteristics for treated water from the TP, reservoirs and community taps were the same. The samples from the rivers also had the same physical characteristics except the smell where some had and earthy smell while others had a fishy smell.

Table 4: Physical characteristics of water samples

Sample Source Category	Appearance	Odour	Colour
Samples obtained from Rivers	Turbid	Earthy Fishy	Pale yellow Pale brown
Samples obtained from GWCL Treatment plants	Clear	Odourless	Colourless
Samples obtained from GWCL Reservoirs	Clear	Odourless	Colourless
Samples obtained from Community Taps	Clear	Odourless	Colourless
Samples obtained from Boreholes and Wells	Clear	Odourless	Colourless

Table 5: Results of Measurements of pH, Temperature and Residual Chlorine of treated water samples taken at the time of sampling from GWCL sources

Sample	pH	Temperature (°C)	Residual chlorine (mg/L)
SH1	7.5	32.5	1.0
SH2	6.8	30.0	0.8
SH3	8.03	28.2	0.45
SH4	7.8	27.3	0.3
SH5	6.86	30.9	0.2
SH6	6.5	31.8	0.4
B1	8.13	28.4	0.72
B2	7.9	26.2	0.6
B3	7.9	29.8	0.41
B4	6.2	35.0	0.65
B5	7.1	33.3	0.88
B6	7.4	31.3	0.11
BF1	6.4	32.4	0.1
BF2	6.62	32.0	0.6
BF3	6.44	32.2	0.3
BF4	6.2	29.8	0.2
BF5	6.41	31.9	0.4
E1	4.9	28.0	2.1
E2	6.2	29.8	0.3
E3	6.4	24.8	0.1
E4	6.1	31.2	0.7
E5	6.1	30.4	0.1
E6	5.9	29.1	0.3
E7	7.1	28.3	0.4

Table 5, continued

Sample	pH	Temperature (^{o}C)	Residual chlorine (mg/L)
W1	6.7	28.3	0.78
W2	6.6	28.8	1.6
W3	6.4	29.2	0.65
W4	6.6	27.8	0.9
K1	6.8	28.4	0.4
K2	5.9	29.2	0.5
K3	6.4	29.0	1.0
K4	7.0	28.9	0.7
K5	6.4	26.9	1.3
K6	6.9	28.6	0.44
K7	6.5	27.9	0.38
K8	6.6	28.8	0.5

SH-Sekyere Hemang; B-Brimsu; BF-Baifikrom; E-Essakyir; W-Winneba; K- Kwanyako

Detection of Acid-fast bacteria from water sources

Detection of AFB from the 55 water samples involved culturing on Lowenstein-Jensen media and Ziehl-Neelsen microscopy. The results from these studies are shown below.

Culturing on Lowenstein-Jensen media for the detection of Acid-fast bacteria in water sources

Figure 4 shows the number of samples with and without growths at the two incubation temperatures and Table 6 shows the different sources and the number of samples with growths at the two incubation temperatures out of the 110 slants for each temperature. A total of 55 water samples were cultured on Lowenstein-Jensen slants in replicates at 30°C and 37°C. A total of 32 growths (or 58.2%) out of 110 replicates were observed on the LJ slants at 30°C whiles 27 (or 43.6%) out of 110 were observed at 37°C. Twenty-one samples yielded growth at both temperatures while 6 samples yielded growth at 37°C only and 11 samples had growths at 30°C only. Seventeen samples had no growth at all.

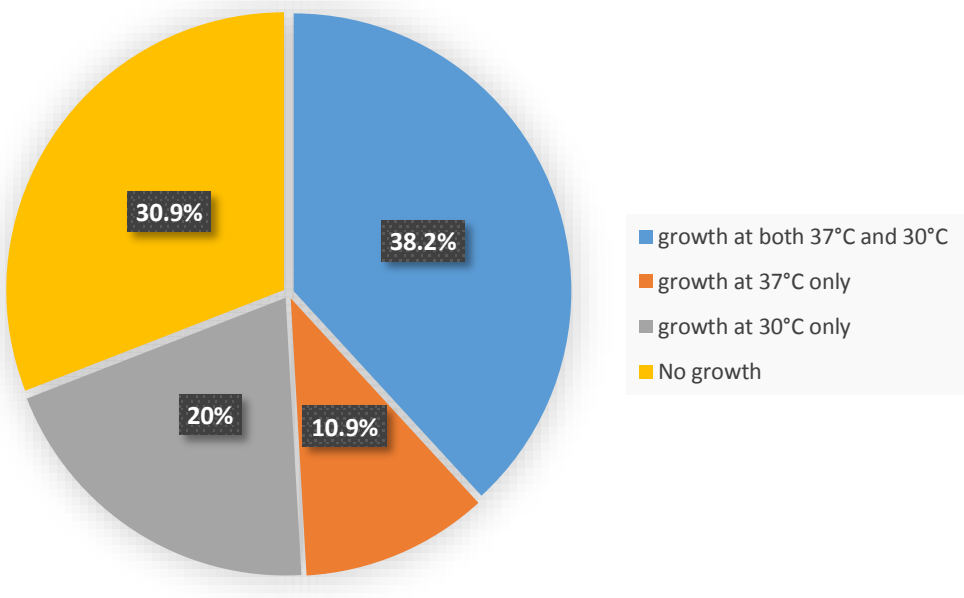

Figure 4: Results of samples with and without growth of AFB on Löwenstein-Jensen slants.

Table 6: Distribution of growths at the two incubation temperatures for the samples obtained from the various sources

Sample Source Category	37°C	30°C
Samples obtained from GWCL Treatment plants	7	7
Samples obtained from GWCL Reservoir	2	3
Samples obtained from River source	6	8
Samples obtained from Community tap	9	10
Samples obtained from Boreholes and wells	3	4

Morphological features observed

Colony morphology varied from smooth to rough with either a yellow or cream coloured colony. Plate 1 shows cultures and the morphology of colonies. Two samples had mixed cultures with morphologically different colonies.

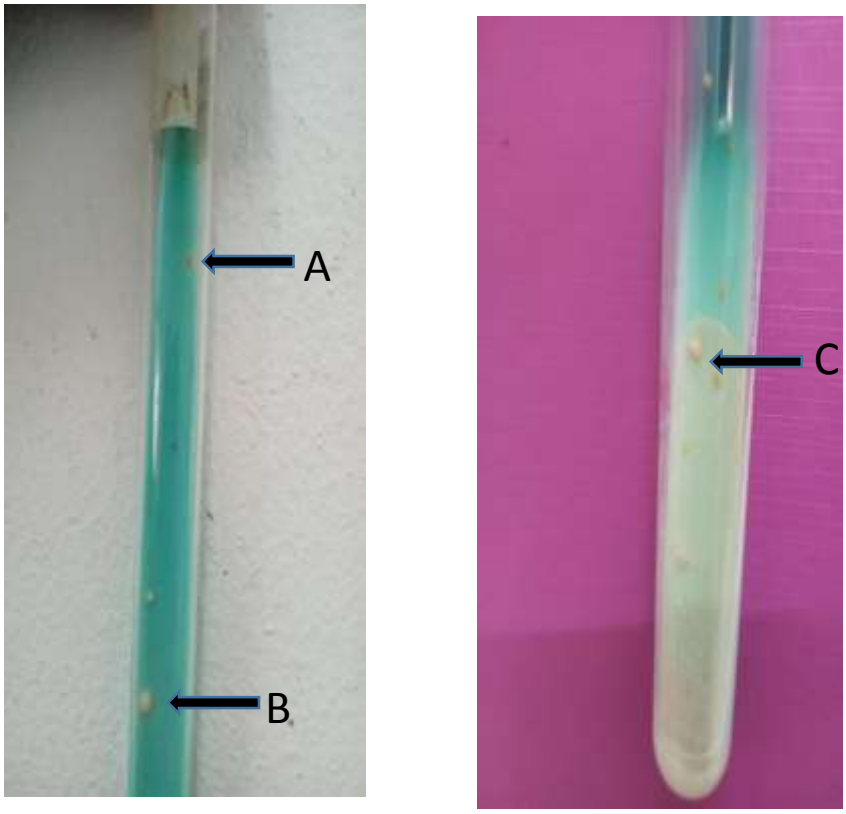

Plate 1: Colony morphology on Lowenstein-Jensen media: A- smooth round yellow colony; B- smooth round cream colony; C rough round cream colony.

Microscopic Examination for the detection of Acid Fast Bacteria (AFB)

Post culture microscopy using the Ziehl-Neelsen staining technique was done to confirm isolates which were acid-fast. Out of the 55 water samples cultured at the two temperatures, 20 isolates obtained from samples incubated at 37^{o}C were positive for the presence of AFB and 22 isolates obtained from samples incubated at 30^{o}C were also positive for the presence of AFB. Displayed in Table 7 are the numbers of acid-fast-positive isolates for each of the sources of water. Seven (7) isolates and 10 isolates incubated at 37^{o}C and 30^{o}C respectively were negative for Ziehl-Neelsen microscopy. Table 7 gives the details of the results from microscopy for the confirmation of the presence of AFB.

Table 7: Number of AFB positive isolates from the various water sources at the two incubation temperatures through microscopy

Sample Source Category	37°C	30°C
Samples obtained from River	3	4
Samples obtained from GWCL Treatment plants	5	4
Samples obtained from GWCL reservoir	2	2
Samples obtained from Community tap	7	8
Samples obtained from Boreholes and wells	3	4

Capilia TB-Neo assay for the categorization of Isolates into MTBC or NTM

Table 8 shows the number of isolates categorized as NTM for each of the water sources. Samples obtained from community tap had the highest number of isolates of 7 and 8 at incubation temperatures of 37°C and 30°C respectively. The lowest number of isolates were recorded for samples obtained from GWLC.

Table 9 shows the categorized isolates and their sources in percentages. Samples obtained from community taps had the highest percentage of 42.9% and reservoir sample category had the lowest with 7.1%. The names of the sample

sources categorized as NTM are shown in Appendix B. Plate 2 shows results for 3 samples; UWM 2, 18 and 36 which were categorized as NTM, a reaction buffer control and a negative control. At 37ºC, all the 20 isolates which were confirmed as AFB after microscopy were identified as NTM using Capilia TB-Neo assay and also at 30ºC, all the 22 AFB confirmed by microscopy were identified as NTM.

Table 8: Distribution of water sources and isolates realised at the two incubation temperatures characterised as NTM by the Capilia TB-Neo Assay

Samples Source Category	37ºC	30ºC
Sample obtained from River	3	4
Sample obtained from GWCL Treatment plants	5	4
Sample obtained from GWCL reservoir	2	2
Sample obtained from Community tap	7	8
Sample obtained from Boreholes and Wells	3	4

Plate 2: Capilia TB-Neo kit showing results for reaction buffer control, negative control (TGH 001) and samples (UWM 2, 18 and 36**)**

Twenty isolates at 37^{o}C and 22 isolates at 3^{o}C out of the 110 cultures for each of the two incubation temperatures were categorized as NTM and the isolates categorized as NTM were used for the molecular aspects of the study.

Table 9: Samples categorized as NTM by the Capilia TB-Neo assay.

Sample Source Category	Sample points (%)
River source	14.3
Treatment plant	17.9
Reservoir	7.1
Community tap	42.9
Ground water source	17.8

Polymerase Chain Reaction (PCR) based on the amplification of the *rpoB* gene

Deoxyribonucleic acid (DNA) extraction from all isolates which were categorized as NTM was followed by PCR-amplification of a 360 bp fragment, 171bp of variable region and 189 bp of conserved region of the *rpoB* gene. Out of the 20 and 22 isolates which were categorized as NTM at 37 ^{o}C and 30 ^{o}C respectively, 10 (50%) and 11 (50%) isolates at 37 ^{o}C and 30 ^{o}C respectively were successful with the amplification of the *rpoB* gene. Table 10 shows the number of isolates from each water source in which the 360 bp fragment was amplified and the culture isolates which amplified the *rpoB* gene. Samples from community tap had the highest percentage of isolates with 40% and 36.3% of isolates at 37 ^{o}C and 30 ^{o}C respectively. Samples from GWL treatment plant had the lowest percentage of isolates with 10% and 9.1% of isolates at 37 ^{o}C and 30 ^{o}C respectively. The samples in the categories which amplified the *rpoB* gene are shown in Table 11. Figure 6 shows the resulting bands after PCR was run for eight samples using a 100bp marker.

Table 10: Number of positive isolates for the amplification of the rpoB gene

Sample Source Category	37°C (%)	30°C (%)
Sample obtained from River	20	18.2
Sample obtained from GWCL Treatment plants	10	9.1
Sample obtained from GWCL reservoir	20	18.2
Sample obtained from Community tap	40	36.3
Sample obtained from Boreholes and Wells	10	18.2

Table 11: Isolates which amplified the 360bp region of the *rpoB* gene and their sources

Sample source category	Sample	Sampling point
River source	Ochi Nakwa	Essakyir
	Ochi Amissah	Baifikrom
	Kakum	Brimsu new
Treatment plant	Winneba	Winneba
	Kwanyako old	Kwanyako old
	Baifikrom	Baifikrom
Reservoir	Mawukpor	Sekyere Hemang
Community tap	Gomoa Adam	Essakyir
	Kwanyako	Kwanyako
	Asanaadze	Brimsu old
	Kojo Badu	Winneba
	Atabaadze	Sekyere Hemang
	Baifikrom	Baifikrom
Groundwater source (well, (W)/borehole,(B)	Asanaadze (B)	Brimsu
	Saltpond (W)	Baifikrom
	Abrodziwuram (W)	Sekyere Hemang

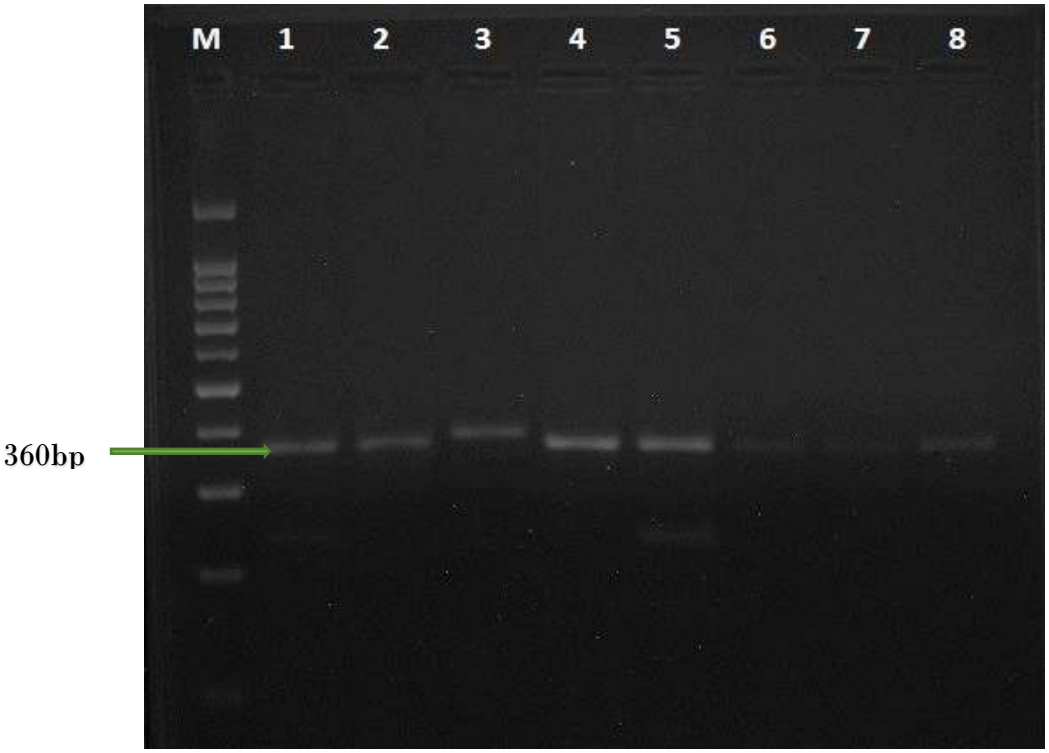

Figure 5: A Representative 2% agarose gel electrophoregram of PCR products of the rpoB gene of isolates. The presence of the 360 bp DNA band indicates the presence of NTM. Lane M represents the 100 bp marker and Lanes 1-8 represent the sample isolates

Restriction fragment length polymorphism for the identification of NTM species.

Amplified products on the *rpoB* gene were separately subjected to digestion with the restriction endonucleases *Msp*I and *Hae*III. Figure 6 and 7 show the resulting bands after gel was run for seven samples using 50bp and100bp markers. From figures 6 and 7, the fragment sizes can be estimated. Table 12 shows the sample isolates with their corresponding fragment sizes for *Hae*III and *Msp*I digestions.

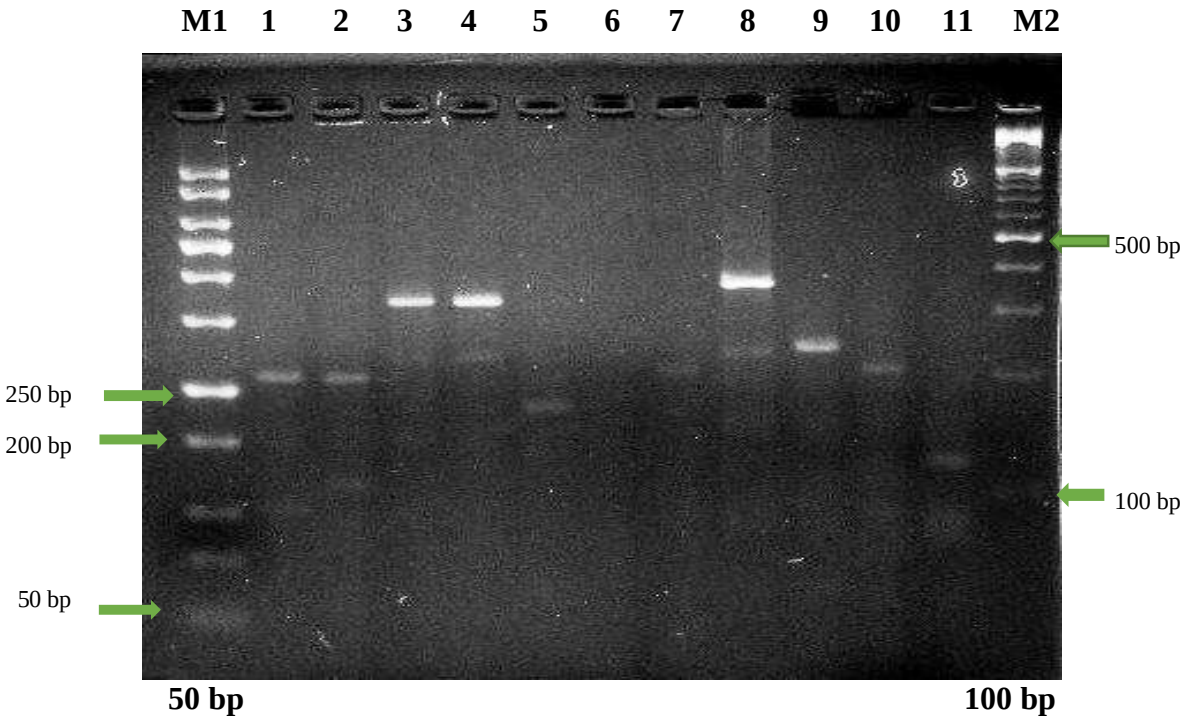

Figure 6: Results of PRA for the digestion of PCR products with HaeIII restriction enzyme run on 4% agarose gel stained with Ethidium bromide. Lanes: M1 and M2, DNA size markers (50 bp and 100 bp ladders respectively); 1, 2, 3, 4, 5, 7, 8, sample isolates; 6, negative control; 9, 10 and 11 positive controls.

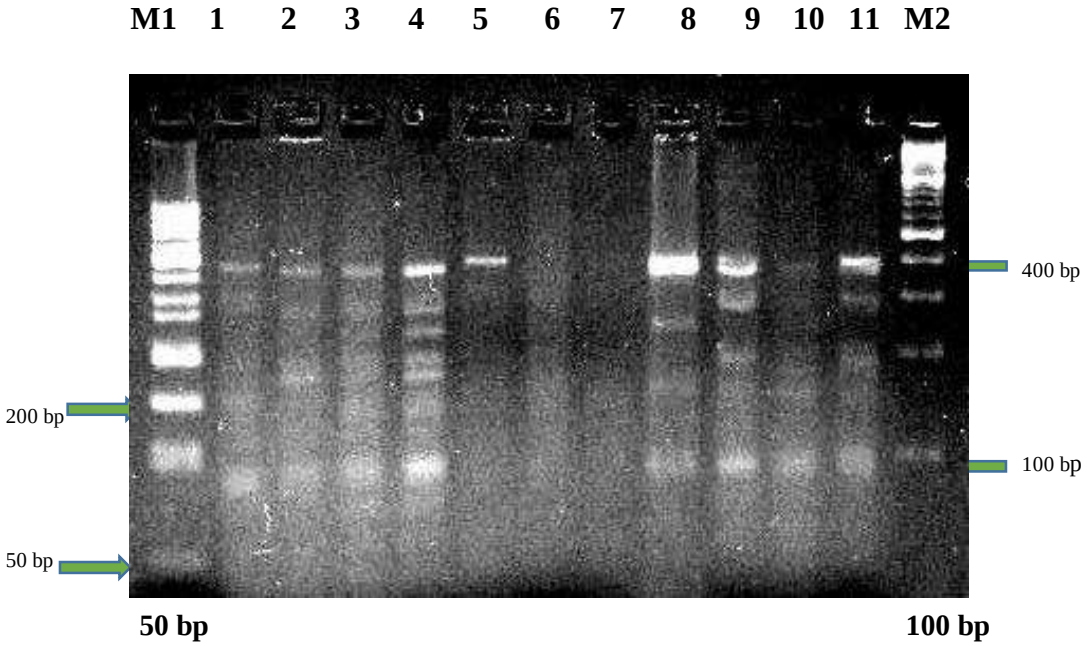

Figure 7: Results of PRA for the digestion of PCR products with MspI restriction enzyme run on 4% agarose gel stained with Ethidium bromide. Lanes: M1 and M2, DNA size markers (50 bp and 100 bp ladders respectively); 1, 2, 3, 4, 5, 7, 8, sample isolates; 6, negative control; 9, 10 and 11 positive controls.

Table 12: Fragments sizes of sample isolates from the digestion with *Hae*III and *Msp*I restriction enzymes.

Sample	Fragments sizes in base pairs (bp)		Possible NTM present
	*Hae*III	*Msp*I	
1	95/150/210	90/155/270/300/410	*M. celatum* *M. gordonae* type II *M. genavense*
2	110/150/210	90/175/295/375	*M. simae* *M. genavense*
3	150/230/330	70/90/140/175/210/225/260/375	*M. gordonae* type II
4	150/230/330	70/90/140/175/210/225/260/375	*M. gordonae* type II
5	55/175	365	*M. kansasii* V strain
7	210	140	*M. kansassi* type I *M. kansassi* II *M. celatum* type I *M. celatum* type 1
8	230/330	90/175/225/365	*M. ulcerans* *M. phlei* *M. gordonae* type II

CHAPTER FIVE

DISCUSSION

In this chapter, the results obtained from sampling through to identification of the organisms recovered are discussed. Comparisons between the results of this study and relevant previous researches are also made to ascertain the conformity of this work to previous knowledge or otherwise.

Results for culture and microscopy showed that some samples contained AFB which were subsequently categorized as NTM by the Capilia TB-Neo assay. Polymerase chain reaction of the *rpoB* gene and digestion of PCR products with *Msp*I and *Hae*III finally confirmed the occurrence of NTM in the water samples by identifying the recovered NTM at the species level. Summary and conclusion of the work are also found in this chapter with recommendations based on the results of the research.

Water samples and their sources

A total of 55 water samples were used for this study. Twelve (21.8%) of the 55 samples were from the rivers which supply the GWCL treatment plants (TP) with untreated water. Six out of the 9 TPs in the region were included in this study and thus the 6 rivers which supply them with untreated water were also included. These rivers are: Kakum which supplies the Brimsu TP with raw water, Pra supplies Sekyere Hemang TP, Ochi Nakwa supplies Essakyir TP, Ochi Amissah supplies Baifikrom TP and Ayensu supplies both Winneba and Kwanyako TPs. Samples from these rivers were taken at the point where the rivers flow into the coagulation or flocculation tanks in the treatment plants. Sixteen (29.1%) of the total number of samples used for the study were taken from GWCL treatment plants. These samples were taken at the point where

water is pumped from the treatment plant to communities and reservoirs after treatment. Brimsu and Kwanyako TPs have the old and new processing plants so for each of these two TPs, samples were taken from both old and new processing plants. The GWCL reservoirs which were included in the study were Mawukpor reservoir which stores treated water from Sekyere Hemang TP and Mbroboto reservoir which stores treated water from Essakyir TP. These two reservoirs gave 4 (7.3%) samples of the total number of samples for the study. Sixteen water samples (29.1%) were taken from community taps in towns which are supplied with treated water by the GWCL. The 16 samples were taken from taps in the following towns: Asanaadze and Brimsu towns which are supplied with treated water by Brimsu TP, Ataabadze and Kissi which are supplied with treated water by Sekyere Hemang; Baifikrom, Saltpond and Mankessim get their water from Baifikrom TP; Gomoa Adam, Antseadze and Kotukwa get their water from Essakyir TP; Kojo Badu, Victoria Road-Winneba and Buduburam get their water from Winneba TP and Salem, Potsin and Kwanyako get their treated water from Kwanyako TP.

A total of 7 (12.7%) samples were taken from groundwater; well and borehole. Water from these sources are not treated but people in these communities with these sources of water drink the untreated water. Five out of the seven samples in this source category were taken from wells and the other two from boreholes. People in the towns where the well water samples were taken have access to treated water but still drink water from these wells because of two major reasons. The main reason they gave was that water from the wells looked cleaner and clearer than the treated water GWCL was supplying them and others also said the location of the community tap which supplies them with

treated water from GWCL was very far from their homes. Communities which drink water from boreholes also gave the reason that borehole water was clearer than treated water from GWCL and it also tasted better. Borehole water samples were taken from the following towns: Asanaadze which is supplied with treated water by Brimsu TP and Eduafo supplied with treated water by Kwanyako TP. Well water samples were taken from the following towns: Abrodziwuram and Kissi (supplied with treated water by Sekyere Hemang TP), Baifikrom and Saltpond (Supplied with water by Baifikrom TP) and Victoria Road in Winneba.

Water quality parameters

The provision of drinking water that is not only safe but also acceptable in appearance, taste and odour is of high priority. Water that is aesthetically unacceptable will undermine the confidence of consumers which sometimes may lead to the use of unwholesome sources of water. The World Health Organization (WHO) has provided a standard for drinking water quality in their 'Guidelines for Drinking-water Quality' publication which is currently in its fourth edition (WHO, 2011). This publication provides guidelines for a vast array of chemical, microbial, and radiological contaminants commonly found in drinking water. It also provides a framework for achieving safe drinking water by implementing health-based targets, creating a water safety plan, and maintaining water surveillance. According to the WHO drinking water guidelines, drinking water must not contain chemicals, inorganic substances or organisms that may be harmful to human health. Drinking water should also be at reasonable temperature and be free of unappealing odours, taste and colour. The guideline defines drinking water as water which is safe to drink over a life time that is, it constitutes no significant risk to health. The water quality

parameters considered in this study were appearance, odour, colour, temperature, pH and residual chlorine value. Results for these characteristics are discussed below.

Appearance of water samples (transparency)

The appearance of water, whether clear or turbid is a characteristic consumers look out for to judge the safety of the water. Turbidity in water is caused by suspended particles or colloidal matter that obstructs light transmission through the water. It may also be caused by inorganic or organic matter or a combination of the two. Microorganisms (bacteria, viruses and protozoa) are typically attached to particulates, and removal of turbidity by filtration significantly reduces microbial contamination in treated water (WHO, 2011). Treated water samples from GWCL, well and borehole water samples were clear with no visible suspended particles but raw water samples from rivers were turbid with muddy suspensions. The difference in transparency of treated water from GWCL and raw water from rivers which are treated for drinking was very clear. From muddy turbid raw water, GWCL treatment methods were able to produce clear transparent potable water. Borehole samples were also very clear and transparent and this is the main reason why the communities with these boreholes drink the water.

Turbidity can seriously interfere with the efficiency of disinfection by providing protection for organisms, and much of water treatment is directed at removal of particulate matter before disinfection. Removal of particulate matter by coagulation, sedimentation and by filtration is an important barrier in achieving safe drinking water. It is clear that the procedure followed by GWCL in removing particulate matter is effective. Notwithstanding, these

procedures do not deal with the microbial contents of the water so it is possible to have microbial contamination in very clear treated water. Achieving low turbidity by filtration (before disinfection) of water from surface sources and ground waters is strongly recommended by WHO to ensure microbial safe water.

Odour of water samples

Odour can originate from natural inorganic and organic chemical contaminants and biological sources or processes (e.g. aquatic microorganisms), from contamination by synthetic chemicals, from corrosion or as a result of problems with water treatment (e.g. chlorination) (WHO, 2011). They may also develop during storage and distribution as a result of microbial activity. Potable water should be odourless. All treated water samples taken from GWCL at the various sampling points were odourless. Well and borehole water samples were also odourless. Samples from rivers (raw water) on the other hand had fishy and earthy odour which if were used for drinking would have been unacceptable. The difference in odour of treated and raw water/river water samples showed that the GWCL treatment process was able to eliminate this bad odour and make treated water odourless which is acceptable by consumers. Odour in drinking water may be indicative of some form of pollution or of a malfunction during water treatment or distribution and because these samples were odourless, consumers may have assumed that water from these sources were free from all contaminations but the study has shown that even with this odourless state, some of the water sources contained microorganisms which can be harmful.

Colour of water samples

According to the World Health Organizations' Guidelines for drinking water quality (WHO, 2011), drinking water should ideally have no visible colour, however organic materials, metals such as iron, and corrosion of pipes may cause slight discoloration of water. Treated water samples from GWCL, well and borehole samples were colourless. Samples from rivers were either pale brown or yellow and this colour may be due to the presence of coloured organic matter associated with the humus fraction of soil (WHO, 2011). Colour is also strongly influenced by the presence of iron and other metals, either as natural impurities or as corrosion products. It may also result from the contamination of the water source with industrial effluents. Again, the change in colour of river or raw water from pale yellow and brown to a colourless state is an indication that the treatment methods of GWCL were able to take away the substances which were giving the raw water the yellow and brown colour, leaving the water colourless.

Temperature of water samples

The WHO does not have any specific guidelines for water temperature but it is noted that higher water temperatures are less pleasing to consumers and warm water encourages microorganism growth and this may increase problems related to taste, odour, colour and corrosion. The rate of chemical reactions in water also generally increases at higher temperatures. Water, particularly groundwater, with higher temperatures can dissolve more minerals from the rocks. According to the Dutch drinking water act (Moerman et al., 2014), the drinking water temperature should remain lower than 25 °C at the customers' tap. However, the drinking water temperature is difficult to control as it is a

function of multiple factors, including: water temperature in the distribution network, climatic conditions and room temperature. The highest temperature recorded for the water samples was 33.3°C from Brimsu treatment plant and the lowest was 24.8 °C from Essakyir community tap. Two point eight percent of GWCL samples recorded temperatures below 25°C, 68.6% of samples recorded temperatures in the range of 25 °C to 30 °C and 28.6% recorded temperatures above 30 °C. In our parts of the world, the temperatures are usually high so it is expected that the water samples would have high temperatures. The high temperatures of the water may give microorganisms a conducive environment for proliferation and because of this, there should be measures put in place to ensure proper disinfection of water to bring to a minimal the growth of these microorganisms.

pH of water samples

The pH of drinking water according to WHO and Ghana Standards Authority (GSA) should be between 6.5 and 8.5 (FDA-Ghana, 2018). Although pH usually has no direct impact on consumers, it is one of the most important operational water quality parameter. Careful attention to pH control is necessary at all stages of water treatment to ensure satisfactory water clarification and disinfection. For effective disinfection with chlorine, the pH should preferably be less than 8; however, lower pH water is more likely to be corrosive (WHO, 2011). The pH of the water entering the distribution system must be controlled to minimize the corrosion of water mains and pipes in household water systems and also ensure the water is as neutral in pH as possible**.** According to WHO, the optimum pH required will vary in different supplies according to the composition of the water and the nature of the construction materials used in the

distribution system, but it is usually in the range 6.5–8.5. Extreme values of pH can result from accidental spills, treatment breakdowns and insufficiently cured cement mortar pipe linings or cement mortar linings applied when the alkalinity of the water is low. The highest pH recorded was 8.13 for water sample from Brimsu old treatment plant and the lowest was 5.9 for water sample from Mbroboto reservoir. The highest is within the range and thus it is normal but the lowest is acidic and below the standard range. Acidic water with a pH of less than 6.5 is more likely to be contaminated with pollutants, making it unsafe to drink and it can also corrode (dissolve) metal pipes (Cirino, 2018). It is possible that water from the Mbroboto reservoir is contaminated because it has been stored for some time. All water samples taken from GWCL were within the WHO recommended range for pH except the sample from Mbroboto reservoir which was acidic and below the standard range by WHO.

Residual chlorine value of water samples

Chlorine is added to drinking water during the treatment process as a disinfectant to kill pathogens. Chlorination can be achieved by using liquefied chlorine gas, sodium hypochlorite solution or calcium hypochlorite granules and on-site chlorine generators. Ghana Water Company Limited treatment plants use sodium hypochlorite and liquefied chlorine gas for disinfection. The WHO suggests a health-based target of less than 5 mg/L. Chlorine does have an effect on the taste of the water and can be detected by consumers at concentrations as low as 0.3 mg/L. In order to ensure drinking water remains free of pathogens and is not re-contaminated during the distribution process, it is important to have a residual level of free chlorine at the point of use. A minimum level of 0.2 mg/L of free residual chlorine is suggested. The WHO

also remarks that "for effective distribution, there should be a residual chlorine concentration of ≥0.5 mg/L after at least 30 minutes contact time at pH <8.0." At the point of delivery, the minimum residual concentration of free chlorine should be 0.2 mg/L. The highest residual chlorine level recorded for the water samples from GWCL was 2.1 mg/L for Essakyir treatment plant and a lowest value of 0.1 mg/L for Baifikrom treatment plant and Essakyir community tap (Gomoa Adam). The 0.1 mg/L value for Baifikrom treatment plant is not the best because as the water travels in the distribution process, the residual chlorine depreciates so if the value is low from the treatment plant, by the time the water reaches the community tap or reservoir, there will be no chlorine left in the water to prevent the growth of microorganisms. The 0.1 mg/L value for the Gomoa Adam community tap is below the minimum standard value and this may be due to depletion of the chlorine as a result of the water travelling a long distance or the chlorine bubbling out of distribution or supply outlets along the way. Most of the values recorded in the current study were in the range recommended by WHO.

As water leaves the treatment plant and travels through the distribution system, the residual free chlorine concentration does deteriorate. This may be due to water age or due to contaminants entering the pipes. Overall, 17.1% of all samples had a free residual chlorine concentration ≤ 0.2 mg/L and 32.7% had values within 0.3-0.5 mg/L range which is the WHO guideline to ensure pathogens are killed. In addition, 45.7% of samples had values within the range of 0.6-2.1 mg/L which are above the WHO recommended range but not above the 5 mg/L health-based limit. When free chlorine is not available for

disinfection, pathogens that may enter water from contact with unwashed hands or unsafe storage containers are then able to infect humans and cause illness.

Elimination of background flora with 4% NaOH solution in the decontamination process

There is no selective medium for the culturing of mycobacteria, so samples which are to be cultured for mycobacteria have to be decontaminated first before culturing so that the associated flora consisting of bacteria and fungi, can be eliminated. Mycobacteria are more resistant to these decontamination treatments than bacteria and fungi, but they are not fully resistant. Decontamination procedure using 4% NaOH has been used to remove or reduce the growth of other microorganisms present in the water samples. Although 4% NaOH is widely used to decontaminate water samples, some authors have shown that quite a good percentage of NTM survive pre-treatment with 4% NaOH (Edirisinghe *et al.*, 2014). Many studies have evaluated many decontamination procedures for the isolation of mycobacteria but none has been universally accepted. For instance, in a study by Kamala *et al.*, 1994, decontamination procedures for isolation of non-tuberculous mycobacteria from soil and water were evaluated, six decontamination methods were evaluated and it was found that treatment with 3% Sodium dodecyl Sulfate (SDS) in combination with 1% sodium hydroxide (NaOH) was the most effective decontamination method for soil as well as water samples. When this procedure was used by Parashar et al., (2004) in a study reported that the decontamination method could not remove any contaminants. There is evidence that even the mildest decontamination methods such as the widely used N-acetyl-cysteine/NaOH method can kill about 33% of the mycobacteria

(Somoskovi *et al*., 2002).Therefore, it can be assumed that, the water samples which had low mycobacterial concentrations than the limit of sensitivity of the isolation method, probably gave a negative result on culturing. The environmental sample being used and even the part of the world being studied are to be considered before selecting an appropriate decontamination procedure.

Isolation and detection of Acid-Fast Bacilli (AFB) from water sources

Culturing of samples on Löwenstein-Jensen (LJ) medium and Ziehl-Neelsen microscopy were the methods used in isolating and detecting AFB in water samples. Water samples after decontamination with 4% NaOH solution were cultured on LJ medium and the percentage recovery was 69.1% (38 samples out of 55 samples). Seventeen samples (30.9%) did not show growth and of the seventeen, 7 samples were from treatment plants, 4 from community taps, 2 from river sources, 2 from ground waters; 1 well and 1 borehole water samples. Twenty-one (38.2%) samples had growth at both incubation temperatures and 6 (10.9%) samples had growth at only 37 ^{o}C while 11 (20%) samples had growths at 30^{o}C. Samples with growth of AFB on LJ slants are discussed below based on treatments plants.

Brimsu Treatment Plant

All the samples taken from Brimsu TP (New processing plant) did not show growth when cultured. This can be an indication that the treatment method used for this processing plant is very effective. The first sample from Brimsu TP (Old processing plant) did not grow but the second sample taken from the same place after two weeks grew on LJ medium. Samples taken from Kakum River, the raw water which feeds this TP had growth when samples were cultured. The first sample from the Kakum River had growth at only 30^{o}C but

the second sample had growth at both temperatures. It can be said that the Kakum River is contaminated with AFB. The old processing plant did not grow during the first sampling but the water from the community tap which receives water from this plant had growth at both temperatures. This can be as a result of contamination along the line from the plant to the community tap or the concentration of NTM in the sample from the old processing plant was low so it could not be recovered but conditions in the distribution system favoured the proliferation of NTM so their numbers increased at the point of use. On the other hand, the second sample from the old processing plant had growths at 37°C but the community tap had no growth and this can also mean that the conditions from the plant to the tap did not favour the proliferation of the mycobacteria or the concentration of mycobacteria in the water source was low so they could not stand the decontamination procedure. These results show that water quality can change along the path from treatment to the point of use The borehole sample from Asanaadze which is drank by the people in the community also had growth of AFB at 37 °C.

Sekyere Hemang Treatment Plant

Water samples from the Pra River had growth at 37 °C for the first sampling but no growth for the second sampling. The first batch of samples for this TP all had growths: raw water, TP water, community tap water and reservoir water. The mycobacteria might have escaped the treatment processes and travelled through the distribution system to the point of use. For the second batch of samples, the river source had no growth but the TP sample and the reservoir sample all had growths. There could have been contamination after the treatment process. Water samples from Abrodziwuram and Kissi wells all

had growths. Conditions in the earth where underground water is found do not favour the growth of microorganisms so the contamination of these source of water may have occurred when the water was exposed to the environment.

Essakyir Treatment Plant

Ochi Nakwa River samples had growth at both temperatures. The sample from Ochi Nakwa River cultured at 30^{o}C gave a mixed culture with two different colonies: yellow and cream colonies. For the first batch of samples, samples from the TP had no growth even though AFB was seen in the river source. The community taps all had growths. First sample from Mbroboto reservoir had growth but the second sample did not.

Baifikrom Treatment Plant

All samples from Baifikrom had growths when cultured. The sample from a well in Saltpond had growths at both temperatures and at 30 ^{o}C it gave a mixed culture with three different colonies: yellow, cream and white colonies. The residual chlorine value for water from the treatment plant was 0.1mg/L which is below the recommended value. This may point to the fact that the disinfection process in this treatment plant was not effective allowing for the growth of AFB.

Winneba Treatment Plant

Sample from the well in Winneba had no growth. Samples from the Ayensu River had growth for the first sampling but no growth for the second set of samples. Samples from the TP correlated with samples from the river source in terms of growth on LJ medium. The second batch of samples had no growth for the river source and TP but the community Tap sample had growth.

Kwanyako Treatment Plant

Samples from Ayensu River had growth for first sampling but no growth for the second sampling and this correlate with the results for Winneba TP.

Thirty one samples out of the 38 samples with growth of AFB on LJ media were microscopy positive for AFB which means seven samples which had growth on LJ medium were negative for ZN microscopy. Samples from Ayensu, Ochi Nakwa, Sekyere Hemang TPs, Pra, Brimsu old TP, Mawukpor reservoir and Essakyir TP gave negative culture results for microscopy even though they had growth at culturing.

Mycobacterial culture is more sensitive and highly specific than smear examination, and is even considered the gold standard for diagnosis of TB (Agrawal *et al*., 2016). A negative AFB smear may mean that no mycobacteria is present or that the mycobacteria were not present in sufficient numbers to be seen under the microscope. False negatives are a problem in microscopy and a smear negative sample may still grow mycobacteria since the culture media allows low numbers of bacteria that cannot be seen in a microscopic examination to multiply and be detected. Positive AFB smears indicate the presence of mycobacteria however, a culture must be performed to confirm and identify the species of mycobacteria present.

Categorization of culture isolates as MTBC or NTM

Samples which were microscopy positive were all categorized as NTM by the Capilia TB-Neo assay. This is a confirmation that the microscopy results were accurate and that mycobacteria were truly present in these samples. Thirty-one samples out of the total 55 samples which contained AFB were further categorized as NTM by the Capilia TB-Neo assay. The sample from Pra River

was positive for AFB culture on LJ medium but the isolates were not categorized as NTM. This indicates the presence of other mycobacteria other than NTM.

Amplification of the *rpoB* gene in NTM

The *rpoB* gene, encoding the beta-subunit of RNA polymerase, has emerged as a core gene candidate for phylogenetic analyses and identification of mycobacteria, especially when studying closely related isolates. DNA sequence analysis of the *rpoB* gene has been suggested in previous studies as a more suitable tool for identification of NTM than others like the 16S rRNA gene analysis (Ben Salah *et al*., 2008), and promising results have been obtained both among rapidly growing mycobacteria (RGM) (Adekambi *et al*., 2008) and within the *M. avium* complex. Seventeen samples out of the 31 samples categorized as NTM amplified the 360 bp region of the *rpoB* gene. In all, 30.9% (17 samples) of the total 55 samples used for the study had the gene of interest. That is 17 out of the 31 isolates categorized as NTM contained the gene of interest.

Identification of species recovered

Samples 1, 2, 3, 4 and 8 (as represented in Figures 6 and 7) are from borehole water sources and 5 and 7 are from river soures. Comparison with the algorithm proposed by Lee *et. al*. (2000) revealed that samples 1, 2 and 7 after *Hae* III digestion yielded fragment bands at 210 bp which indicates that they belonged to the following *mycobacteria: M. kansassi type* I, *M. kansassi type* II, *M. celatum* I, *M. celatum* II, *M. gordonae type* I, *M. ulcerans, M. phlei, and M. scrofulaceum.* Additionally, for sample 1 there was a band at 95 bp clearly indicating that it was either *M. celatum* (*M. celatum I* or *M. celatum II*) or *M.*

gordonae type II. For sample 2, there was an additional band at 110 bp, tentatively indicating it to be *M. simae*. On the other hand for *Hae* III digestion, both samples 1 and 2 had additional bands at 150 bp, which generally is indicative of *M. genavense.* These observations clearly indicated that these strains could be mixed. For *Hae* III digestion, Samples 3, 4 and 8 generated bands at 330 bp indicating, on face value, *M. gordonae* II mixed with a novel strain. Sample 5, after *Hae* III digestion gave bands at 55 bp and 175 bp, clearly placing it as *M. kansasii* V strain.

Samples 1 and 2 are isolates from Saltpond borehole but they yielded different morphological features on culture. The colonies of these isolates were different in colour. The colony of sample 1 was yellow while 2 was cream in colour. Despite the difference in colour, the digestion results with *Hae*III indicated that both were the same NTM: *M. genavense.* Sample 3 was also from the same source but colony was white in colour. Sample 3 was identified as *M. gordonae* II. This shows that this water source was contaminated with two different NTM species. Samples 4 and 8 are isolates from Abrodziwuram borehole and they were also identified as *M. gordonae* II. Both isolates gave cream colonies on culturing. Samples 5 and 7 were isolated from the culture of the water sample from Ochi Nakwa River. The colony of Sample 5 was cream while that of 7 was yellow. Sample 5 was identified as belonging to M. kansasii V strain while Sample 7 was identified as belonging to *M. kansassi type I, M. kansassi type II, M. celatum I, M. celatum II, M. gordonae type I, M. ulcerans, M. phlei, or M. scrofulaceum*.

The most common clinical manifestation of NTM disease is lung disease and lymphatic. Skin/soft tissue and disseminated disease also occur in patients

with pre-disposing conditions. (Griffith *et al*., 2007). *Mycobacterium simiae* can cause lung infection, osteomyelitis, peritonitis, and pyelonephritis and disseminated infection in immunocompromised patients. *M. scrofulaceum, M. gordonae, M. genavense, M. celatum, M. kansasii and M. simiae* have been described as causing pulmonary or disseminated disease in AIDS patients. *M. scrofulaceum* usually causes cervical lymphadenitis in children but also rarely causes extra nodal infection. The presence of these mycobacteria is a public health concern because these organisms when found in large numbers in the environment can cause serious outbreaks of diseases.

Limitations observed in RFLP analysis based on Lee et al. (2000) algorithm

Although Lee et al. included known NTM and Asiatic strains to generate diagnostic fragment patterns (Lee *et. al*., 2000), the strains in this work were all obtained from the environment in Ghana. Fragments below 50 bp could not be conclusively determined and evaluated because owing to logistical, time and budgetary constraints, an appropriate molecular marker incorporating fragments below 50 bp could not be procured and included in the work. Pronounced fragment bands which could not successfully lead to an unambiguous classification could indicate new strains. However, BLAST sequencing could be employed to unequivocally determine if these strains could be novel. Apparent discordant results obtained after digestion with these endonucleases may indicate mixed growths particularly in light of the observation of uniform morphological features on LJ medium for most of the samples.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

This chapter contains the summary of the whole research and conclusions based on the findings made from the research. It also contains series of recommendations which seek to improve future researches and add knowledge to the existing one.

Summary

This study sought to find the occurrence of nontuberculous mycobacteria in some selected water sources in the Central Region of Ghana. Fifty-five water samples were examined by acid-fast staining, LJ medium culture, and PCR-RFLP for the occurrence of NTM. The water quality parameters checked for the water samples included appearance, odour, temperature, pH and residual chlorine value. Most of the samples from GWCL met the WHO standards for these parameters. Notwithstanding, NTM were identified in some of the water sources. This study confirmed the presence of Acid fast mycobacteria in the selected water sources by culture and microscopy and Capilia TB-Neo assay categorized sample isolates as nontuberculous mycobacteria. PCR-RFLP led to the identification of the species contaminating these water sources. Sixty-nine point one percent of total number of samples had growth on LJ slants and 56.4% were identified by microscopy as AFBs. Isolates identified as AFBs by microscopy were all categorized as NTM. 30.9% of samples amplified the 360bp region of the *rpoB* gene. Restriction digestion with *Msp*I and *Hae*III resulted in restriction fragments which were compared to Lee et al (2000) algorithm for species identification. The study identified NTMs including *M. gordonae* II, *M. kansassi type* I, *M. kansassi* type II, *M. celatum* I,

M. celatum II, *M. gordonae* type I, *M. ulcerans, M. simae, M. phlei, M. scrofulaceum* and *M. genavense* .

Conclusion

Nontuberculous mycobacteria were isolated from 16 water sources in the Central Region of Ghana and this is an indication that these mycobacteria can thrive in water. Culture confirmed the presence of AFB and Capilia TB-Neo assay confirmed these AFBs as NTMs. Only few of the NTMs were identified to the species level. A number of isolates could not be identified by the PCR-RFLP of the *rpoB* gene and this calls for the construction of a more diverse database and the use of other molecular techniques which will increase the sensitivity and specificity of investigation. Most of the NTMs isolated were from borehole water sources even though the physical characteristics of this source of water was good and acceptable by consumers. Findings from this study show that the water sources in the Central Region harbour NTM which can result in a variety of diseases especially in immunocompromised individuals.

Recommendations

Based on the findings from this study, the following recommendations are being made for the relevant stakeholders and policy makers for consideration and implementation.

1. Maintenance of the distribution system of GWCL should be improved to prevent contamination during distribution.
2. Most of the reservoirs of GWCL looked abandoned and unattended to and this may be the points of contamination along the distribution chain. Reservoir should be emptied and cleaned frequently.

3. Measurement of other water quality parameters should be included in the routine measurements done by GWCL. There are more parameters outlined by WHO for water quality and only few are included in the routine measurement done by GWCL. Aside from microbial contamination detected by coliform presence in samples, water can also be contaminated by other microorganisms, metals or other chemicals. Samples from various locations in the distribution system could be taken and analyzed for these other contaminants and compared to health guidelines provided by the WHO.

Suggestions for Further Research

1. Sample size should be expanded to include more areas in the distribution system.
2. A need for sub-culture and pre- and post-culture when screening water samples during water treatment.
3. Further research should employ DNA-free RNase in DNA isolation steps to improve PCR amplification and hence fragment band signal strength during electrophoresis.
4. Molecular markers for fragments below 50 bp should be included.
5. BLAST Sequencing should be included in further work to unambiguously characterize apparently novel NTMs.
6. More information is required on the latency and dormancy periods of NTM diseases. Further research can focus on finding the latency and dormancy of NTM to facilitate the identification of environmental sources which lead to disease outbreaks.

7. Information and results generated could be used to compile a comprehensive data base on NTM in water sources in Ghana.

REFERENCES

Aboagye, S. Y., Danso, E., Ampah, K. A., Nakobu, Z., Asare, P., Otchere, I. D. & Yeboah-Manu, D. (2016). Isolation of nontuberculous mycobacteria from the environment of Ghanaian communities where Buruli ulcer is endemic. *Applied and Environmental Microbiology, 82*(14), 4320-4329.

Adékambi, T., Berger, P., Raoult, D. & Drancourt**,** M. (2006). *rpoB* gene sequence-based characterization of emerging non-tuberculous mycobacteria with descriptions of *Mycobacterium bolletii* sp. *Mycobacterium phocaicum* sp. and *Mycobacterium aubagnense* sp. *International Journal of Systematic Evolutionary Microbiology, 56* (1), 133-43.

Adékambi, T., Shinnick, T.M., Raoult, D. & Drancourt, M. (2008). Complete *rpoB* gene sequencing as a suitable supplement to DNA-DNA hybridization for bacterial species and genus delineation. *International Journal of Systematic and Evolutionary Microbiology, 58*, 1807–1814.

Agrawal, M., Bajaj, A., Bhatia, V. & Dutt, S. (2016). Comparative Study of GeneXpert with ZN Stain and Culture in Samples of Suspected Pulmonary Tuberculosis. *Journal of Clinical Respiratory Medicine.* 10(5), 9-12.

Aldous, W.K., Pounder, J.I., Cloud, J.L. & Woods, G.L. (2005). Comparison of six methods of extracting Mycobacterium tuberculosis DNA from processed sputum for testing by quantitative real-time PCR. *Journal of Clinical Microbiology, 43*(5), 2471-3.

American Thoracic Society (ATS). (1997). Diagnosis and treatment of disease caused by nontuberculous mycobacteria. *American Journal of Respiratory and Critical Care Medicine*, 156, 1-25.

American Thoracic Society (ATS) and the Infectious Diseases Society of America (IDSA). (2007). An Official ATS/IDSA Statement: Diagnosis, Treatment, and Prevention of Nontuberculous Mycobacterial Diseases. *American Journal of Respiratory and Critical Care Medicine*, 175, 367–416.

Amofah, G. K., Sagoe-Moses, C., Adjei-Acquah, C., & Frimpong, E. H. (1993). Epidemiology of buruli ulcer in Amansie west district, Ghana. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *87*(6), 644-645.

Amofah, G., Bonsu, F., Tetteh, C., Okrah, J., Asamoa, K., Asiedu, K., & Addy, J. (2002). Burly ulcer in Ghana: results of a national case search. *Emerging Infectious Diseases*, *8*(2), 167.

Archuleta, R. J., Hoppes, P. Y., & Primm, T. P. (2005). *Mycobacterium avium* enters a state of metabolic dormancy in response to starvation. *Tuberculosis*, *85*(3), 147-158.

Asiedu, K., Raviglione, M. C., Scherpbier, R. (2000). *Buruli ulcer: Mycobacterium ulcerans infection*. Geneva: World Health Organization.

Aubry, A., Chosidow, O., Caumes, E., Robert, J., & Cambau, E. (2002). Sixty-three cases of *Mycobacterium marinum* infection: clinical features, treatment, and antibiotic susceptibility of causative isolates. *Archives of Internal Medicine*, *162*(15), 1746-1752.

Bar-On, O., Mussaffi, H., Mei-Zahav, M., Prais, D., Steuer, G., Stafler, P., & Blau, H. (2015). Increasing nontuberculous mycobacteria infection in cystic fibrosis. *Journal of Cystic Fibrosis*, *14*(1), 53-62.

Bellinger, D. A., & Bullock, B. C. (1988). Cutaneous *Mycobacterium avium* infection in a cynomolgus monkey. *Laboratory Animal Science*, *38*(1), 85.

Bendinger, B., Rijnaarts, H. H., Altendorf, K., & Zehnder, A. J. (1993). Physicochemical cell surface and adhesive properties of coryneform bacteria related to the presence and chain length of mycolic acids. *Applied and Environmental Microbiology*, *59*(11), 3973-3977.

Bennett, S. N., Peterson, D. E., Johnson, D. R., Hall, W. N., Robinson-Dunn, B., & Dietrich, S. (1994). Bronchoscopy-associated *Mycobacterium xenopi* pseudo- infections. *American Journal of Respiratory and Critical Care Medicine*, *150*(1), 245-250.

Beran, V., Matlova, L., Dvorska, L., Svastova, P. & Pavlik, I. (2006). Distribution of mycobacteria in clinically healthy ornamental fish and their aquarium environment. *Journal of Fish Diseases*, *29*(7), 383-393.

Bhamidi, S. (2009). *Mycobacterial Cell Wall Arabinogalactan*. In: Ullrich, M., Ed., Bacterial Polysaccharides: Current Innovations and Future Trends, Horizon Scientific Press.

Bodmer, T., Miltner, E. & Bermudez, L. E. (2000). *Mycobacterium avium* resists exposure to the acidic conditions of the stomach. *FEMS Microbiology Letters*, *182*(1), 45-49.

Bollo, E., Guarda, F., Capuccl1io, M.T. & Galietti, P. (1998) Direct detection of *A1ycobacterium tuberculosis* complex and *M. avium* complex in tissue specimens from cattle through identification of specific rRNA sequences**.** *Zentralbl. Veterinarmed*, *45*, 395-4

Brennan, P. J. & Nikaido, H. (1995). The envelope of mycobacteria. *Annual Review of Biochemistry*, *64*(1), 29-63.

Brooks, R. W., George, K. L., Parker, B. C., Falkinham III, J. O. & Gruft, H. (1984). Recovery and survival of nontuberculous mycobacteria under various growth and decontamination conditions. *Canadian Journal of Microbiology*, *30*(9), 1112-1117.

Burback, B. L. & Perry, J. J. (1993). Biodegradation and biotransformation of groundwater pollutant mixtures by Mycobacterium vaccine. *Applied and Environmental Microbiology*, *59*(4), 1025-1029.

Butler, W. R., & Guthertz, L. S. (2001). Mycolic acid analysis by high-performance liquid chromatography for identification of *Mycobacterium* species. *Clinical Microbiology Reviews*, *14*(4), 704-726.

Cangelosi, G. A., Palermo, C. O., & Bermudez, L. E. (2001). Phenotypic consequences of red–white colony type variation in *Mycobacterium avium*. *Microbiology*, *147*(3), 527-533.

Carter, G., Wu, M., Drummond, D. C., & Bermudez, L. E. (2003). Characterization of biofilm formation by clinical isolates of *Mycobacterium avium*. *Journal of Medical Microbiology*, *52*(9), 747-752.

Chang, C. T., Wang, L. Y., Liao, C. Y., & Huang, S. P. (2002). Identification of nontuberculous mycobacteria existing in tap water by PCR-restriction fragment length polymorphism. *Applied and Environmental Microbiology*, *68*(6), 3159-3161.

Chanda-kapata, P., Kapata, N., Klinkenberg, E., Mulenga, L., Tembo, M., Katemangwe, P., Sunkutu, V., Mwaba, P., Grobusch, M., (2015). Non-tuberculous mycobacteria in Zambia : prevalence, clinical, radiological and microbiological characteristics. *Infectious Diseases*, 1–7

Cirillo, J. D., Falkow, S., Tompkins, L. S., & Bermudez, L. E. (1997). Interaction of *Mycobacterium avium* with environmental amoebae enhances virulence. *Infection and Immunity*, *65*(9), 3759-3767.

Cloud, J. L., Neal, H., & Rosenberry, R. (2002). Identification of *Mycobacterium* spp. by using a commercial 16S ribosomal DNA sequencing kit and additional sequencing libraries. *Journal of Clinical Microbiology*, *40*, 400–6.

Cooksey, R. C., Jhung, M. A., Yakrus, M. A., Butler, W. R., Adékambi, T., Morlock, G. P., & Charles, N. (2008). Multiphasic approach reveals genetic diversity of environmental and patient isolates of *Mycobacterium mucogenicum* and *Mycobacterium phocaicum* associated with an outbreak of bacteraemia at a Texas hospital. *Applied and Environmental Microbiology*, *74*(8), 2480-2487.

Correa, A. G., & Starke, J. R. (1996). Nontuberculous mycobacterial disease in children. *Respiratory Infections*, *1*(4), 262-271.

Covert, T. C., Rodgers, M. R., Reyes, A. L., & Stelma, G. N. (1999). Occurrence of nontuberculous mycobacteria in environmental samples. *Applied and Environmental Microbiology*, *65*(6), 2492-2496.

Cumberworth, V. L., & Robinson, A. C. (1995). *Mycobacterium avium-intracellulare* cervical lymphadenitis in siblings: a case report and review. *The Journal of Laryngology and Otology*, *109*(1), 70-71.

Dailloux, M., Laurain, C., Weber, M., & Hartemann, P. H. (1999). Water and nontuberculous mycobacteria. *Water Research*, *33*(10), 2219-2228.

Dawson, D. J. (1971). Potential pathogens among strains of mycobacteria isolated from house- dusts. *Medical Journal of Australia*, *1*(13), 679-681.

Dawson, D. J., Armstrong, J. G., & Blacklock, Z. M. (1982). Mycobacterial cross-contamination of bronchoscopy specimens. *American Review of Respiratory Disease*, *126*(6), 1095-1097.

De Groote, M. A., Pace, N. R., Fulton, K., & Falkinham III, J. O. (2006). Relationships between Mycobacterium isolates from patients with pulmonary mycobacterial infection and potting soils. *Applied and Environmental Microbiology*, *72*(12), 7602-7606.

Dhople, A. M., Storrs, E. E., & Lamoureux, L. C. (1992). Isolation of Cultivable Mycobacteria from Faces and Lungs of Armadillos Infected with *Mycobacterium leprae*. *International Journal of Leprosy and other Mycobacterial Disease*, 60, 244, 249.

Dick, T., Lee, B.H., & Murugasu-Oei, B. (1998). Oxygen depletion induced dormancy in *Mycobacterium smegmatis*. *FEMS Microbiology Letters*, 163, 159–6

Duker, A. A., Portaels, F., & Hale, M. (2006). Pathways of *Mycobacterium ulcerans* infection: A review. *Environment International*, *32*(4), 567-573.

Eaton, T., Falkinham III, J. O., & von Reyn, C. F. (1995). Recovery of *Mycobacterium avium* from cigarettes. *Journal of Clinical Microbiology*, *33*(10), 2757.

Edirisinghe, E. A. R., Dissanayake, D. R. A., Abayasekera, C. L., & Arulkanthan, A. (2014). Occurrence of nontuberculous mycobacteria in aquatic sources of Sri Lanka. *International Journal of Mycobacteriology*, *3*(4), 242-246.

Embil, J., Warren, P., Yakrus, M., Stark, R., Corne, S., Forrest, D., & Hershfield, E. (1997). Pulmonary illness associated with exposure *to Mycobacterium-avium* complex in hot tub water: Hypersensitivity pneumonitis or infection? *Chest*, *111*(3), 813-816.

Environmental Protection Agency (EPA-US) (2002). *Mycobacteria: Drinking Water Fact Sheet*, EP A-822-F-02-O

Falcão, D. P., Valentini, S. R., & Leite, C. Q. F. (1993). Pathogenic or potentially pathogenic bacteria as contaminants of fresh water from different sources in Araraquara, Brazil. *Water Research*, *27*(12), 1737-1741.

Falkinham III, J. O. (1996). Epidemiology of infection by nontuberculous mycobacteria. *Clinical Microbiology Reviews*, *9*(2), 177.

Falkinham III, J. O. (2002). Nontuberculous mycobacteria in the environment. *Clinics in Chest Medicine*, *23*(3), 529-551.

Falkinham, III, J. O. (2009). Surrounded by mycobacteria: nontuberculous mycobacteria in the human environment. *Journal of Applied Microbiology*, *107*(2), 356-367.

Falkinham III, J. O. (2010). Impact of human activities on the ecology of nontuberculous mycobacteria. *Future microbiology*, *5*(6), 951-960.

Falkinham III, J. O. (2011). Nontuberculous mycobacteria from household plumbing of patients with nontuberculous mycobacteria disease. *Emerging Infectious Diseases*, *17*(3), 419.

Falkinham III, J. O. (2013). Ecology of nontuberculous mycobacteria—where do human infections come from?. *Seminars in Respiratory and Critical Care Medicine*, *34*(1), 095-102.

Falkinham III, J. O., Norton, C. D., & Le-Chevallier, M. W. (2001). Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare*, and other mycobacteria in drinking water distribution systems. *Applied Environmental. Microbiology*, *67*(3), 1225-1231.

Falkinham III, J. O., Iseman, M. D., de Haas, P., & van Soolingen, D. (2008). *Mycobacterium avium* in a shower linked to pulmonary disease. *Journal of Water and Health*, *6*(2), 209-213.

Feldman, R. A., Long, M. W., & David, H. L. (1974). *Mycobacterium marinum*: A leisure-time pathogen. *The Journal of Infectious Diseases*, *129*(5), 618-621.

Fischer, O. A., Matlova, L., Bartl, J., Dvorska, L., Svastova, P., Du Maine, R., & Pavlik, I. (2003). Earthworms (Oligochaeta, Lumbricidae) and mycobacteria. *Veterinary Microbiology*, *91*(4), 325-338.

Flaig, W., Beutelspacher, H., & Rietz, E. (1975). *Chemical composition and physical properties of humic substances.* Springer, Berlin, Heidelberg.

Forbes, A. B. (2017). Mycobacterial Taxonomy. *Journal of Clinical Microbiology, 55*(22),380-383.

Food and Drugs Authority (FDA-Ghana). (2018). *High alkaline water unsafe for consumption. www.*citinewsroom.com (April 10, 2018)

Freitas, 1, Panetta, IC, Curcio, M, and Ueki, S.Y. (2001). *Mycobacterium avium* complex in water buffaloes slaughtered for consumption. *Rev. Saude Publica* 35, 315---317.

Galassi, L., Donato, R., Tortoli, E., Burrini, D., Santianni, D., & Dei, R. (2003). Nontuberculous mycobacteria in hospital water systems: application of HPLC for identification of environmental mycobacteria. *Journal of Water and Health*, *1*(3), 133-139.

Gangadharam, P. R., & Jenkins, P. A. (1997). *Mycobacteria: I basic aspects* (Vol. 1). Springer Science & Business Media.

Gauthier, D.T. & Rhodes, M.W. (2009). Mycobacteriosis in fishes: A review. *Veterinary Journal, 180,* 33 – 47

Ghana Water Company Limited (GWCL) (2016). *History of water supply in Ghana*. Retrieved from http://www.gwcl.com.gh/pgs/history.php.

Goodwin, C. S., Marshall, B. J., Blincow, E. D., Wilson, D. H., Blackbourn, S., & Phillips, M. (1988). Prevention of nitroimidazole resistance in Campylobacter pylori by co-administration of colloidal bismuth sub citrate: clinical and in vitro studies. *Journal of Clinical Pathology*, *41*(2), 207-210.

Gopinath, K. & Singh, S. (2010). Non-Tuberculous Mycobacteria in TB-Endemic Countries: Are We Neglecting the Danger?. *Neglected Tropical Diseases*, 4(4), 615.

Grange, J. M. (1996). The biology of the genus Mycobacterium. *Journal of Applied Bacteriology*, *81*, 1-9.

Griffith, D. E., Aksamit, T., Brown-Elliott, B. A., Catanzaro, A., Daley, C., Gordin, F., Holland SM, Horsburgh R, Huitt, G, Iademarco M. F, Iseman M, Olivier K, Ruoss S, Fordham von Reyn C, Wallace RJ, Winthrop K. (2007). An official ATS/IDSA statement: Diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. *American Journal of Respiratory and Critical Care Medicine*, *175*(4), 367-416.

Gruft, H., Loder, A., Osterhout, M., Parker, B. C., & Falkinham III, J. O. (1979). Postulated Sources of *Mycobacterium intracellulaire* and *Mycobacterium scrofulaceum* Infection: Isolation of Mycobacteria from Estuaries and Ocean Waters. *American Review of Respiratory Disease*, *120*(6), 1385-1388.

Gubler, J. G., Salfinger, M., & von Graevenitz, A. (1992). Pseudo epidemic of nontuberculous mycobacteria due to a contaminated bronchoscope cleaning machine: Report of an outbreak and review of the literature. *Chest*, *101*(5), 1245-1249.

Halstrom, S., Price, P., & Thomson, R. (2015). Environmental mycobacteria as a cause of human. *International Journal of Mycobacteriology*, *4*(2), 81-91.

Harboe, M., Nagai, S., Patarroyo, M. E., Torres, M. L., Ramirez, C., & Cruz, N. (1986). Properties of proteins MPB64, MPB70, and MPB80 of *Mycobacterium bovis* BCG. *Infection and Immunity*, *52*(1), 293-302.

Harvey, R. W., & Young, L. Y. (1980). Enumeration of particle-bound and unattached respiring bacteria in the salt marsh environment. *Applied Environmental Microbiology*, *40*(1), 156-160.

Hasegawa, N., Miura, T., Ishii, K., Yamaguchi, K., Lindner, T. H., Merritt, S., Matthews, J. D., & Siddiqi, S. H. (2002). New simple and rapid test for culture confirmation of Mycobacterium tuberculosis complex: a multicenter study. *Journal of clinical microbiology*, *40*(3), 908–912. https://doi.org/10.1128/jcm.40.3.908-912.2002

Heitkamp, M. A., Freeman, J. P., Miller, D. W., & Cerniglia, C. E. (1988). Pyrene degradation by a *Mycobacterium* sp.: identification of ring oxidation and ring fission products. *Applied Environmental Microbiology*, *54*(10), 2556-2565.

Hilborn, E. D., Covert, T. C., Yakrus, M. G., Stelma, N. & Schmitt, M. (2002). *Nontuberculous mycobacyeria spp isolated from residents of King county, Washington*. Presented at International Conference on Emerging and Infectious Diseases, Atlanta, GA, February 29-March 3, 2004.

Hoffmann, C., Leis, A., Niederweis, M., Plitzko, J. M., & Engelhardt, H. (2008). Disclosure of the mycobacterial outer membrane: cryo-electron tomography and vitreous sections reveal the lipid bilayer structure. *Proceedings of the National Academy of Sciences*, *105*(10), 3963-3967.

Hoop, R. K., Böttger, E. C., Ossent, P., & Salfinger, M. (1993). Mycobacterioses due to *Mycobacterium genavense* in six pet birds. *Journal of Clinical Microbiology, 31*(4), 990-993.

Hosek, J., Svastova, P., Moravkova, M., Pavlik, I., & Bartos, M. (2006). Methods of mycobacterial DNA isolation from different biological material: a review. *Veterinarni Medicina, 51*(5), 180-192.

Hom, B., Forshaw, D., Cousins, D., and Irwin, PJ. (2000) Disseminated Mycobacterium avium infection in a dog with chronic dianhoea Aust. Vet. J. 78, 32(}-3

Howard, S. T., Rhoades, E., Recht, J., Pang, X., Alsup, A., Kolter, R., & Byrd, T. F. (2006). Spontaneous reversion of *Mycobacterium abscessus* from a smooth to a rough morphotype is associated with reduced expression of glycopeptidolipid and reacquisition of an invasive phenotype. *Microbiology, 152*(6), 1581-1590.

Ichiyama, S., Iinuma, Y., Hasegawa, Y., Shimokata, K., Kawahara, S. & Matsushima, T. (1997). Large-restriction-fragment analysis of *Mycobacterium kansasii* genomic DNA and its application in molecular typing. *Journal of Clinical Microbiology*, *35*(3), 596–599.

Iivanainen, E. K., Martikainen, P. J., Väänänen, P. K., & Katila, M. L. (1993). Environmental factors affecting the occurrence of mycobacteria in brook waters. *Applied Environmental Microbiology*, *59*(2), 398- 404.

Iivanainen, E. K., Martikainen, P. J., Räisänen, M. L., & Katila, M. L. (1997). Mycobacteria in boreal coniferous forest soils. *FEMS Microbiology Ecology*, *23*(4), 325-332.

Iivanainen, E., Sallantaus, T., Katila, M. L., & Martikainen, P. J. (1999). Mycobacteria in runoff waters from natural and drained peatlands. *Journal of Environmental Quality*, *28*(4), 1226-1234.

Jacobsen, E., Gurevich, I. & Schoch, P. (1996). Pseudoepidemic of nontuberculous mycobacteria in a community hospital. *Infection Control Hospital Epidemiology, 17* (6), 348.

Jarzembowski, J. A., & Young, M. B. (2008). Nontuberculous mycobacterial infections. *Archives of Pathology and Laboratory Medicine*, *132*(8), 1333-1341.

Jenkins, P. A. (1991). Mycobacteria in the environment. *Society for Applied Bacteriology Symposium Series,* 20, 137.

Johnson, P. D. R., Stinear, T., Small, P. L. C., Pluschke, G., Merrit, R. W., Portaels, F., Huygen, K., Hayman, J. A. and Asiedu, K. (2005). Buruli ulcer (*M. ulcerans* Infection): New insights, new hope for disease control. *Plos Medicine.* 2(5), 173.

Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S., & Madden, T.L. (2008). NCBI BLAST: a better web interface. *Nucleic Acids Research 36,* 5–9. https://doi.org/10.1093/nar/gkn201.

Kahana, L. M., Kay, J. M., Yakrus, M. A., & Waserman, S. (1997). *Mycobacterium avium* complex infection in an immunocompetent young adult related to hot tub exposure. *Chest, 111*(1), 242-245.

Kajioka, R., & Hui, J. (1978). The pleiotropic effect of spontaneous single-step variant production in *Mycobacterium intracellulare*. *Scandinavian Journal of Respiratory Diseases*, *59*(2), 91-100.

Katoch, V. M. (2004). Infections due to non-tuberculous mycobacteria (NTM). *Indian Journal of Medical Research*, *120*, 290-304.

Kamala, T., Paramasivan, C.N., Herbert, D., Venkatesan, P., & Prabhakar, R. (1994). Isolation and Identification of environmental mycobacteria in the *Mycobacterium bovis* BCG trial area of South India. *Applied Environmental Microbiology*, 60, 2180–2183

Kaufinan, A.C., Greene, C.E., Rakich, P.M., & Weigner, D.D. (1995). Treatment of localized *Mycobacterium avium* complex infection with clofuzimine and doxycycline in a cat *Journal of American Veterinary Medicine. Association*, 207, 457.

Kazda, J., Pavlik, I., Falkinham III, J. O., & Hruska, K. (2010). *The ecology of mycobacteria: impact on animal's and human's health*. Springer Science & Business Media.

Kirschner, P., Teske, A., Schröder, K. H., Kroppenstedt, R. M., Wolters, J., & Böttger, E. C. (1992). *Mycobacterium confluentis* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, *42*(2), 257-262.

Kumar, V., Sachan, T. K., Sharma, P., & Rawat, K. D. (2015). Ultrastructural morphologic changes in mycobacterial biofilm in different extreme condition. *Ultrastructural Pathology*, *39*(1), 38-48.

Le Dantec, C., Duguet, J. P., Montiel, A., Dumoutier, N., Dubrou, S., & Vincent, V. (2002). Chlorine disinfection of atypical mycobacteria isolated from a water distribution system. *Applied Environmental Microbiology*, *68*(3), 1025-1032.

Lee, H., Park, H.J. Cho, S.N., Bai, G.H and Kim, S.J. (2000) Species identification of mycobacteria by PCR-restriction fragment length polymorphism of the *rpoB* gene. *Journal of Clinical Microbiology 38,* 296-297

Lee, B. Y., Clemens, D. L., & Horwitz, M. A. (2008). The metabolic activity of *Mycobacterium tuberculosis*, assessed by use of a novel inducible GFP expression system, correlates with its capacity to inhibit phagosomal maturation and acidification in human macrophages. *Molecular Microbiology*, *68*(4), 1047-1060.

Leifsson, P. S., Olsen, S. N., & Larsen, S. (1997). Ocular tuberculosis in a horse. *Veterinary Record*, *141*(25), 651-654.

Leite, C. Q. F., Anno, I. S., Leite, S. R., Roxo, E., Morlock, G. P., & Cooksey, R. C. (2003). Isolation and identification of mycobacteria from livestock specimens and milk obtained in Brazil. *Memorias do Instituto Oswaldo Cruz*, *98*(3), 319-323.

Liu, J. U. N., Rosenberg, E. Y., & Nikaido, H. (1995). Fluidity of the lipid domain of cell wall from *Mycobacterium chelonae*. *Proceedings of the National Academy of Sciences*, *92*(24), 11254-11258.

Liu, J., Barry, C. E., Besra, G. S., & Nikaido, H. (1996). Mycolic acid structure determines the fluidity of the mycobacterial cell wall. *Journal of Biological Chemistry*, *271*(47), 29545-29551.

Lloyd, J. B., Whittington, R. J., Fitzgibbon, C., & Dobson, R. (2001). Presence of *Mycobacterium avium* subspecies *paratuberculosis* in suspensions of ovine trichostrongylid larvae produced in faecal cultures artificially contaminated with the bacterium. *Veterinary Record*, *148*(9), 261-263.

Mangione, E. J., Huitt, G., Lenaway, D., Beebe, J., Bailey, A., Figoski, M., & Yakrus, M. A. (2001). Nontuberculous mycobacterial disease following hot tub exposure. *Emerging Infectious Diseases*, *7*(6), 1039.

Mansfield, K. G., & Lackner, A. A. (1997). Simian immunodeficiency virus–inoculated macaques acquire *Mycobacterium avium* from potable water during AIDS. *Journal of Infectious Diseases*, *175*(1), 184-187.

Marsollier, L., Robert, R., Aubry, J., Saint André, J. P., Kouakou, H., Legras, P., & Carbonnelle, B. (2002). Aquatic insects as a vector for *Mycobacterium ulcerans. Applied Environmental Microbiology*, *68*(9), 4623-4628.

Martin, G., & Schimmel, D. (2000). *Mycobacterium avium* infections in poultry--a risk for human health or not?. *DTW. Deutsche Tierarztliche Wochenschrift*, *107*(2), 53-58.

McCarthy, C.M. & Schaefer, l. O. (1974). Response of *Mycobacterium avium* to ultraviolet irradiation. *Applied Microbiology, 28*,151-15

Merritt, R. W., Benbow, M. E., & Small, P. L. (2005). Unraveling an emerging disease associated with disturbed aquatic environments: the case of Burly ulcer. *Frontiers in Ecology and the Environment*, *3*(6), 323-331.

Merritt, R. W., Walker, E. D., Small, P. L., Wallace, J. R., Johnson, P. D., Benbow, M. E., & Boakye, D. A. (2010). Ecology and transmission of Burli ulcer disease: a systematic review. *PLoS Neglected Tropical Diseases*, *4*(12), 911.

Miltner, E. C., & Bermudez, L. E. (2000). *Mycobacterium avium* Grown in *Acanthamoeba castellanii* is protected from the effects of antimicrobials. *Antimicrobial agents and chemotherapy*, *44*(7), 1990-1994.

Moermana, A., Blokkera, M., Vreeburga, J. & van der Hoekb, J. P. (2014). Drinking Water Temperature Modelling in Domestic Systems. *Procedia Engineering*, *89*, 143 – 150

Montgomery, P. L. (1985). Modular multiplication without trial division. *Mathematics of Computation*, *44*(170), 519-521.

Morimoto, K., Iwai, K., Uchimura, K., Okumura, M., Yoshiyama, T., Yoshimori, K., & Kudoh, S. (2014). A steady increase in nontuberculous mycobacterioses mortality and estimated prevalence in Japan. *Annals of the American Thoracic Society*, *11*(1), 1-8.

Nishiuchi, Y., Maekura, R., Kitada, S., Tamaru, A., Taguri, T., Kira, Y., & Ito, M. (2007). The recovery of *Mycobacterium avium-intracellulare* complex (MAC) from the residential bathrooms of patients with pulmonary MAC. *Clinical Infectious Diseases*, *45*(3), 347-351.

Odiawo, G. O., & Mukurira, J. M. (1988). Avian cerebral tuberculosis. *The Veterinary Record*, *122*(12), 279-280.

O'Grady, D., Flynn, O., Costello, E., Quigley, F., Gogarty, A., McGuirl, O'Rourke, I. & Gibbons, N. (2000) Restriction fragment length polymorphism analysis of *Mycobacterium avium* isolates from animal and human sources. *International Journal of Tuberculous Lung Diseases*, *4*, 278—28

Parasher, D., Chauhan, D. S., V. D. Sharma, D. V., Chauhan, A., Chauhan, S. V. S. & Katoch, V. M. (2004). Optimization of Procedures for Isolation of Mycobacteria from Soil and Water Samples Obtained in Northern India. ***Applied Environs. Microbial***. *70*, 3751–3753.

Parker, B. C., Ford, M. A., Gruft, H., & Falkinham III, J. O. (1983). Epidemiology of infection by nontuberculous mycobacteria: IV. Preferential aerosolization of *Mycobacterium intracellulare* from natural waters. *American Review of Respiratory Disease*, *128*(4), 652-656.

Paull, A. (1973). An environmental study of the opportunist mycobacteria. *Medical Laboratory Technology*, *30*, 11–9.

Pavlik, I., Svastova, P., Bartl, J., Dvorska, L., & Rychlik, I. (2000). Relationship between IS901 in the *Mycobacterium avium* Complex Strains Isolated from Birds, Animals, Humans, and the Environment and Virulence for Poultry. *Clini*cal. *Diagnosis Laboratory of Immunology.*, *7*(2), 212-217.

Pfyffer, G. E., Brown-Elliott, B. A., & Wallace Jr, R. J. (2003). Mycobacterium: general characteristics, isolation, and staining procedures. *Manual of Clinical Microbiology, (8th ed.). ASM Press, Washington, DC*, 532-559.

Piddington, D. L., Kashkouli, A., & Buchmeier, N. A. (2000). Growth of *Mycobacterium tuberculosis* in a defined medium is very restricted by acid pH and Mg2+ levels. *Infection and Immunity*, *68*(8), 4518-4522.

Pond, C. L., & Rush, H. G. (1981). Infection of white carneaux pigeons (Columbia livia) with *Mycobacterium avium*. *Laboratory Animal Science*, *31*(2), 196-199.

Portaels, F., Elsen, P., Guimaraes-Peres, A., Fonteyne, P. A., & Meyers, W. M. (1999). Insects in the transmission of *Mycobacterium ulcerans* infection. *The Lancet*, *353*(9157), 986.

Ramasoota, P., Chansiripornchai, N., Källenius, G., Hoffner, S. E., & Svenson, S. B. (2001). Comparison of *Mycobacterium avium* complex (MAC) strains from pigs and humans in Sweden by random amplified polymorphic DNA (RAPD) using standardized reagents. *Veterinary Microbiology*, *78*(3), 251-259.

Realini, L., De Ridder, K., Hirschel, B., & Portaels, F. (1999). Blood and charcoal added to acidified agar media promote the growth of *Mycobacterium genavense*. *Diagnostic Microbiology and Infectious Disease*, *34*(1), 45-50.

Rodgers, M. R., Blackstone, B. J., Reyes, A. L., & Covert, T. C. (1999). Colonisation of point of use water filters by silver resistant non-tuberculous mycobacteria. *Journal of Clinical Pathology*, *52*(8), 629.

Ryan, K. J., & Ray, C. G. (2004). Mycobacteria. *Sherris medical microbiology: An introduction to infectious diseases.* (4th ed.) McGraw-Hill, New York.

Sabater, J. F., & Zaragoza, J. M. (1993). A simple identification system for slowly growing mycobacteria. Identification of 25 strains isolated from surface water in Valencia (Spain). *Acta microbiologica Hungarica*, *40*(4), 343-349

Sanford, S. E., Rehmtulla, A. J., & Josephson, G. K. (1994). Tuberculosis in farmed rheas (Rhea americana). *Avian Diseases*, 193-196.

Saubolle, M. A., Kiehn, T. E., White, M. H., Rudinsky, M. F., & Armstrong, D. (1996). *Mycobacterium haemophilum*: microbiology and expanding clinical and geographic spectra of disease in humans. *Clinical Microbiology Reviews*, *9*(4), 435-447.

Scammon, L., Froman, S., & Will, D. W. (1964). Enhancement of virulence for chickens of battey type of mycobacteria by pre-incubation at 42 C. *American Review of Respiratory Disease*, *90*(5), 804-805.

Schaefer, W. B., Davis, C. L., & Cohn, M. L. (1970). Pathogenicity of transparent, opaque, and rough variants of *Mycobacterium avium* in chickens and mice. *American Review of Respiratory Disease*, *102*(4), 499-506.

Schnitzer, M. (1982). Organic matter characterization: Methods of soil analysis Part 2. *Chemical and Microbiological Properties*, 581-594.

Schröder, F. H., Hermanek, P., Denis, L., Fair, W. R., Gospodarowicz, M. K., & Pavone- Macaluso, M. (1992). The TNM classification of prostate cancer. *The Prostate*, *21*(S4), 129-138.

Schulze-Robbecke, R, Feldmann, C, Fischeder, R, Janning, B., Exner, M & Wahl, G. (1995) Dental units: an environmental study of sources of potentially pathogenic mycobacteria Tuber. LWlg. Dis. 76,318-32

Shane, S. M., Camus, A., Strain, M. G., Thoen, C. O., & Tully, T. N. (1993). Tuberculosis in commercial emus (Dromaius novaehollandiae). *Avian Diseases*, 1172-1176.

Sharma, I. M., Petchiappan, A. & Chatterji, D. (2015). Quorum sensing and biofilm formation in mycobacteria: role of c-diGMP and methods to study this second messenger. *Life*, *66*(12), 823–834.

Shelton, B.G., Flanders, WD. and Monis, GK (1999) Mycobacterium sp. as a possible cause of hypersensitivity pneumonitis in machine workers. Emerg. Inject. Dis. 5,270---273

Slosarek, M., Kubin, M., & Jaresova, M. (1993). Water-borne household infections due to *Mycobacterium xenopi*. *Central European Journal of Public Health*, *1*(2), 78-80.

Sniadack, D. H., Ostroff, S. M., Karlix, M. A., Smithwick, R. W., Schwartz, B., Sprauer, M. A., & Good, R. C. (1993). A nosocomial pseudo-outbreak of *Mycobacterium xenopi* due to a contaminated potable water supply: lessons in prevention. *Infection Control and Hospital Epidemiology*, *14*(11), 636-641.

Somoskovi, A., Mester, J., Hale, Y. M., Parsons, L. M., & Salfinger, M. (2002). Laboratory diagnosis of nontuberculous mycobacteria. *Clinics in Chest Medicine*, *23*(3), 585-597.

Steadham, J. E. (1980). High-catalase strains of *Mycobacterium kansasii* isolated from water in Texas. *Journal of Clinical Microbiology*, *11*(5), 496-498.

Steadham, K. A., & Falkinham, J. O. (2006). Effect of growth in biofilms on chlorine susceptibility of *Mycobacterium avium* and *Mycobacterium intracellulare*. *Applied Environmental Microbiology*, *72*(6), 4007-4011.

Stelmack, P. L., Gray, M. R., & Pickard, M. A. (1999). Bacterial adhesion to soil contaminants in the presence of surfactants. *Applied Environmental Microbiology*, *65*(1), 163-168.

Stine, T. M., Harris, A. A., Levin, S., Rivera, N., & Kaplan, R. L. (1987). A pseudo epidemic due to atypical mycobacteria in a hospital water supply. *Jama*, *258*(6), 809-811.

Strahl, E. D., Gillaspy, G. E., & Falkinham, J. O. (2001). Fluorescent acid-fast microscopy for measuring phagocytosis of *Mycobacterium avium, Mycobacterium intracellulare,* and *Mycobacterium scrofulaceum* by Tetrahymena pyriformis and their intracellular growth. *Applied Environmental Microbiology*, *67*(10), 4432-4439.

Takigawa, K., Fujita, J., Negayama, K., Terada, S., Yamaji, Y., Kawanishi, K., & Takahara, J. (1995). Eradication of contaminating *Mycobacterium chelonae* from bronchofibrescopes and an automated bronchoscope disinfection machine. *Respiratory Medicine, 89*(6), 423-427.

Thomson, R., Tolson, C., Sidjabat, H., Huygens, F., & Hargreaves, M. (2013). *Mycobacterium abscessus* isolated from municipal water-a potential source of human infection. *BMC Infectious Diseases*, *13*(1), 241.

Thorel, M.F., Huchzermeyer, H., Michel, A.L. (2001). *Mycobacterium avium* and *M. intracellulare* infection in mammals. *Review Science and Technology Office Internationale des Epizooties*, 20, 204-218.

Tortoli, E. (2012). Phylogeny of the genus Mycobacterium: many doubts, few certainties. *Infection, Genetics and Evolution*, *12*(4), 827-831.

Tortoli, E., Cichero, P., Piersimoni, C., Simonetti, M. T., Gesu, G., & Nista, D. (1999). Use of BACTEC MGIT 960 for recovery of mycobacteria from clinical specimens: Multicenter study. *Journal of Clinical Microbiology*, *37*(11), 3578-3582.

Torvinen, E., Meklin, T., Torkko, P., Suomalainen, S., Reiman, M., Katila, M. L., & Nevalainen, A. (2006). Mycobacteria and fungi in moisture-damaged building materials. *Applied Environmental Microbiology*, *72*(10), 6822-6824.

Torvinen, E., Torkko, P., Nevalainen, A., & Rintala, H. (2010). Real-time PCR detection of environmental mycobacteria in house dust. *Journal of Microbiological Methods*, *82*(1), 78-84.

Tuffley, R. E., & Holbeche, J. D. (1980). Isolation of the *Mycobacterium avium-M. intracellulare-M. scrofulaceum* complex from tank water in Queensland, Australia. *Applied Environmental Microbiology*, *39*(1), 48-53.

Usui, Y., Kaga, A., Sakai, F., Shiono, A., Komiyama, K. I., Hagiwara, K., & Kanazawa, M. (2013). A cohort study of mortality predictors in patients with acute exacerbation of chronic fibrosing interstitial pneumonia. *BMJ Open*, *3*(7), e002971.

van der Werf, M. J. K¨odm¨on C., & Katalini´c-Jankovi´c, V. (2014). Inventory study of non-tuberculous mycobacteria in the European Union. *Infectious Diseases*, *14*, 62.

van der Wielen, P. W., & van der Kooij, D. (2013). Nontuberculous mycobacteria, fungi, and opportunistic pathogens in unchlorinated drinking water in The Netherlands. *Applied Environmental Microbiology*, *79*(3), 825-834.

van Ingen, J., Boeree, M. J., van Soolingen, D., Iseman, M. D., Heifets, L. B., & Daley, C. L. (2012). Are phylogenetic position, virulence, drug susceptibility and in vivo response to treatment in mycobacteria interrelated?. *Infection, Genetics and Evolution*, *12*(4), 832-837.

Van Ingen, J. (2013). Diagnosis of nontuberculous mycobacterial infections. *Seminars in Respiratory Critical Care Medicine*, *34*, 103-109.

van Ingen, J. (2017). Mycobacteria. In *Infectious Diseases*, 1645-1659.

Wallace, R. J., Silcox, V. A., Tsukamura, M., Brown, B. A., Kilburn, J. O., Butler, W. R., & Onyi, G. (1993). Clinical significance, biochemical features, and susceptibility patterns of sporadic isolates of the *Mycobacterium chelonae*-like organism. *Journal of Clinical Microbiology*, *31*(12), 3231-3239.

Wang, Y., Ogawa, M., Fukuda, K., Miyamoto, H., & Taniguchi, H. (2006). Isolation and identification of mycobacteria from soils at an illegal dumping site and landfills in Japan. *Microbiology and Immunology*, *50*(7), 513-524.

Wayne, L. G. (1994). Dormancy of *Mycobacterium tuberculosis* and latency of disease. *European Journal of Clinical Microbiology and Infectious Diseases*, *13*(11), 908-914.

Wendt, S. L., George, K. L., Parker, B. C., Gruft, H., & Falkinham III, J. O. (1980). Epidemiology of infection by nontuberculous mycobacteria: III. Isolation of potentially pathogenic mycobacteria from aerosols. *American Review of Respiratory Disease*, *122*(2), 259-263.

Whittington, R. J., Marsh, I. B., & Whitlock, R. H. (2001). Typing of IS 1311 polymorphisms confirms that bison (Bison bison) with paratuberculosis in Montana are infected with a strain of *Mycobacterium avium* subsp. paratuberculosis distinct from that occurring in cattle and other domesticated livestock. *Molecular and Cellular Probes*, *15*(3), 139-145.

Winthrop, K.L., McNelly, E., Kendall, B., Marshall-Olson, A. Morris, C., Cassidy M., Saulson, A. & Hedberg, K. (2010). Pulmonary Nontuberculous Mycobacterial Disease Prevalence and clinical features. *American Journal of Respiratory and Critical Care Medicine*, *182*, 977-982.

Wolinsky, E. (1979). Nontuberculous mycobacteria and associated diseases. *American Review of Respiratory Disease, 119*(1), 107-159.

Won Jin, B., Saito, H., & Yoshii, Z. (1984). Environmental Mycobacteria in Korea; distribution of the Organisms. *Microbiology and Immunology*, 28 (6), 667-677

World Health Organization (WHO). (1996). *Freshwater bodies require protection from pathogens that pose threats to drinking water supplies, recreational waters, agricultural and aqua cultural source waters, and to aquatic ecosystems in general*. World health report: Fighting disease, fostering development. World Health Organization,Geneva.

World Health Organization (WHO). (2004). *Guidelines for drinking-water quality*. World Health Organization, Geneva.

World Health Organization (WHO). (2008). Burli ulcer: progress report. *Weekly Epidemiological Record Relevé Épidémiologique Hebdomadaire*, *83*(17), 145-154.

World Health Organization. (2010). *Working to overcome the global impact of neglected tropical diseases: First WHO report on neglected tropical diseases*. World Health Organization, Geneva.

World Health Organization. (2011). *Water safety in buildings*. World Health Organization, Geneva.

World Health Organization. (2011). *Guidelines for drinking-water quality*, 4. World Health Organization, Geneva.

World Health Organization. (2012). *Treatment of Mycobacterium ulcerans disease (Burli ulcer): guidance for health workers.* World Health Organization, Geneva.

Yajko, D. M., Madej, J. J., Lancaster, M. V., Sanders, C. A., Cawthon, V. L., Gee, B., & Hadley, W. K. (1995). Colorimetric method for determining MICs of antimicrobial agents for *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology*, *33*(9), 2324-2327.

Yeboah-Manu, D., Röltgen, K., Opare, W., Asan-Ampah, K., Quenin-Fosu, K., Asante-Poku, A., Ampadu E., Fyfe, J., Koram, K., Ahorlu, C., Pluschke, G. (2012) Sero-Epidemiology as a Tool to Screen Populations for Exposure to *Mycobacterium ulcerans*. *PLoS Neglected Tropical Diseases*, *6*, 1460.

Zaugg, M., Salfinger, M., Opravil, M., & Lüthy, R. (1993). Extrapulmonary and disseminated infections due to *Mycobacterium malmoense*: case report and review. *Clinical Infectious Diseases*, *16*(4), 540-5

APPENDIX

APPENDIX A

INTRODUCTORY LETTER

GHANA WATER COMPANY LIMITED

Main Bankers: GCB Bank Limited
Societe Generale Ghana
National Investment Bank

My Ref. No.: HO·224/11/SF.1/22

Your Ref. No.: SBS/MBB/Vol.3/82

Central Region
Post Office Box 377
Cape Coast – Ghana
West Africa

10th October, 2017

The Head of Department / Principal Supervisor
Department of Molecular Biology and Biotechnology
College of Agriculture & Natural Sciences
School of Biological Sciences
University of Cape – Coast.

Dear Sir,

RE: INTRODUCTORY LETTER

Reference your letter SBS/MBB/Vol.3/82 and dated 2nd October 2016 on the above subject matter.

We are happy to inform you that Yvonne Edem Quist is permitted to sample water from our Water Treatment Plants (WTPs) within Central Region to enable her research on the topic Occurrence of Non – Tuberculosis Mycobacteria in selected water sources in Central Region of Ghana.

Yvonne Edem Quist will report to the Station Manager on arrival at our WTPs and will be responsible for her personal safety during the water sampling.

We are grateful for choosing Ghana Water Company Limited for the research.

Yours faithfully,

Stephen Amihere – Mensah
(Regional Production Manager)

***For* Regional Chief Manager**

CC: Regional Production Manager
All Station Managers

Hon. Alexander Kwamena Afenyo – Markin (Chairman), Ing. Dr Clifford Abdallah Bruimah (Ag Managing Director), Mr. Joseph Obe Opoku, Mr Micheal Ayesu, Naba Sigri Bewong, Mr Kwame Amporfo Twumasi, Mr Clement A. Kuba, Dr Forster Kum – Ankama Sarpo Madam Maria Aba Lovelace – Johnson, Jemima Toseafa (Mrs)

Registered Office: 28th February Road, (Near Independence Square)
Telephone: 233-302-666781-7 Fax: 233-302-663552
Website: www.gwcl.com.gh E-mail: info@gwcl.com.gh

APPENDIX B

Samples categorized as NTM by the Capilia TB-Neo assay and their sources

Sample source category	Sample	Sampling point (Treatment plant)
River source	Kakum	Brimsu new
	Ochi Nakwa	Essakyir
	Ochi Amissah	Baifikrom
	Ayensu	Kwanyako
Treatment plant	Baifikrom	Baifikrom
	Winneba	Winneba
	Sekyere Hemang	Sekyere Hemang
	Kwanyako old	Kwanyako old
	Kwanyako new	Kwanyako new
Reservoir	Mawukpor	Sekyere Hemang
	Mbroboto	Essakyir
Community tap	Gomoa Adam	Essakyir
	Kwanyako	Kwanyako
	Saltpond	Baifikrom
	Asanaadze	Brimsu old
	Kojo Badu	Winneba
	Kissi	Sekyere Hemang
	Antseadze	Essakyir
	Atabaadze	Sekyere Hemang
	Baifikrom	Baifikrom
	Kotukwa	Essakyir
	Mankessim	Baifikrom
	Victoria road	Winneba
Groundwater source (well, (W)/borehole,(B)	Baifikrom (W)	Baifikrom
	Saltpond (W)	Baifikrom
	Asanaadze (B)	Brimsu old
	Abrodziwuram (W)	Sekyere Hemang
	Kissi	Sekyere Hemang