IMPACT OF WATER AND SEDIMENT QUALITY ON BACTERIAL DENSITIES AND DIVERSITIES IN MARINE POND CULTURE SYSTEM IN MTWAPA CREEK, KENYA

BY

EDWIN KIPYEGON MUTAI

SC/PGB/066/09

A THESIS SUBMITTED TO THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARDOF THE DEGREE OF MASTER OF SCIENCE IN MICROBIOLOGY OF THE UNIVERSITY OF ELDORET, KENYA.

NOVEMBER, 2013

DECLARATION

DECLARATION BY THE STUDENT

This thesis is my original work and has not been presented for a degree or any other award in any other University or any other Institution. No part of this thesis may be reproduced without the prior written permission of the author and/or University of Eldoret.

EDWIN MUTAI (SC/PGB/066/09)…..……. Date ……
Department of Biology
School of Science
University of Eldoret, Eldoret, Kenya

DECLARATION BY THE SUPERVISORS

This Thesis has been submitted for examination with our approval as the university supervisors.

Dr. Moses Ngeiywa …… Date ……
Department of Biology
School of Science
University of Eldoret, Eldoret, Kenya

Prof. David Liti …… Date ……
Department of Biology
School of Science
University of Eldoret, Eldoret, Kenya

DEDICATION

To my late father, Stephen Kalya, who sadly departed too soon.

To my Mother Leah Kalya, Brothers, sisters and Nephew Ken, for their love and support

ABSTRACT

The purpose of the present study was to analyze the impact of water quality and sediment quality on temporal variations of bacterial densities and diversities in marine pond culture system in Mtwapa Creek. The work was based on sediment and water samples collected from grow-out and nurseries ponds from November 2011 to April 2012. The objectives of the study were to; isolate and identify bacterial genera, determine bacterial densities, determine temporal variations of bacterial densities and diversities and determine the influence of variations in water and sediment quality parameters on the densities and diversities of bacteria in marine pond culture system in Mtwapa creek. Cultural methods were used for the identification of bacterial types and the total colony plate counts were used to estimate the bacterial densities. One way ANOVA (Analysis of variance) was used to compare the difference in bacterial densities between water and sediment samples. The RELATE routine technique was then applied to total bacterial count (TBC) data to determine the presence of change in TBCs between sites and among months for the two types of samples; sediment and water samples. For sample types where change occurred, the BIO-ENV routine was used to determine which subset of physico- chemical parameters best described sample-specific changes in TBCs among sites and months. A total of 4 bacterial groups belonging to 22 bacterial families and 30 genera were isolated from the 10 study sites. There was significant difference in bacterial counts between sediment and water samples with the sediment recording higher bacterial counts compared to the water samples. The most dominant bacterial types in the sediments were Actinomyces, Vibrio, Clostridium, and Listonella while Vibrio and Listonella, Clostridium and Norcadia were dominant in water samples. Temporal change in bacterial types was observed at 5 of the 10 sites evaluated. At four of these sites, changes in physico-chemical parameters were significantly correlated with the concurrent temporal change identified in bacterial types. Continuous and constant monitoring is recommended in order to establish and reveal the changes that are likely to take place in the system with mariculture intensification which is likely to change bacteria, chemical and physical conditions in the pond culture system and communities in nearby waters and sediments.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABREVIATIONS

SAL - Salinity

ORP - Oxidation Reduction Potential

OM - Organic Mater

DIFCO – Digestive Ferments Company

WHO - World Health Organization

FAO - Food and Agricultural Organization

GOK - Government of Kenya

KDDP - Kilifi District Development Plan

NGO - Non Governmental Organization

NA - Nutrient Agar

BA - Blood Agar

TCBS - Thiosulphate Citrate Bile Salts

TSI - Triple Sugar Indole

TBC - Total Bacterial Count

CFU - Colony Forming Unit

MRVP - Methyl Red Voges Proscauer

ONPG - OrthoNitroPhenylGluconate

a or aH - (referring to greening of agar) Alpha haemolysis

b Beta - (refers to clear zone or beta haemolysis)

C-F-B grp - Cytophaga- Flavobacteriaceae- Bacteriodetes group

ACKNOWLEDGEMENTS

I would like to thank the almighty God for giving me the strength to have gone this far.

I express my gratitude to my father Kalya (*in memorium*) and mother Leah, who directed me to the Scientific and academic life and encouraged me always, to my brothers, sisters and nephew Ken who were always there when I needed them.

I thank Prof. D. Liti and Dr. M. Ngeiywa for their guidance and as mentors and friends during my research and thesis preparation and also for the financial support.

I express my sincere appreciation to Brendan Muli for his friendship and support during my studies, Brigid who was my long time research partner for moral support and encouragement; Clement, Khan, Raymond, Japhet and Irine who have been true friends and offered their help in one way or another.

Finally I wish to thank University of Eldoret, and Kwetu Training Centre staff for their support.

CHAPTER ONE

INTRODUCTION

1.1 Background

Globally, fish provide about 15% of all the animal proteins consumed, with variations from an average of 23% in Asia to approximately 18% in Africa and around 7% in Latin America (FAO, 2007). With the increasing rate of exploitation of wild fishery recourses and the ever increasing World population especially in Africa, Kenya included, fish protein from the wild will not be able to meet the demand. In order to bridge the fish protein gap, aquaculture is considered an appropriate alternative source to food security and sustenance of livelihood. Marine ecosystem is important in the supply of fish proteins and is a unique habitat for bioprospecting because of the enormous microbial diversity.

The declining Kenya's coastal fishery output has been attributed to destruction and over exploitation of mangrove wetland, therefore, there is need to introduce and practice silvo-aquaculture which is an integrated system involving culture of organisms within the Mangrove forests sustainably. Practiced in this way, best environmental aspects can be tackled while generating income to the community. Silvo-culture of finfish has focused mainly on Milk fish (*Chanos chanos*) and mullet (*Mugil* sp) especially in Asian countries (Primavera, 2000). Although marine finfish culture in Kenya is still at its infancy stages, it has been shown that marine fish can be cultured successfully (Liti , unpublished; Mirera, 2008).

The conservation and utilization of biological diversity requires comprehensive knowledge about the species distribution so as to keep the ecological balance in an

environment (Hewson *et al*., 2007). Recent anthropogenic interventions in marine environment have threatened all lives, including microorganisms. Study of marine microbial biodiversity is of vital importance to the understanding of the different processes of the marine environment including novel microorganisms for screening of bioactive compounds. As the microbial communities have a complex ecosystem process, biodiversity study explores their distribution and their roles in the habitat. Marine microbial diversity can be best studied by a combination of techniques of both conventional and modern approaches for better understanding.

Under natural environmental conditions, coastal waterways are free of obstructions and therefore the water and sediment quality is maintained at optimum due to the constant supply of Sea water and removal of wastes as the water leaves. However, construction of ponds along these waterways creates 'artificial islands', resulting in the congregation of a diverse biological community comprising both vertebrate and invertebrate organisms (Holguin *et al*., 2007). Therefore, one would expect to find a similar congregation of bacteria, viruses, fungi and parasites within the ponds which is the newly- created ecosystem, as well as the natural occurrence of other wild aquatic organisms (Wu *et al*., 2004).

Microbial communities are structured by temporal and spatial variability of physicochemical and biotic parameters, including salinity, pH, Dissolved Oxygen, Redox reactions, terrestrial inputs, Temperature, precipitation, and climate. Terrestrial inputs create gradients of nutrients, pollutants, and other matter that affect habitats (Hewson *et al*., 2007). Microbes themselves also exert influence on their habitats by consuming, producing, and sequestering a variety of compounds. Hence, in the pond

environment, gradients of materials important to micro- and macro- organisms alike are often controlled by processes carried out by microbes. Importantly, bacterial communities readily respond at extremely faster rates (compared to other benthic organisms) to environmental and pollution changes thus important as bioindicators of environmental health. If the bacterial community structure is determined by the environment they live in, then pollution loading or organic enrichment is expected to shift their composition, and a counter shift toward the original community should be evident after the abatement of pollution discharge (Yoza *et al*., 2007).

As humans continue to alter the environment, climate change will impact marine microbial communities and the biogeochemical cycles in which they participate (Kathiseran *et al*., 2001). Human and fish health relies on a number of critical equilibria that marine microorganisms broker, including the balance between viruses and their hosts in the oceans, the balances that keep harmful algal blooms in check, the processes that control nutrient concentrations in marine waters, and others. Interactions marine microorganisms have with larger organisms are either symbiotic or pathogenic. Beneficial microbial symbioses enable many invertebrate species to take advantage of habitats that would otherwise be unavailable to them. They may also enjoy the benefits of bioactive compounds microbes may produce to prevent biofouling or to ward off predators (Gillan *et al*., 2005).

1.2 Problem Statement

There is a growing awareness of the importance of near-shore coastal areas as regions integral to carbon cycle and as receiving grounds of anthropogenic input and for which there is paucity of data relating to bacterial diversity (Han *et al*., 1999). The

conservation and utilization of biological diversity requires comprehensive knowledge about the species distribution dynamics so as to keep the ecological balance in an environment. Recent anthropogenic interventions in marine environment such as aquaculture have also threatened all lives, including microorganisms. A great deal of research on the biogeography of marine microorganisms has been carried out (Kuenen *et al*., 2008), but a lot about the effects of these bacteria is still unknown especially on their potential to cause not only fish pathogens but also human pathogens, therefore more work is needed to elucidate and understand their complexity.

The marine environment is characterized by the hostile parameters such as high pressure, salinity, low temperature, absence of light, etc. and marine heterotrophic bacteria have adapted themselves to survive in this environment. Oligotrophy is also one more adaptation because of the small amount of available nutrient. However, heterotrophic bacterial action promotes organic degradation, decomposition and mineralization processes in sediments and in the overlying water, and releases dissolved organic and inorganic substances (Purushothaman, 1998). These heterotrophic bacteria comprise the bulk of microbial populations inhabiting the water column of oceans and are responsible for much of the biological transformation of organic matter and production of carbon dioxide (Sherr and Sherr, 1996).

Aquaculture leads to localized, high inputs of organic carbon to the underlying sediments (McGhie *et al*., 2000; Caroll *et al*., 2003), which in turn increase the benthic oxygen demand. Sediments may become anoxic, causing microbial oxidation of carbon to switch to alternative electron acceptors such as Sulphate. The shift from aerobic to anaerobic bacteria results in change in diversity and could result in

introduction of bacterial types pathogenic to both fish and humans. Consequently, the chemical and physical nature of the sediment may be greatly altered by the interacting effects of organic particles, microbial metabolism and altered invertebrate faunal communities. Microbial groups important in nutrient cycling in coastal marine waters, such as the nitrifying bacteria, have been studied in relation to fish farming (McCaig *et al*., 1999). Little is known about the impact of fish farming on overall microbial diversity and how this diversity shifts over time. Without an understanding of the microbial community densities and distribution and their reaction to organic perturbation, it is difficult to predict its effect on nutrient cycling and ecosystem function.

1.3 Justification

Bacterial communities are vulnerable to natural and anthropogenic disturbances such as global climate change, pollution, heavy metal contamination, organic pollution and enrichment. Interactions of bacterial communities and settlement chemical cues with supply side ecology and marine invertebrate larval settlement have been observed. Larvae of benthic marine invertebrates recognize suitable settlement substrata by using various environmental cues, including biofilms composed of complex matrices of macromolecular deposits and microbial constituents (Thiyagarajan *et al*., 2006). Because bacteria are key players in the marine ecosystem, as they directly affect many aspects of Marine microenvironment including the health of fish and other animals living in the marine ecosystem, thus the study will help in understanding the challenges in fish health.

The study will help contribute to the understanding of bacterial quantities and diversity in relation to water and sediment quality and the effects of anthropogenic activities on the distribution of bacterial species in the marine environments, and thus appreciate their complexity which is crucial in the conservation and utilization of biological diversity in keeping the ecological balance in the environment.

The effects that localized, high inputs of organic carbon to the underlying sediments, and the alteration of chemical and physical nature of the sediment by the interacting effects of organic particles, microbial metabolism and altered bacterial communities will be known. Microbial groups important in nutrient cycling in coastal marine waters and the overall impact that fish farming has on microbial diversity and how this diversity shifts over time will also be known thus will be useful in maintenance of a health ecosystem.

1.4 Objectives

1.4.1 Broad objective

To determine the impact of water and sediment quality on temporal variations of bacterial densities and diversities in pond culture system in Mtwapa creek.

1.4.2 Specific objectives

1. To identify bacterial genera in the marine pond culture system in Mtwapa creek.
2. To determine bacterial densities in the marine pond culture system in Mtwapa creek.

3. To determine temporal variations patterns of different bacterial genera in marine pond culture system in Mtwapa creek.
4. To determine the influence of variations in water and sediment quality parameters on the diversities of bacteria in marine pond culture system in Mtwapa creek.

1.4.3 Null hypotheses

1. Bacterial genera in the marine pond culture system in Mtwapa creek are not diverse.
2. Bacterial densities in the marine pond culture system in Mtwapa creek are lower than the set acceptable levels.
3. There is no temporal variation in bacterial densities and diversities in marine pond culture system in Mtwapa creek.
4. Water and sediment quality parameters have no influence on the diversities of bacteria in marine pond culture system in Mtwapa creek.

CHAPTER TWO

LITERATURE REVIEW

2.1 Water quality and micro-organisms

Marine fish farming has experienced an almost exponential growth over recent decades and is the fastest growing food production system Worldwide (FAO, 2007). Very soon, fish consumed from fish farming is expected to match the fish consumed from capture fisheries. For a correct environmental management, it is important to have a good knowledge about the processes that regulate the effects of aquaculture on the ecosystem.

The fish pond environment consists of the water column and surface sediment in the ponds, and the mangrove ecosystem which is found in between the creek water and the ponds. The water from the creek moves through the mangrove ecosystem before getting into the ponds. The ponds are usually constructed in such a way that there is regulated inflow and out flow of water which is controlled by a sluice gate at the main channel entry. Water enters the ponds when the tide level rises above 3.4m but when the tide is below 3.4m no water enters the ponds. This means that the ponds are subjected to periods of no water exchange alternating with periods where there is water exchange during the high spring tide. The length of these periods varies with behavior of tides (Liti, unpublished). Mangroves are unique inter-tidal ecosystems of the tropics, which support genetically diverse groups of aquatic and terrestrial organisms. This ecosystem is ideally situated at the inter-phase between the terrestrial and marine environment and supports a rich and diverse group of microorganisms. There are different groups of bacteria which get nourished by detritus and in turn help

the mangrove ecosystem in different ways (Holguin *et al*., 2001). These bacteria perform various activities in the mangrove ecosystem like photosynthesis, nitrogen fixation, methanogenesis, agarolysis, production of antibiotics and enzymes (arylsuphatase, L-glutaminase, chitinase, L-asparaginase, cellulose, proteasae, and phosphatase), which result in the high productivity of these systems (Kathiresan and Bingham, 2001).

Environmental problems have resulted from the conversion of wetland habitats to aquaculture ponds (Hall *et al*., 1999). These include nutrient, sediment and organic waste accumulation leading to deterioration of water quality (Holmer *et al*., 2005), one of the important factors that determine the viability of fish farming. The water quality problem is associated with both physical and chemical problems such as too high or too low dissolved oxygen (DO) concentrations, and high concentrations of nitrogenous compounds (ammonium-N and nitrate-N) and hydrogen sulphide (Mmochi *et al.,* 2001). Excretion of nitrogenous compounds by cultured fish and microbial decomposition of organic matter are the main source of ammonium, nitrates, nitrites, phosphates and other inorganic substances (Neori *et al*., 1989; Hall *et al*., 1992). Furthermore, high concentrations of carbon dioxide (CO_2), ammonia and other nitrogenous compounds are introduced into the water column after phytoplankton blooms collapse.

The greatest source of wastes in aquaculture is the organic matter (OM) that comes from fertilizing the ponds using organic manure and feeding of cultured fish (Gowen and Bradbury, 1987). This OM is relatively rich in organic carbon and nutrients such as nitrogen and phosphorus (Holmer *et al*., 2005) and is released in two forms,

particulate and dissolved. Particulate wastes come from uneaten feed, organic fertilizer and fish feces, while dissolved wastes come from fish feces and excretions derived from fish metabolism, such as urea (Brown et al., 1987; Sanz-Lazaro *et al*., 2011). The impact fish farming has, is usually more obvious in the sediments than in the water column, where particulate wastes tends to accumulate in the proximity of fish farms.

The accumulation of OM on the surface of the sediment enhances sediment metabolism, and so does the sediment oxygen uptake, since oxygen is the electron acceptor which aerobic bacteria uses for respiration (Holmer and Kristensen, 1992). Oxygen concentration in the sediment pore water is limited and depends largely on the exchange rate with water from the sediment surface, which has higher oxygen content. In the pore water, if the oxygen supply is less than the consumption of oxygen, oxygen levels decrease resulting in hypoxia or even anoxia. This produces profound changes in the sediment metabolism (Murray *et al*., 2008). Since aerobic bacteria can no longer mineralize the OM, this, in turn, becomes mineralized by anaerobic bacteria (Roszak and Colwel, 1987). Anaerobic bacteria have a less efficient metabolism and use other electron acceptors for respiration, and therefore, OM tends to accumulate in the seabed at even higher rates (Zhang *et al*., 2008).

Of the different types of anaerobic metabolic pathways, sulfate reduction is the most important in conditions of organic enrichment (Jorgensen, 1982; Middelburg and Lewin, 2009). Sulphate reduction uses sulphate as the electron acceptor and produces sulphides as a by-product. Sulphides can be dissolved in the pore water and have detrimental consequences for the organisms inhabiting the sediment as well as those

living in the water column. Sulphate is a compound abundant in sea water and so it is very unlikely to be depleted. However, if the metabolic rate is very high, the sulphate can be exhausted leading to methanogenesis. This anaerobic metabolic pathway produces a by-product, methane which is also toxic (Middelburg and Lewin, 2009). Although OM is considered the most important source of pollution derived from fish farming, other pollutants may also have adverse effects on the benthic communities, such as metals, mainly Copper, Zinc and Cadmium, as well, as chemotherapeutic substances (Sanz-Lazaro *et al*., 2011). These contaminants may have interactive effects (Murray *et al.,* 2008) and can also produce changes in diversity and structure of benthic and pelagic communities including bacteria.

2.2 Marine Bacterial Diversity

Marine microbes hold a position of unique importance in the biosphere. They were the original form of life on earth and today marine micro-organisms are a primary support for the biogeochemical cycles that continue to make life possible. A great deal of research has been carried out to elucidate the biogeography and metabolism of these organisms, but many unknowns persist. Uppermost on this list of questions is what effects human induced changes will have on the services marine microbes perform for the planet. The ocean is filled with microorganisms that dwell there permanently and other microbes that have been carried there from terrestrial environments.

Definition of marine bacteria is based on their ability to grow only at certain sea water concentrations (Rheinheimer, 1980). Many marine bacteria require sodium, potassium and magnesium ions. Some of them also require chloride ions and ferric ions (Reice,

1994). Generally obligate marine bacteria are capable of growing exclusively in seawater. All bacteria that live in seawater are not true marine bacteria. Near coastal waters, 95% of the bacterial population is salt tolerant forms; only 5% is true marine forms (Sanz-Lazaro *et al*., 2008). In the open ocean and in deep sea the true marine forms dominate. The oceans contain 10^{22} liters of water and as there are 10^{7} to 10^{9} bacteria per litre, significant portion of the world's bacteria (10^{30}) lives in the water column of the oceans (Woese and Fox, 1977). Large proportions of the remaining bacteria live in the ocean sediments, which cover 70% of the world's surface (Rheinheimer, 1980). The ultimate source of energy for them is sunlight, but light is available only at the surface waters, the majority of bacteria in water and sediments would be expected to experience various conditions for different periods. Most oceans are oligotrophic *i.e.* the concentration and the rate of supply of available organic matter is generally low.

Bacterial forms are highly versatile in their nutritional requirement. It is very difficult to classify the bacteria and many systems have been adopted since their discovery. A classification system based on phylogenic similarity has been proposed and it has wide acceptance (Woese and Fox, 1977). These grouping split the previously unified prokaryotes into archaebacteria and the eubacteria. The bacteria are further categorized based on their mode of obtaining carbon and energy. They are classified as autotrophs and heterotrophs. Autotrophs, based on the source of energy used are further grouped into photoautotrophs (sunlight) and chaemoautotrophs (oxidation of reduced inorganic substances). Chemoheterotrophic bacteria which represent large and heterogenous groups have been further divided according to morphological and physiological characteristics. Since the bacterial forms exhibit high degree of

metabolic diversity even within taxonomic groups and some individual bacteria has the ability to utilize variety of nutritional products depending upon environmental conditions (Fenical and Jensen, 1993)

2.3 Gram positive Bacteria

Most of the marine bacteria belong to gram negative group (Zobell and Upham, 1944). Gram positive bacteria are less than 10% of the total bacterial population. Gram positive bacteria do occur at higher percentage in sediments. The actinomycetes (Order Actinomycetales) and related diverse group are Gram positive filamentous forms. The other gram-positive genera like Arthrobacter and endospore producing forms *Bacillus* and *Clostridium* (family *Bacillaceae*) have also been isolated. Especially *Bacillus* species readily grow in medium containing nutrients. Besides spore formers, non spore forming cocci (family *Micrococceae*) and rods are found in marine environs (Wietz, 2010a).

2.4 Gram negative Bacteria

The gram negatives represent the largest and diverse group of marine chemoheterotrophic prokaryotes. These forms are grouped on morphological characters as rods and cocci and their affinity for oxygen. Majority of bacteria belong to the families *Pseudomonadaceae* and *Vibrionaceae*. They can easily be isolated and cultured. They are rarely distinct morphologically but are biochemically and ecologically diverse and the genera include *Photobacterium* and *Vibrio* (Snieszko, 1986). The bacteria in *Pseudomonadaceae* are collectively called *Pseudomonas* and they belong to genus *Pseudomonas* but also include other genera like *Xanthomonas* and *Altetromonas* (Baumann and Baumann, 1981). Most marine bacteria are aerobic or facultatively anaerobic because large parts of the ocean are well oxygenated.

Obligatory anaerobic forms do occur in marine environment. One such genus is *Desulfovibrio*, a sulfur reducing group (Zubkov *et al*., 2002).

Many gram negative heterotropic bacteria having distinct morphological features are usually associated with surfaces and not found in open ocean waters. The gliding bacteria in the orders of Cytophagales and Beggiatoales are filamentous in nature (Wietz *et al*., 2010b). Most of them are chemoheterotrophs. They are frequently found attached to the surfaces and occur as mats. These orders include the aerobic genus *Leucothrix* and *Beggiatoa*. The *Myxobacteria* (order Myxococcales) are motile gliding forms and their occurrence in the marine environment is not well documented. *Spirochaetes* belonging to the order Spirochaetales, are large and coiled bacteria that occur as free living form in marine environment and as symbionts in crystalline style of certain mollusks (Johnson, 1981). These Spirochaetes belonging to the genus *Cristispira*, has never been successfully cultured. The spiral and curved bacteria of the Spirillaceae which are Gram negative rods can be distinguished from spirochaetes in that they move using flagella. They are common in marine environment (Huys *et al*., 2002). They also include the genus *Bdellovibrio*, a parasitic bacterium that parasitizes other bacterial forms. In addition to gram negative bacteria, there is a group called the mollicutes the smallest forms characterized by lack of defined cell wall (formerly called as mycoplasmas) which occur as parasites in invertebrates (Boyle *et al.*, 1987).

2.5 Factors affecting marine bacterial communities

Marine bacterial community is a major component of microbial food webs, biogeochemical cycles and energy flow. Their biodiversity is structured and determined by the temporal and spatial variability of physicochemical and biotic

parameters and thus, can reflect local environmental conditions (Urakawa *et al*., 1999; Zhang *et al*., 2008). Therefore, any shift in nutrient, environmental and pollution profiles in the benthic-pelagic ecosystems will directly impact bacterial community that in turn further affects nutrient cycles and other related communities including those pathogenic to fish. Besides, bacterial communities are vulnerable to natural and anthropogenic disturbances such as global climate change, pollution, heavy metal contamination, organic pollution and enrichment (Han *et al*., 1999; Gillan *et al*., 2005; Duran *et al*., 2008). Bio-communicative compounds mediate the settlement of invertebrate larvae (Urakawa *et al*., 1999; Zhang *et al*., 2008). Interactions of bacterial communities and settlement chemical cues with supply side ecology and marine invertebrate larval settlement have been observed. Larvae of benthic marine invertebrates recognize suitable settlement substrata by using various environmental cues, including biofilms composed of complex matrices of macromolecular deposits and microbial constituents (Thiyagarajan *et al*., 2006). Thus, bacteria are key players in the marine ecosystem as they directly affect many aspects of Marine microenvironment including the health of fish and other animals living in the marine ecosystem.

The most important factor in defining marine microbial habitats is the distance over which these organisms interact with their environments (Thiyagarajan *et al*., 2006). The habitat attributes that are apparent to the naked eye are usually less important to a marine microbe than the microscopic and submicroscopic facts, including concentrations of nutrients, the presence of gels and particulate matter, metal concentrations, light levels, pH, ultraviolet exposure and solar flux, temperature, oxygen saturation, and redox (Richmond, 1997). Hence, the scale at which marine

microbial habitats are most relevant is very small, but defining the boundaries of these habitats is difficult to accomplish in a controlled laboratory experiment and is even more difficult to define for a microbial cell embedded in the environment. In general, marine microbes in and near sediments interact with and intercept reductants diffusing from the sediments below and oxidants diffusing from the water column above (Hewson *et al*., 2007). Despite their importance, our knowledge of the bacteria that inhabit sediments and water column is very limited, especially in the heterogeneous marine ecosystems and marine pond culture systems (Wu *et al*., 2004).

The detection of bacterial diversity and their spatio-temporal variation in surface sediments is also of great practical and scientific relevance, especially in coastal ecosystems. Recently, the analysis of changes in surface sediment bacterial community has been used for detecting and monitoring the biological effects of human activities in the marine environment (Zhang *et al*., 2008). If the bacterial community structure in soft-benthic habitat is determined by their environment, then pollution loading or organic enrichment is expected to shift their composition, and a counter shift toward the original community should be evident after the abatement of pollution discharge (Yoza *et al*., 2007). Construction of ponds and rearing of fish especially with intensification have unknown effects on these bacterial communities and therefore the current study will come up with some of that knowledge.

Distribution of bacteria depends on changes in water temperature, salinity and other physicochemical parameters. Bacteria also serve as an important source of food for a variety of marine organisms. Thus, bacteria not only maintain the pristine nature of

the environment, but also serve as biological mediators through their involvement in the biogeochemical processes (Alavandi, 1990).

2.6 Breakdown of organic matter

Bacteria play a role in the cycle of matter in water, as they are able to breakdown all natural organic compounds into the components from which they have originated (Rheinheimer, 1980). Decomposition of protein takes place by proteolytic bacteria, e.g. *Pseudomonas* and other eubacteria. Cellulose is decomposed by cellulolytic bacteria, e.g. *Cytophaga, Sporocytophaga* (Gooday, 1990). Chitin, which is synthesized by several marine organisms as extracellular material from algae, cell walls of some chlorophytes (Mulisch, 1993), exoskeletons, including molts from copepods and other marine invertebrates (Gooday, 1990) is a structural polysaccharide. However, it is not degraded easily as there is a report on chitin preservation in fossils. However, this biopolymer is degraded by chitinolytic or chitinoclastic bacteria, e.g. *Bacillus, Pseudomonas* and *Vibrio,* by their mexoenzyme chitinase (Kirchman and White, 1999). Pectins are also decomposed by numerous bacteria in anaerobic condition, e.g. *Clostridium pectinovorum* and the end-products are pectic acid and methanol (Gooday, 1990).

2.7 Mineral cycle

The metabolic diversity of marine microorganisms allows them to assume many roles in the biogeochemical cycles that other organisms cannot complete. The element carbon which forms the basis of all organic matter undergoes a constant cycle in nature by various heterotrophic bacteria. Nitrogen, a constituent of the protein, is cycled in aquatic environment by several bacteria (Kirchman and White, 1999).

Nitrification is an aerobic process, whereas denitrification is the process used by facultative anaerobic bacteria. Fixation of molecular nitrogen is carried out intracellularly by various bacteria, e.g. *Azotobacter* and *Clostridium*. Microbial oxidation progresses to sulphate, which represents the terminal step of mineralization of organic sulphur compounds and serves as a source of sulphur for plants (Stankiewics *et al*., 1997). This process is called sulphurication. Sulphate-reducing bacteria such as *Desulfovibrio* and *Desulfomonas*

2.8 Sedimentation

Bacteria play an important part in the formation of sediments through their metabolic activities (Rheinheimer, 1980). Due to bacterial activity in the sediments, the total amount of organic matter gradually diminishes and its composition is changed (Stankiewics *et al*., 1997). As the compounds which are more easily attacked are broken down first, the proportion of substances which decompose with difficulty increases in the deeper layers. Thus, the bacteria colonize suspended particles which change their size and shape and consequently their sedimentation rate (Rheinheimer, 1980).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area and study site

The Kenyan Coast is situated immediately south of the equator; it covers a distance of about 500 km while the actual length of the seafront is about 600 km. The coastline forms part of the western border of the Indian Ocean and has an almost continuous fringing coral reef. Other features of the Kenyan coast include mangrove forests and estuaries as well as a number of islands to the south, which protect several embayments and harbours (GOK, 2011). Approximately three million people inhabit the Kenyan coastal areas, at a density of 300–400 persons/km^2. The marine environment provides this population with employment and food in the form of shell and finfish. Fish contributes over 70% of the protein consumed by the coastal inhabitants (Richmond, 1997).Artisanal fishery lands 95% of the total marine catch, contributes 6% to the coastal economy, and is the main source of livelihood for more than 60,000 households (GOK, 2011). Mariculture in the Kenyan coast at the moment is still at its infancy stage.

Kilifi County is located in coast Region. It lies between latitude 3 ° and 4 ° south and between longitudes 39 °and 40 ° east. It covers an area of about 4779.2 km^2 and borders Malindi County to the north, the Indian Ocean to the east, Mombasa County to the south, Kwale County to the south west and Taita Taveta County to the west. The county is categorized into seven administrative divisions namely: Bahari, Kikambala, Chonyi, Jaribuni, Bamba,Vitengeni and Ganze. According to the 2009 population and Housing Census, there are 38,610,097 people in Kenya. Of these, 3,325,307 people reside in the Coast province and out of these, 1,109,735 live in

Kilifi County (Table 1). Thus the Coast Province supports 8.6%. of the National population. The coastal population is growing at a rate of 2.9% pa which is almost equal to the National average of 3.0% pa (GOK-2009 Census). The population in the province has increased by 22.6% from between 1999 to 2009. Around 40.6% of the Coastal population lives in urban areas while the remaining 59.4% resides in rural areas. The divisions with the highest densities in Kilifi County correspond to the economic activities undertaken in the area. Bamba, Vitengeni and Ganze are sparsely populated due to harsh living condition while Bahari and Kikambala have highest densities due to the major towns that provide employment and the high agricultural production in these divisions.

Table 1: Population distribution in Coast Province according to the 2009 Population and Housing Census

Parameter	**Mombasa**	**Lamu**	**Kwale**	**Kilifi**	**Taita Tafeta**	**Tana River**	**Total**
Pop size	939,370	101,539	649,931	1,109,735	284, 659	240,075	3,325,307
Pop density	4,292	16	79	88	17	6	40
Urban pop	915,101	13,243	62,529	237,415	92,844	27,837	1,348,969

(Source GOK, 2011)

The county receives a bi-modal rainfall pattern with 66% reliability. Amount of rainfall ranges from 700 mm–1200 mm per annum with the long rains being experienced from April to June, and short rains from October to December. The altitude ranges from 0 – 310 m above sea level (ASL) while mean temperatures vary from 22 – 34^{o}C. About 80.6% of the households depend on agriculture for income generation with an average farm size is 6 acres (s/s) and 20 acres for large size farms.

(Source: www.googlemaps.com)

Figure 1: Map of Kenya coast showing the study sites where the study was carried out (Mtwapa creek).

3.2 Facility design

The study was carried out in ponds constructed at Kwetu Training Centre - Mtwapa creek. The fish pond culture system consists of the water column and surface sediment in the ponds. The water from the creek moves through the mangrove ecosystem before getting into the ponds. The ponds were constructed in such a way that there is regulated inflow and out flow of water which is controlled by a sluice gate at the main channel entry. Water enters the ponds when the tide level rises above 3.4m but when the tide is below 3.4m no water enters the ponds. This means that the ponds are subjected to periods of no water exchange alternating with periods where there is water exchange during the high spring tide. The length of these periods varies with behavior of tides but on average water exchange takes place between 10-14 days.

3.3 Pond layout and design

Proper site selection was evaluated after considering recommended soil quality, tidal characteristics, water supply and mangrove vegetation coverage. Two existing Nurseries ponds were renovated with the following dimensions:

- Nursery one (18m by 7m)
- Nursery six (25m by 11m)

Four nursery pond units with the following dimensions were constructed:

- Nursery two (6m by 3m)
- Nursery three to five (14m by 6m)

(Source: www.googlemaps.com)

Figure 2: map showing the study sites in Mtwapa Creek, Kenya

Existing five grow out ponds measuring 29 × 38m were also used during the study. After completion, the facilities were cured through drying and water flushing in readiness for stocking. Water was then let into the ponds and nurseries through screens at the gate and overflow pipes.

3.4 Soil and water quality sample collection and analyses

3.4.1 Sample collection and transport to the laboratory

After completion of Nursery pond construction, the average pH of the soil was taken every month using a soil pH meter. Water quality parameters including Temperature,

Dissolved Oxygen (DO), pH, Salinity, Total dissolved Solids (TDS), Conductivity and Oxidation reduction potential (ORP) were taken using a multi parameter meter and recorded in tables. For the existing grow out ponds, and after filling the nursery ponds with water, average water quality parameters were recorded every day throughout the study period. Water samples were collected in clean reagent bottles while sediment samples were collected in clean polythene bags. Water and sediment samples were collected randomly from four points in 5 Nurseries and 5 grow out ponds. The four samples for each sample type from each sampling site was then mixed and homogenized to have one uniform sample. This gave a total of one sample for each sample type from each sampling site therefore a total of ten water samples and ten sediment samples. All the samples were then transported to the laboratory for bacteriological analysis to detect, identify and quantify bacteria. The sampling was done every month for a period of six months beginning from November, 2011 to April, 2012.

3.4.2 Bacteriological culture techniques: Microscopy, culture and identification

The culture techniques used in this study were those described by Buller *et al* (2004)

3.4.2.1 Total Bacterial Count (TBC).

TBCs were performed using Nutrient agar (NA) with 2%NaCl. Samples were stored at 4°C during transport and while waiting to be analyzed in the laboratory.

3.4.2.2 Water and sediment processing

Serial dilutions were done for both water and sediment samples as follows; 10^{-1}, 10^{-2}, 10^{-3}.

10^{-1} = 900 µl of sterile distilled water + 100 µl of sample

10^{-2} = 900 µl of sterile distilled water + 100 µl of dilution 10^{-1}

10^{-3} = 900 µl of sterile distilled water + 100 µl of dilution 10^{-2}

The procedure involved pipeting 100 µl (or 0.1 ml) of each dilution into the centre of an agar plate using a separate plate for each dilution. Each inoculum was then spread evenly over each plate using a sterilized inoculating cotton swap, ensuring that the inoculum was spread evenly over the plate so that individual colonies could be obtained for counting. Plates were then placed in an incubator with temperatures set at room temperature for incubation. Counting of bacterial colonies in the plates was done after 24 and 48 hours. To facilitate this, a felt pen was used to mark each colony from the back of the petri dish to assist during counting. Appropriate colonies with pure growth were selected for subculture. These were given an individual number (e.g. #1, #2, etc). Selected colonies were circled and numbered on the underside of the Petri dish using a felt pen, and then picked off and sub cultured to fresh plates. They were sub cultured to Nutrient Agar (NA) with 2% NaCl, Triple Sugar Indole (TSA), Blood Agar (BA) and Thiosulphate Citrate Bile Salt Agar (TCBS). They were then incubated for a further 24 h or as required for sufficient growth. Pure subculture growths were then used for the inoculation of biochemical identification sets.

3.4.3 Calculation to obtain CFU/ml

If 100 µl had been placed on to a plate and then lawn inoculated, this was a plate dilution of 10^{-1}. If 10 µl was placed on a plate, this would be a plate dilution of 10^{-2} and the bacterial count = N, the CFU/ml was calculated using formula: CFU/ml = N × dilution × plate dilution. Where, if 268 colonies are counted from the 10^{-2} dilution and 100 µl was inoculated to the plate, the count = $268 \times 10^2 \times 10 = 2.68 \times 10^5$ CFU/ml.

3.4.4 Identification tests

3.4.4.1 Microscopic and cultural characteristics

Microscopic and colony appearance, plus the results of the primary tests of Gram reaction, oxidase and catalase were observed and recorded in a table (Appendix 4).

3.4.4.2 Inoculation of Biochemical Identification Sets

The subculture plate was examined and, when growth was pure, it was used to inoculate biochemical identification sets. NaCl was added to the set at a final concentration of 2%. The set was then incubated at 25°c for a minimum of 48 h. To inoculate the tubes, a sterile Pasteur pipette was plunged three-quarters of the way into the tubed media, releasing 3–4 drops of inoculum as the pipette is withdrawn. After inoculating all the tubes, one drop of the inoculum was placed on to a purity plate and streaked out for isolated colonies. After incubation, they were checked for pure growth (Appendix II, schematic for using biochemical identification tables). Plate and tubed media were then incubated at 25°C for 2–5 days.

Positive sugar fermentation results were recorded at 24 h. All liquid media were incubated for at least 48 h. This applied in particular to Aesculin, Citrate and MRVP. The results were then recorded on a table (Appendix 4).

Table 2: Biochemical identification tests and their reactions

Biochemical test	**Test reaction**
1. Carbohydrate fermentation test	Colour change from red to yellow was observed for positive test in. Phenol red was used as the pH indicator.
2. Aesculin test	Dark black colour was observed for positive
3. ADH	Change of colour from green-brown to bright purple (Møller's method) was observed for positive test.
4. Citrate	Change of colour from green to blue indicated a positive test
5. DNase test	Plates were flooded with 1M HCl, left for 1–2 min and poured off plate. Plate was then examined over a dark coloured tile for zones of clearing around bacterial growth for positive test
6. ODC, LDC test	Colour change from green-brown to purple/grey indicated a positive test.
7. H2S test	Blackening of medium especially along the inoculum line indicated a positive test
8. Indole test	3–7 drops of Kovács reagent was added and formation of upper pink layer in tube indicated a positive test.
9. Motility tube test	Motile bacteria grew and spread through the semi-solid gel. Spreading was seen as diffuse growth emanating from the line of inoculation in motility – tube test method. Non-motile bacteria could not migrate from the line of inoculation
10. MR (Methyl Red) test	3–5 drops of Methyl Red reagent was added after 48 h incubation. Persistence of red colour indicated a positive test. A negative reaction was incubated for 3 days before making final conclusion.

11. VP test	200 ml of medium from MRVP tube was removed and placed into a 0.6 ml microfuge tube and one drop each of VP I and VP II reagents added. After 10–20 min, development of pink-red colour indicated a positive reaction. A negative aliquot was incubated for a further 24 h and test repeated.
12. Nitrate test	5 drops of each reagent, Nitrate A and Nitrate B was added. Formation of a red colour indicated a positive test. For negative reactions a match-head size amount of zinc dust was added. Formation of a pink colour confirmed a negative nitrate result, whereas no further colour with Zn indicated a positive reaction.
13. Oxidative fermentation test	Formation of yellow colour (glucose fermentation) in tubes was recorded for OF medium (oxidative fermentative). Growth seen in one or both tubes was recorded. Growth in the tube without paraffin oil (open tube) indicated an oxidative organism. Growth in both the open and covered tubes indicated the organism was a facultative anaerobe and grows with and without oxygen.
14. ONPG test	Yellow colour indicated a positive test. Clear or no colour was recorded as negative
15. TCBS agar	Growth of yellow (Y) or green (G) colonies, or 'no-growth' was recorded for TCBS (Thiosulphate-citrate-bile salts-sucrose agar)

Nutrient agar with 2% NaCl and supplemented with 30% glycerol (Bowman and Nichols, 2002) in micro tubes was used to store the bacterial isolates in the freezer at 4°c.

3.5 Data analysis

Data were entered in MS Excel spreadsheets then exported to PRIMER version 6.1 software package, Plymouth, United Kingdom (Clarke and Warwick, 2001) where verification to check for outliers and statistical analyses were performed.

One way ANOVA (Analysis of variance) was used to determine the difference in bacterial densities between water and sediment samples (P value<0.05). Mean (± SE) values for total bacterial counts from each study site and sample type were determined for each Month. Bacterial quantities were then compared for the different samples and sites and to other studies, and Corliform bacteria to General acceptable levels of fecal and total Coliforms set by Hongkong, Kenya, Malaysia, Norway, Philippines and the United States (Appendix 1).

Total Bacterial Counts and diversity data were analyzed using two nonparametric multivariate routines available in the PRIMER version 6.1 software package, Plymouth, United Kingdom (Clarke and Warwick, 2001). The underlying ordination method used in these multivariate routines is non metric multidimensional scaling (MDS). The reason for using MDS over other ordination techniques was that MDS did not make any assumptions about the form of the data or the interrelations of the samples, and it had greater ability than other ordination methods to represent complex relations in low dimensional space. Multidimensional scaling (MDS) ordination was subsequently used to map the spatio-temporal relationships in the similarities for all the ten sites and six sampling dates. Hierarchical clustering into sample groups (CLUSTER) ordination was performed by non-metric Multidimensional scaling (MDS) to summarize patterns in data aggregation, transformation and resemblance. Univariate Shanon diversity indices for the sample types (Sediment and water) were then performed to summarize Totals of each bacterial type, Total times isolated, and its richness. The RELATE routine was then applied to Total Bacterial Count data to determine the presence of change in Total Bacterial Counts among sites and among months for the two types of samples; sediment and water samples. Secondly, for sample types where change occurred, the BIO-ENV routine was used to determine

which subset of physico- chemical parameters best described sample-specific changes in total bacterial counts among sites and months.

First, the RELATE (test for seriation) routine was applied to bacterial data to determine the presence of temporal change in bacterial diversity among months. Second, at sites where temporal change occurred, the BIO-ENV routine was used to determine which subset of physico- chemical parameters best described site-specific changes in bacterial diversity. At sites where temporal patterns were detected based on the RELATE test for seriation, the BIO-ENV routine was applied to determine which subset of available physico- chemical parameters best matched site-specific temporal change.

The RELATE routine (test for seriation) was used to estimate the degree of site-specific temporal change (Clarke and Warwick, 2001) in bacterial diversity among Months, where temporal change is defined as the extent to which Bacterial diversity departs from a predefined starting point; in this case, the first Month a sample was collected. The RELATE routine computes the strength between two independently derived matrices as the Spearman Rank correlation coefficient (Kendall, 1970). In this case, the two matrices were one of bacterial diversity and the other of temporal distances (that is, Months) among samples. Under this analysis, if changes in bacterial diversity exactly matched the temporal sequence (for example, Bacterial diversity of the November sample was most similar to December, less similar to January, and so on with April being the most dissimilar), Spearman's rho (hereafter, rho) was close to 1; whereas, it was near zero when there was no apparent temporal pattern. In this report, rho greater than 0.9 indicated a very strong temporal change, 0.8 to 0.9

indicated a strong change, 0.7 to 0.8 indicated a relatively strong change, 0.5 to 0.7 indicated a moderate change, and less than 0.5 indicated a weak or no change. Additionally, the RELATE routine incorporates a significance test derived by permutation to test the third null hypothesis of complete absence of temporal change (Clarke and Warwick, 2001). Herein, rho values were considered significant (that is, the null could not be rejected) when less than 5 percent of 999 randomly permutated rho values were greater than the real rho value (p value less than 0.05). Because diversity change was evaluated among equally spaced temporal distances (Months), the presence of a temporal change at a site (significant rho value) indicated a linear change in Bacterial diversity among Months.

Before the analysis, Bray-Curtis similarity values among samples were calculated from presence-absence bacterial data (Clarke and Warwick, 2001). Bray-Curtis similarity values measured the degree of similarity in community structure between pairs of samples. Values ranged between zero and one, where one indicated an exact match in community composition and zero indicated completely different communities between two samples. A fourth-root transformation was applied to the relative abundance bacterial count data in order to down weight the most dominant species (that is, to prevent the similarity values calculated from these data to be driven by mostly the highly abundant species), while retaining information about rare species. The time-sequential matrix among samples was calculated directly in the RELATE routine under the “result of seriation” option in the Primer software (Clarke and Warwick, 2001).

At sites where temporal patterns were detected based on the RELATE test for seriation, the BIO-ENV routine was applied to determine which subset of available

physico- chemical parameters best matched temporal bacterial diversity. BIO-ENV also uses rank Spearman's rho to determine the strength of association between two similarity matrices (Clarke and Warwick, 2001); in this case, between the bacteria and concurrently collected physico-chemical parameters. All possible subsets of physico-chemical parameters were evaluated for the best match (that is, highest rho value) with the bacteriological data. The subset with the highest rho value (closest to 1) for each site was determined to best describe bacterial diversity among months. The significance of the best subset (Global BEST test) was calculated through permutation as described by Clarke and Gorley (2006), testing the fourth null hypothesis of no relation between the best subset of physico-chemical parameters and the associated bacteria. The best subset of physico-chemical parameters was considered significant (not due to chance) when less than 5 percent of 999 randomly permutated rho values were greater than the real value ($p < 0.05$). Because the BIO-ENV analysis was specific to sites with temporal change, a significant BIO-ENV finding identified the subset of selected physico-chemical parameters that best matched observed linear changes in bacterial diversity among Months.

Before analysis, the physico-chemical parameters data were $\log_{10}$ transformed to improve their normality and then normalized so that each variable had a mean of zero and a standard deviation of one. This normalization was necessary to ensure that each variable took on values over a similar range because different classes of physico-chemical parameters were measured on different scales. After transformation and normalization, Euclidean distance values were calculated for the physico-chemical parameters data (Clarke and Warwick, 2001). Bray-Curtis similarity values were calculated from fourth-root transformed relative abundance Bacterial count data.

CHAPTER FOUR

RESULTS

4.1 Bacterial diversity

The results of bacterial occurrence are shown in Table 3. A total of 30 bacterial Genera belonging to 22 families and 4 bacterial groups were isolated from the Sediment and water samples. Most bacteria were common in sediment and water samples but a few either occurred in water or sediment samples. *Flexibacter* and *E. coli* were present in the water sample and absent in the the sediment samples. *Carnobacterium* was present in the sediment but was absent in the water samples.

Table 3: bacterial types with their bacterial groups, families, genera and where possible species isolated from the pond culture system during the study period.

Bacterial group	Family	Genera (Bacterium)
1. Proteobacteria	1. *Vibrionaceae*	-*Vibrio*
		-*Enterovibrio*
		-*Salinivibrio*
		-*Listonella*
		-*Photobacterium*
	2. *Moraxellaceae*	-*Acinetobacter*
	3. *Hallomonadaceae*	-*Hallomonas*
	4. *Enterobacteriaceae*	-*Eschirichia coli*
	5. *Rhodobacteraceae*	-*Roseobacter*
	6. *Pasteurellaceae*	-*Actinobacillus*
	7. *Neisseriaceae*	-*Chromobacterium*
	8. *Pseudomonadaceae*	-*Pseudomonas*

	9. *Pseudoalteromonadaceae*	*-Pseudoalteromonas*
	10. *Colwelliaceae*	*-Colwellia*
2. Actinobacteria	1. *Actinomycetaceae*	*-Actinomyces*
	2. *Nocardiaceae*	*-Nocardia*
		-Rhodococcus
	3. *Micrococcaceae*	*-Arthrobacter*
	4. *Corynebacteriaceae*	*-Corynebacterium*
3. C-F-B grp	1. *Flavobacteriaceae*	*-Aquorivita*
		-Chryseobacterium
		-Flavobacterium
	2. *Flexibacteraceae*	*-Flexibacter*
	3. *Sphingobacteriaceae*	*-Pedobacter*
4. Firmicutes	1. *Staphylococcaceae*	*-Staphylococcus*
	2. *Streptococcaceae*	*-Lactococcus*
		-Streptococcus
	3. *Clostridiaceae*	*-Clostridium*
	4. *Bacillaceae*	*-Bacillus*
	5. *Leuconostocaceae*	*-Carnobacterium*

4.2 Bacterial densities

4.2.1 Total Bacterial densities

The results of means for Total Bacterial densities by substrate, the P-value and their significance are presented in table 4. Analysis of variance (ANOVA) (95 percent LSD intervals) of the Total bacterial counts between the Sediment and water samples revealed that there was significant difference ($P<0.05$) in TBC between the sediment and water samples during the study period with the sediment recording generally higher counts up to tenfold than those in the water samples. Highest bacterial quantities were realized in March 2012, while the lowest quantities were in November 2011 for water and sediment samples respectively. The bacterial quantities for January, February and March for water samples and December, April and March for

sediment samples were more similar with higher counts in Pond 3, Pond 4, Nursery 1 and Pond 5 respectively. Bacterial quantities in November, April, and February in sediment samples and December, April and March in water samples were similar with higher loads equally distributed in Pond 3, Pond 4 (P4), Nursery 1 (N1) and Pond 5 (P5). This is shown in fig. 2.

Table 4: Means for Total Bacterial densities by Substrate in Marine pond culture system during the study period (95.0 percent LSD intervals, P-value 0.05)

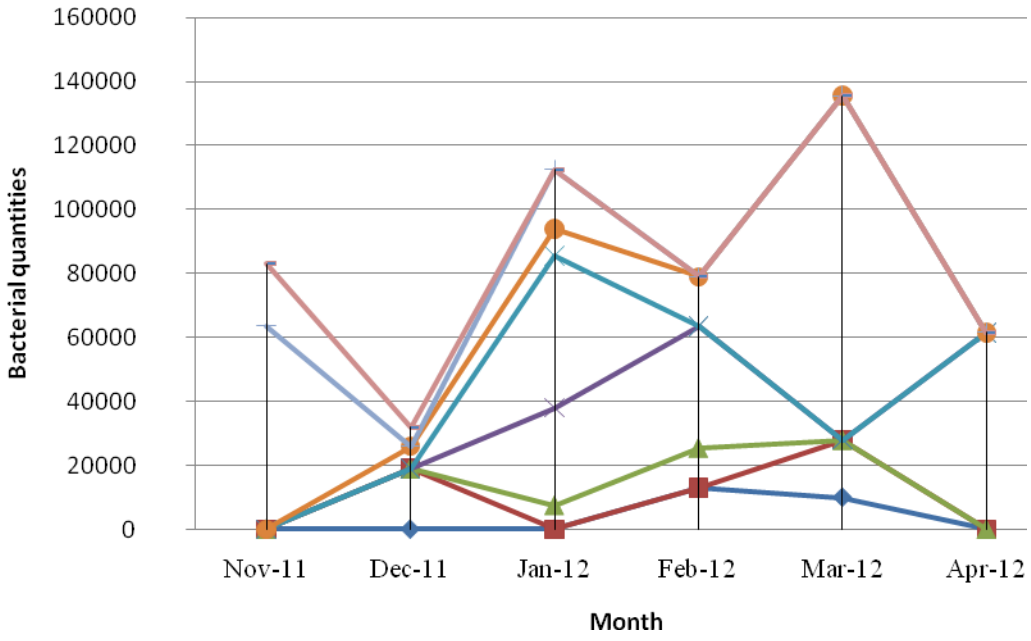
Date	Sediment (Mean±SE, n=10)	Water(Mean±SE, n=10)	P-Value	Significance
November	67350±6121.79	5760±6121.79	<0.01	Significant
December	85400±7461.16	7025±7461.16	<0.01	Significant
January	96150±9644.5	6935±9644.5	<0.01	Significant
February	144250±9644.5	9479±9644.5	<0.01	Significant
March	239950±24442.1	15580±24442.1	<0.01	Significant
April	118500±5560.45	15990±5560.45	<0.01	Significant

Results of seasonal variation of bacterial genera in Sediment and water samples of Nursery 3 are shown in figure 2 (a) and (b) respectively. *Clostridium* and *Arthrobacter* were the most dominant bacterial genera in both water and sediment samples, while *Vibrio* which was more dominant in sediment samples occurred in

lower numbers in the water samples. *Pseudoalteromonas* was low in both the samples. *Roseobacter, Carnobacterium, Arthrobacter* and *Chryseobacterium* were significantly higher in the Sediments while *Actinomyces* was higher in the water samples. Most bacterial genera exhibited a gradual increase in quantities from November with a peak in March and then a drop in April.

4.2.2 Bacterial Genera densities

(a)



(b)

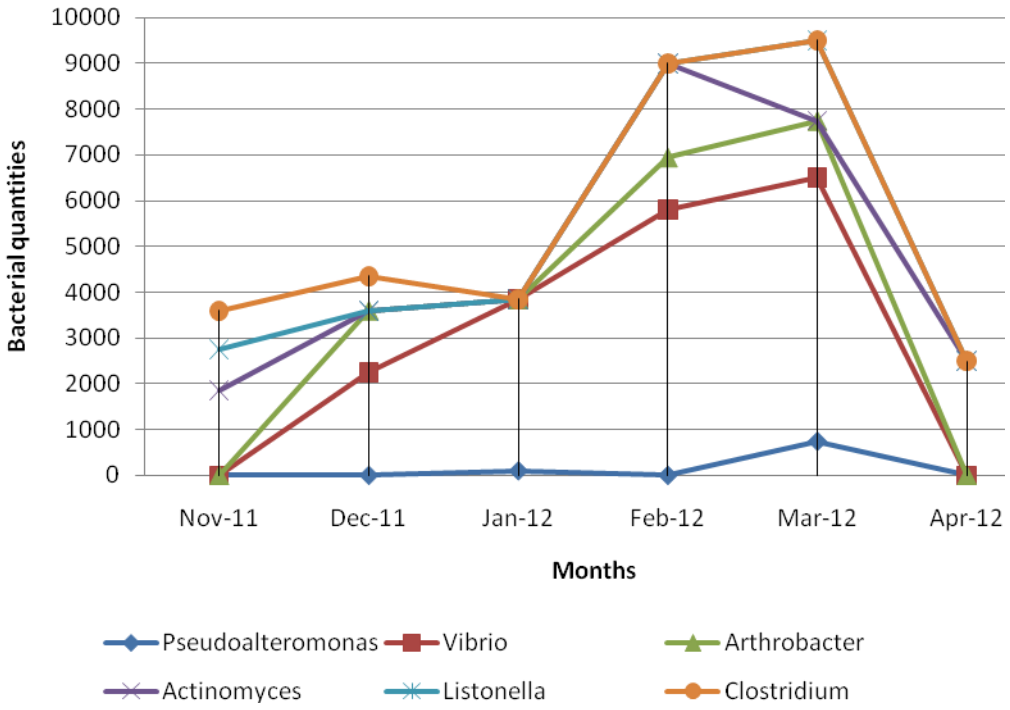
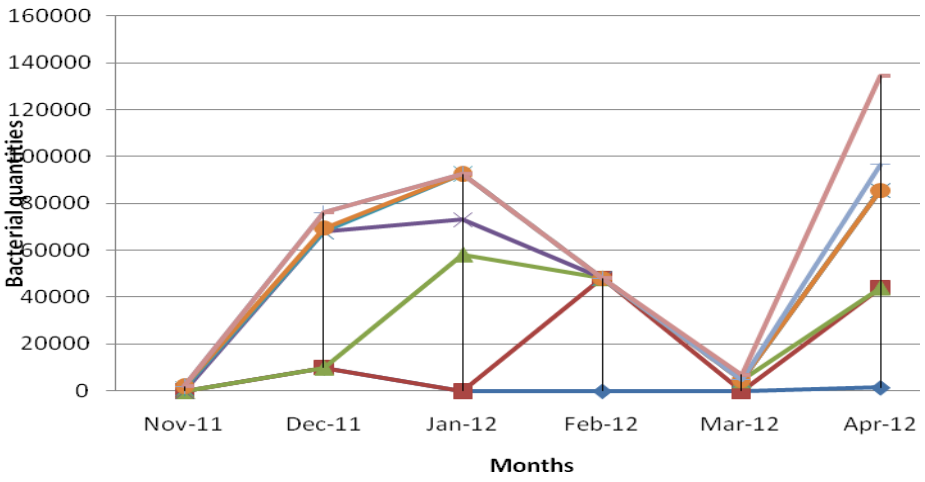


Figure 3: Seasonal variation of bacterial genera in Nursery 3 ((a) sediment and (b) water samples) during the study period.

Results of seasonal variation of bacterial genera in Sediment and water samples of Pond 6 are represented in Figure 3 (a) and (b) respectively. In both water and sediment samples, *Listonella* and *Flavobacterium* occurred in higher numbers while *Actinobacillus* was present but in lower counts. *Bacillus*, *Corynebacterium*, *Actinomycetes* were found in higher numbers while *Streptococcus* and *Staphylococcus* occurred in moderate counts in the sediment samples. In the water samples, *Norcadia*, *Roseobacter* and *Vibrio* occurred in significantly high counts. In the Sediment samples, most bacterial groups exhibited higher counts in March, January and April, with low counts in November and March. In the water samples, fairly low counts was observed from November to February, then high counts in March and a drop in April.

(a)



(b)

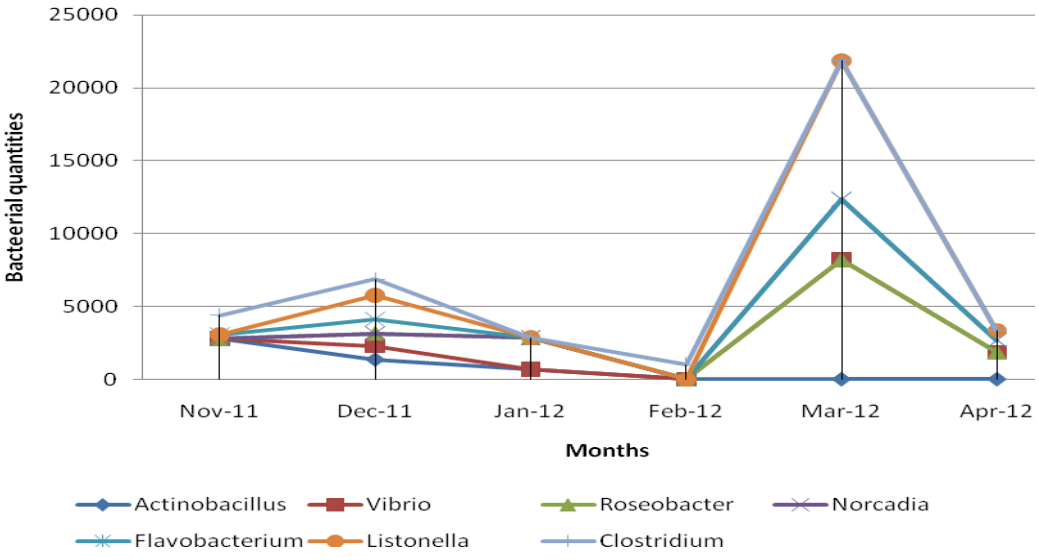


Figure 4: Seasonal variation of bacterial genera in Pond 6 ((a) sediment and (b) water samples) during the study period.

4.3 Temporal change in bacterial densities and diversity

4.3.1Total bacterial densities

Results of correlation coefficients, percent significance level, and the number of Months/sites associated with the strength of multivariate seriation of bacterial quantities among Months and sites are shown in table 5. Total Bacterial Counts did not differ among Sampling Sites in both the Sediment and Water samples (Rho values 0.261 and 0.38 respectively) neither was temporal change realized in water (Rho 0.326) and Rho 0.011 thus BIOENV was not tested for best Physico-chemical parameters. Water samples recorded fairly high Rho values compared to the sediment samples that is, variability among months and sites was notably higher in water samples than in the sediment samples.

Table 5: Correlation coefficients, percent significance level, and the number of Months/sites associated with the strength of multivariate seriation of bacterial densities among Months and sites (RELATE test for seriation) in Mtwapa creek during the study period.

Factor	Sample type	No. of months/sites	Spearman's Rho	% significance level
Among sites	Sediment	10	0.261	5
	Water	10	0.387	7.3
Among Months	Sediment	6	0.011	46.1
	Water	6	0.326	3

4.3.2 Temporal Change in bacterial diversity

Results of correlation coefficients, percent significance level, and the number of months associated with the strength of multivariate seriation of bacterial community structure among months is shown in table 6. Temporal change in bacterial diversity was present at 5 of the 20 sample types evaluated. Out of the 5 sites, only Pond 4 (water sample) showed relatively strong temporal change (rho = 0.744, p <0.003), whereas the strength of temporal change was more moderate at the remaining 4 sites Pond 3 (water sample), Pond 1 (sediment sample), Nursery 4 (sediment sample) and Nursery 3 (water sample) with rho = 0.508; 0.543; 0.553; and 0.612 respectively.

Table 6: Correlation coefficients, percent significance level, and the number of Months associated with the strength of multivariate seriation of bacterial community structure among Months (RELATE test for seriation).

[Sites with significant correlations between patterns in bacterial community structure and equally spaced temporal distances (months) are in **bold].**

Sampling site	Period/ Months	Sample Source/ type	Spearman's rho	% Significance level
Nursery 1	6	Sediment (N1S)	0.415	7.5
	6	Water (N1W)	0.303	12.2
Nursery 3	6	Sediment (N3S)	-0.214	79.4
	6	**Water (N3W)**	**0.612**	**2.4**
Nursery 4	**6**	**Sediment (N4S)**	**0.553**	**5.6**
	6	Water (N4W)	0.239	16.1
Nursery 5	6	Sediment (N5S)	0.046	40.3
	6	Water (N5W)	0.096	35.4
Nursery 6	6	Sediment (N6S)	-0.061	57.3
	6	Water (N6W)	0.216	21.7
Pond 1	**6**	**Sediment (P1S)**	**0.543**	**3.1**
	6	Water (P1W)	0.141	31.6
Pond 3	6	Sediment (P3S)	-0.006	44.1

	6	**Water (P3W)**	**0.508**	**2.7**
Pond 4	6	Sediment (P4S)	0.233	22.2
	6	**Water (P4W)**	**0.744**	**0.8**
Pond 5	6	Sediment (P5S)	-0.118	64.2
	6	Water (P5W)	0.3	12.4
Pond 6	6	Sediment (P6S)	-0.145	68
	6	Water (P6W)	0.278	15.5

Results of the BIO-ENV procedure determining which physico-chemical parameters best described observed multivariate temporal change (seriation) in bacterial diversity are shown in table 7. Physico-chemical parameters significantly determined temporal change among bacterial diversity at 4 of the 5 sites (sites N3W, P1S, P3W, and P4W) where seriation was present. At these sites, the strength of association between the best subset of physico-chemical parameters and bacterial diversity was rho values 0.943 at site N3W, 0.844 at P1S, 0.808 at P4W and 0.544 at site N4S.

Table 7: The physico-chemical parameters that best described observed multivariate temporal change (seriation) in bacterial diversity at Mtwapa Creek during the study period.

[Four subsets for each site with the highest Spearman's rho are presented, for those that were significant (that is < 5 percent of 999 permutated rho values were greater than the real rho value), are in **bold**]

Sampling site	Sample Source/type	Subset No.	Spearman's rho	Variables included	Environmental parameters (best)
Nursery3	**Water(N3W)**	**1**	**0.943**	**2,4**	**DO_2,TDS**
		2	**0.886**	**1,2,4,5**	**DO_2, PH, Temp, Cond**
		3	**0.829**	**1,2,4,6**	**DO_2, PH, Cond., SAL**
		4	**0.829**	**2,4,5**	**PH, TDS, Cond.**
Nursery4	Sediment(N4S)	1	0.406	1,2,5	DO_2, PH, Cond.
		2	0.377	1	DO_2
		3	0.348	1,5	DO_2, Cond.
		4	0.058	2	PH
Pond1	**Sediment(P1S)**	**1**	**0.844**	**4,6**	**TDS, SAL**
		2	**0.794**	**2,4,5,6**	**PH, TDS, Cond., SAL**
		3	**0.788**	**5**	**Cond.**
		4	**0.788**	**5,6**	**Cond., SAL**
pond3	**Water(P3W)**	**1**	**0.544**	**2,4**	**PH, TDS**
		2	0.47	1,2,4,5	DO_2, pH, TDS, Cond.
		3	0.47	1,2,4,6	DO_2, PH, TDS, SAL
		4	0.455	2,4,5	PH, TDS, Cond.
pond4	**Water(P4W)**	**1**	**0.808**	**2**	**PH**
		2	**0.782**	**1,2,4**	**DO_2, PH, TDS**
		3	**0.782**	**1,2,5**	**DO_2, PH, Cond.**
		4	**0.782**	**1,2,6**	**DO2, PH, SAL**

CHAPTER FIVE

DISCUSSION

The study focused on addressing temporal bacterial abundances, diversity and the impact of physico-chemical parameters in the marine pond culture system in Mtwapa creek. Linkages of environmental parameters with temporal bacterial diversity change underlined that external factors have an influence on bacterial distribution. The two compartments, sediment and the overlying water column, shared a large proportion of microbial assemblages, because the sediment is constantly flushed by the overlying water, trapping detritus and living cells from the water column (Boudreau *et al*., 2001).

Since only a small fraction of marine bacteria can be cultured (Staley and Konopka, 1985), culture-based studies indicated that the marine pond culture system is dominated by a few major groups of bacteria commonly found in marine waters. *Proteobacteria* and *Bactereoidetes* were generally the most abundant in water samples confirming previous results on the basis of fluorescence *in situ* hybridization and 16S rRNA gene-based clone libraries (Eilers *et al*., 2000; Zubkov *et al*., 2002). They were represented by the genera such as *Vibrio, Actinobacillus, Roseobacter, Aquorivita and Flavobacterium* which dominates bacterioplankton communities worldwide (Morris *et al*., 2002) and can constitute almost 50% of all prokaryotes in some locations (Wietz *et al*., 2010a). Other microbial groups isolated, included marine Firmicutes (Fuerst, 1995) and *Actinobacteria* (Wietz *et al*., 2010b). *Actinobacteria* were the most abundant in sediments samples similar to results by Bull and Stach, (2007) than in the water although other groups like *Proteobactria* and

Bacteriodetes were found to be significantly abundant. Firmicutes had an almost equal distribution in both the sediment and the water samples.

The high abundance of Cytophaga- Flavobacteriaceae- Bacteriodetes group (CFB) (mainly *Flavobacteria* subgroup) in this study suggested that this bacterial assemblage played an important role in biochemical degradation processes in the marine pond culture environment. CFB abundance might be related to availability of polymeric organic matter from seasonal terrestrial input or alga-derived metabolites and detritus.

Although antagonistic effect of various bacteria on others was not the focus of this study, some bacterial groups were found which exhibited inhibitory effect on other isolates during culture and these included Actinobacteria, the *Vibrionaceae* and *Pseudoalteromonas*. (Plate)The potential of marine bacteria to produce antimicrobial compounds has been known for decades (Burkholder *et al*., 1966; Gauthier and Flatau, 1976; Nair and Simidu, 1987). Subsequent studies have also shown that the marine environment indeed comprises a multitude of bacterial species producing bioactive metabolites (Jensen and Fenical, 2000; Debbab *et al*., 2010). Despite the only small fraction of microbes that can be cultured, the isolation of bacteria is therefore still a valuable approach (Giovannoni and Stingl, 2007) and promising way to isolate bioactive strains with desired physiological traits (Bull, 2004).

Generally, the multi-dimensional scaling (MDS) ordinations of bacterial quantities for each sampling month revealed a site-specific pattern. For instance, bacterial loads of water and sediment samples formed separate groups on the right and left side of the

MDS ordinations respectively. The bacterial quantities in each site also differed with time with the quantities increasing gradually from November 2011 to a maximum in March and a slight drop in April. These patterns were attributed to the seasonal climatic change. In November, rains were received and this could have contributed to the washing of pyrite soils from the surface into the ponds. Microorganisms are involved in the conversion of the pyrites in anoxic conditions to form sulphurous acid which results in the lowering of the acidity in the pond culture system and their fore impacting on the bacterial densities (Murray *et al*., 2008). From December to March, no rains were received and therefore the pH was maintained at optimum thus the gradual bacterial density increased in both the water and sediment samples. In April, the first rains were received and thus the bacterial densities dropped slightly. Such MDS grouping of bacterial quantities were largely supported by the graphical monthly representation of their distribution as shown in fig. 3 and 4. Thus, the study clearly indicated that the bacterial quantities distribution most likely respond to climatic and environmental change.

The estimates for bacterial groups (normally isolated from marine environments) genera richness in both sediment and water samples in this study were greater than those indicated by Torsvike *et al*. (1996). This probably could be due to the fact that the impact of organic loading from aquaculture to the pond culture system is not as great as that seen in other regions where production is more intensive. Currently the kind of farming system being used here is more of free range system whereby only organic fertilizers in small amounts, bamboos and coconut rafts were used to increase primary productivity and thus development of algae which is food for the fish. This result suggests that all sediments possessed a large range of functional redundancy

that contributed to their ability to maintain diverse bacterial types. The results of this study support the idea that bacterial community structure is determined by the environment and random succession events (Reice, 1994), rather than proceeding in an easily predictable manner.

Several Authors have reported loss of recovery of faecal corliform in sea water due to microbial die-off and entry of micro organisms into a viable and non-culturable state (Roszak *et al.*, 1987; Barcina *et al.*, 1997; Dioniso *et al.*, 2002). The presence of feacal corliforms in marine pond system may have been due to contamination from personnel working in the ponds, surface run-off from rain water or from creek water contaminated by fishing boats which finally finds its way into the ponds. The E. coli counts were found to be low; they were not isolated completely from the sediment samples while in the water samples they were only isolated 3 times with a total count of 625×10^3 thus an indication that feacal contamination in the culture system is insignificant. *Eschirichia coli* is normally found in human and animal intestines and is the most reliable indicator of feacal contamination in water which indicates the possible presence of pathogens (Gieldreich and Clarke, 1996).

Seasonal variation in bacterial diversity was observed only at five out of ten sites where the study was undertaken. Although samples were obtained from the sediment and water sample, in no instance was temporal change of bacterial diversity detected in both of the samples. In all cases, only one of either sample showed temporal change in bacterial diversity among months. Additional time of data collection may uncover other directional changes in bacterial diversity, especially at those sites with non significant rho values (closest to 0.5). However, it is unlikely that major temporal

change will be detected unless major environmental changes occur for example, changes in water-quality conditions significant enough to restructure the current micro –organism populations and their diversity.

Although environmental variables described temporal change in bacterial type structure at four sites, in one site (Nursery 4), temporal change was present but unexplained by the available variables (Table 5). In most cases (N1S, N1W and P5W), rho values were greater than 0.3 (near moderate) and percent significance levels were weak (about 7.5 to 12.4 percent). Because the significance level of the Global BEST test in the BIO-ENV procedure is strongly related to the number of data points being fit (Clarke and Warwick, 2001), additional years of data may reveal more conclusive results, as long as the descriptive variables of temporal change in bacterial type structure are measured.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

A total of 30 bacterial genera belonging to 22 bacterial Families and 4 main bacterial groups were isolated from the 10 sites where the study was undertaken in Mtwapa Creek, Kwetu during the period between November 2011 and April 2012. The most dominant bacterial types in the sediments were *Actinomyces, Vibrio, Clostridium,Listonella, Arthrobacter,* Bacillus and *Roseobacter* while the least dominant bacterial types were *Aquorivita*, *Salinivibrio, Colwellia* and *Lactococcus. Flexibacter and Eschirichia coli* were never isolated in sediment samples. The most dominant bacterial types in water samples included; *Vibrio, Listonella, Clostridium, Norcadia, Actonomyces, Actinobacillus, Pseudoalteromonas, Flavobacterium, Arthrobacter,* and *Acinetobacter* while *Bacilllus, Flexibacter, Pedobacter* and *Eschirichia coli* was the least dominant.

There was significant difference in bacterial counts between Sediment and water samples with the Sediment recording higher bacterial counts as compared to the water samples. Seasonal variation of bacterial types was observed only at five out of the ten sites where the study was undertaken. Out of the five sites where temporal change in bacterial diversity was present, physico-chemical parameters including $DO_{2,}$ pH, TDS, ORP, Salinity, and Conductivity, explained the change. In one site (Nursery 4), temporal change was present but unexplained by the available variables.

6.2 Recommendations

- Continuous and constant monitoring of the pond culture system is required in order to establish and reveal the changes that are likely to take place in the system. All the above that comes about due to aquaculture intensification may change bacteria, chemical and physical conditions of bacteria in the pond culture system and communities in nearby waters and sediments.
- After each culture cycle, the ponds should be completely drained and cured by drying to kill all the pathogenic organisms which consist of pathogenic bacteria.
- The six-months of bacterial diversity monitoring are not sufficient to trace the long term trends in correlation between environmental gradient and bacterial diversity. In order to have a better understanding of the relationships among pond management processes especially with aquaculture intensification in relation to bacterial diversity, a longer monitoring time is needed.
- Relatively few bacterial types were observed in this study using traditional culture methods than those observed by studies using non-cultivated molecular methods, therefore future studies should incorporate non -cultivated molecular methods.
- Substantial efforts in the future should target on identifying potentially novel bacterial species. Furthermore, bacterial activity experiments on these potential novel species are also suggested. The studies should also target on specific functional bacterial groups to reveal their roles in ecosystem recovery processes and to explore potential application of these bacteria on environmental pollution monitoring.

REFERENCES

Alavandi, S. V. "Relationship between heterotrophic bacteria and suspended particulate matter in the Arabian Sea (Cochin)". *Indian J. Mar. Sci*. 30 (1990): 89–92.

Barcina, I, Lebaron P, and Vives-Rigo J. "Survival of allochthonous bacteria in aquatic systems: a biological approach". *FEMS microbial. Ecol*. 23 (1997):1-9.

Baumann, P. and Baumann, L. "The marine gram-negative eubacteria: genera *Photobacterium, Beneckea, Alteromonas, Pseudomonas* and *Alealigenes"*. In: Stave, M.P., Stolp, H., Truper, H.G., Balows, A. and Schlegel, H.G. (Eds.), "The prokaryotes", Vol. II, *Springer-Verlag, Berlin*, (1981) 1302-1331.

Boudreau B.P, Huettel M, Forster S, Jahnke RA, McLachlan A, Middelburg JJ. "Permeable marine sediments: overturning an old paradigm". *EOS*. 82 (2001):133–136.

Boyle, P., Maki, J .S. and Mitchell, R. "Mollicute identified in novel association with aquatic invertebrate". *Curl. Microbiol* 15 (1987): 85-89.

Brown, J.R., Gowen, R.J., Mclusky, D.S. "The effect of salmon farming on the benthos of a Scottish Sea Loch". *J. Exp. Mar. Biol. Ecol.* 109 (1987): 39–51.

Bull, A.T. *How to look, where to look. In Microbial Diversity and Bioprospecting*. Bull, A T (ed) ASM Press, Washington DC., 2004.

Bull, A.T. Stach, J.E.M. "Marine actinobacteria: new opportunities for natural product search and discovery". *Trends Microbiol 15* (2007): 491–499.

Buller, N. B. "Bacteria from Fish and other Aquatic Animals" *A practical identification manual.* (Eds) CABI publishing, Wallingford, Oxfordshire, United Kingdom., 2004.

Burkholder, P., Pfister, R., Leitz, F. "Production of a pyrrole antibiotic by a marine bacterium". *Appl Microbiol* 14 (1966): 649-653.

Carroll, M.L., Cochrane S., Fieler R., Velvin R., White P. (2003) Organic enrichment of sediments from salmon farming in Norway*: environmental factors, management practices, and monitoring techniques.* Aquaculture 226*:* 165–180.

Clarke, K.R., and Gorley, R.N. Primer version 6—*User manual/tutorial*, Primer-E: Plymouth, United Kingdom, 2006. 190.

Clarke, K.R., and Warwick, R.M. "Change in marine communities"–*An approach to statistical analysis and interpretation.* 1st ed. Plymouth, United Kingdom, Plymouth Marine Laboratory, 2001. 172.

Debbab, A., Aly, A.H., Lin, W.H., Proksch, P. "Bioactive Compounds from Marine Bacteria and Fungi". *Microb Biotechnol* 3 (2010):544-563.

Dioniso L .P .C., Rosado E.G., Lopez-Cortes L., Castro D. and Borrego J.J. "Microbiological and sanitary quality of recreational sea waters of southern Portugal, water, Air and Soil pollution". *Environmental Pollution* 138 (2002): 319-334.

Duran, R., Ranchou-Peyruse M., Menuet V., Monperrus M., Bareille G., Gon M.S., Salvado J.C., Amouroux D., Guyoneaud R., Donard O.F.X., and Caumette P. "Mercury methylation by a microbial community from sediments of the Adour Estuary (Bay of Biscay, France)". *Environmental Pollution* 156 (2008): 951–958.

Eilers H., Pernthaler J., Glockner F.O., Amann R. "Culturability and *in situ* abundance of pelagic bacteria from the North Sea". *Appl Environ Microbiol*. 66 (2000):3044–3051.

FAO, The State of World Fisheries and Aquaculture 2006; Food and Agricultural Organization: Rome, Italy, 2007.

Fenical, W. and Jensen, P.R. "Marine microorganisms: Anew Biomedical resource". *In*: Attaway, D.H. and Zaborsky, O.R. *(Eds.), Marine Biotechnology, Vol. l. Pharmaceutical and bioactive natural products,* Plenum press, New York, 1993. 500.

Fuerst, J.A. "The planctomycetes: emerging models for microbial ecology, evolution and cell biology". *Microbiol* 141 (1995): 1493-1506.

Gauthier, M.J., and Flatau, G.N. "Antibacterial activity of marine violet-pigmented *Alteromonas* with special reference to production of brominated compounds". *Can J Microbiol.* 1976. 1612-1619.

Gieldreich E.E and Clerke N.A. "Bacterial pollution indicators in the intestinal tract of fresh water fish". *Appl. Microbial*. 14 (1996):429-437.

Gillan, D., Danis B., Pernet P., Joly G. and Dubois P. "Structure of sediment-Associated MicrobialCommunities along a Heavy-Metal Contamination Gradient in the Marine Environment". *Applied Environmental Microbiology* 71 (2005): 679–690.

Giovannoni, S. and Stingl, U. "The importance of culturing bacterioplankton in the 'omics' age". *Nat Rev Microbiol* 5(2007):820-826.

GOK state of the Coast Report: *Towards integrated management of Coastal and Marine Resources in Kenya*. NEMA, Nairobi, 2009. 88.

GOK: Integrated Coastal Zone Management Action for Kenya, 2011-2015. *Towards an integrated Kenya's coastal and marine resources*. NEMA, Nairobi, 2011. 90.

Gooday, G. W. "The ecology of chitin degradation". *Adv. Microbiol. Ecol.* 1 (1990): 387–430.

Gowen, R.J., Bradbury, N.B. "The ecological impact of salmonid farming in coastal waters-A review". *Oceanogr. Mar. Biol.* 25 (1987): 563–575.

Hall, P.O.J., Holby, O., Kollberg, S. & Samuelsson, M.O. "Chemical flux and mass balances in a mariculture fish cage farm. IV. Nitrogen". *Mar. Ecol. Pro. Ser.* 89 (1992): 81–91.

Han, J.B., Y.L. Mu and L.M. Wang. "Advances in research of marine aquaculture and coastal water pollution". *Fish Science* 18 (1999): 40–43.

Hewson, I., Jacobson M.M.E. and Fuhrman J.A. "Diversity and biogeography of bacterial assemblages in surface sediments across the San Pedro Basin, Southern California Borderlands". *Environmental Microbiology* 9(2007): 923– 933.

Holguin, G., Vazquez, P. and Bashan, Y. "The role of sediment microorganisms in the productivity, conservation, and rehabilitation of mangrove ecosystems: an overview". *Biol. Fertil. Soils* 33 (2001): 265–278.

Holmer, M., Kristensen, E. "Impact of marine fish cage farming on metabolism and sulfate reduction of underlying sediments". *Mar. Ecol. Prog. Ser.* 80 (1992): 191–201.

Holmer, M., Wildfish, D., Hargrave, B. "Organic enrichment from marine finfish Aquaculture and effects on sediment biogeochemical processes". *In* Hargrave, B.T .*Environmental Effects of Marine Finfish Aquaculture*, Ed.; Springer: Berlin, Germany, 2005. 181–206.

Huys, G., Denys, R. and Swings, J. "DNA:DNA re-association and phenotypic data indicate synonymy between *Aeromonas enteropelogenes* Schubert *et al*. 1990

and *Aeromonas trota,* Carnahan *et al*. 1991". *International Journal of Systematic and Evolutionary Microbiology* 52 (2002): 1969–1972.

Jensen, P.R. and Fenical, W. "Marine Microorganisms and Drug Discovery: Current Status and Future Potential". *In* Fusetani, N (ed). Basel: Karger *Drugs from the sea*, 2000. 6-29.

Johnson, R.C. "Introduction to spirocheates". *In*: Starr, M.P., Stolp, H., Truper, H.G., Balows, A. and Schlegel, H.G. *(Eds.), The Prokaryotes*, Vol. I, Springer-Verlag, Berlin, 1981. 582-591.

Jorgensen, B.B. "Mineralization of organic-matter in the sea bed–The role of sulfate reduction". *Nature*. 296 (1982): 643–645.

Kathiresan, K. and Bingham, B. L. "Biology of mangroves and mangrove ecosystems". *In* Southward, A. J. *et al. Advances in Marine Biology* eds Academic Press, UK, 2001. 81–251.

Kendall, M.G. "Rank correlation methods *(4th ed.)":* Griffin, London, 1970. 166.

Kirchman, D. L. and White, J. "Hydrolysis and mineralization of chitin in the Delaware Estuary". *Aquat. Microb. Ecol*. 18 (1999): 187–196.

Kuenen, J.G." Anammox bacteria: from discovery to application". *Nat Rev Microbiol* 6 (2008): 320-326.

Liti , D. M., Jonathan, M. M., Mirera, D. (2008) unpublished.

McCaig, A.E., Phillips, C.J,. Stephen, J.R, Kowalchuk G.A., Harvey S.M., Herbert, R.A., Embley, T.M., Prosser, J.I. Nitrogen cycling and community structure of proteobacterial beta-subgroup ammonia-oxidizing bacteria within polluted marine fish farm sediments. *Appl Environ Microbiol* 65*:* (1999) 213–220. PubMed, ChemPort, Web of Science®

McGhie, T.K., Crawford, C.M., Mitchell, I.M., O'Brien, D. The degradation of fish-cage waste in sediments during fallowing. *Aquaculture*187*:* (2000) 351–366.

Middelburg, J.J., Duarte, C.M., Gattuso, J.P. "Respiration in coastal benthic communities". *In* Del Giogio, P.A., Williams, P.J., *Respiration in Aquatic Ecosystems*; Eds., Oxford University Press: Chippenham, UK, 2005. 206–224.

Middelburg, J.J., Levin, L.A. "Coastal hypoxia and sediment biogeochemistry". *Biogeosciences* 6 (2009): 1273–1293.

Mirera, H. O. D. "In the Face of Poverty Mangrove Wetlands are Lifelines: Viability Indicators in Silvofishery Initiatives along the Kenyan Coast Assessing Polyculture of Milkfish (Chanos chanos) and Mullets (Mugil mugil)" in Obura, D.O., Tamelander, J., & Linden, O. (Eds) Ten years after bleaching - facing the consequences of climate change in the Indian Ocean.CORDIO Status Report 2008. *Coastal Oceans Research and Development in the Indian Ocean/Sida-SAREC. Mombasa. http//:www.cordioea.org* (2008): 420-422.

Mmochi, A. J., Mozes, N. Kite-Powell, H. L., Dubi, A. M., Gordin, H., Jiddawi, N., Kissil, G., Msuya, F. & Mwangamilo, J. "Design and preliminary results of an integrated mariculture pond system (IMPS) at Makoba, Zanzibar, Tanzania". *In:* Richmond, M.D. & Francis, J. (eds) *Marine Science Development in Tanzania and Eastern Africa*. Proceedings of the 20th Anniversary Conference on Advances in Marine Sciences in Tanzania, 28 June to 1 July 1999, Zanzibar, Tanzania. WIOMSA Book Series No. 1. WIOMSA, 2001. 431–450.

Morris, R.M., Rappe, M.S., Connon, S.A., Vergin, K.L., Siebold, W.A., Carlson, C.A., Giovannoni, S.J. "SAR11 clade dominates ocean surface bacterioplankton communities". *Nature* 420 (2002): 806-810.

Mulisch, M. "Chitin in protistian organisms: distribution, synthesis and deposition". *Eur. J. Protistol*. 29 (1993): 1–18.

Murray, L., Bulling, M., Mayor, D., Sanz-Lázaro, C., Paton, G., Killham, K., Solan, M. "Interactive effects of biodiversity, copper and a chemotherapeutant on marine benthic function". *In Proceedings of the World Conference on Marine Biodiversity (MARBEF)*, 11–15 November 2008, Valencia, Spain, 2008

Nair, S., and Simidu, U. "Distribution and Significance of Heterotrophic Marine Bacteria with Antibacterial Activity". *Appl Environ Microbiol* 53 (1987): 2957-2962.

Neori, A., Krom, M.D., Cohen, I. and Gordin, H. "Water quality conditions and particulate chlorophyll *a* of new intensive seawater fishponds in Eilat, Israel: daily and diel variations". *Aquaculture* 80 (1989): 63–78.

Primavera, J.H. A critical review of shrimp pond culture in the Philippines. Rev. Fish. Sci.. 1: (2000): 151-201.

Purushothaman, A. "Microbial diversity". *In* Swaminathan M. S. *Proceedings of the Technical Workshop on Biodiversity of Gulf of Mannar Marine Biosphere Reserve*, Research Foundation, Chennai, 1998. 86–91.

Reice SR." Non equilibrium determinants of biological community structure". *AM sci* 82 (1994): 424-435.

Rheinheimer, G. In" *Aquatic Microbiology"*, John Wiley, New York, 1980. 235.

Richmond, D.M. (Ed.) *A guide to the sea shores of eastern African and the western Indian Ocean islands*. Sida Department for Research Cooperation/ SAREC, 1997. 448.

Roszak, D.B and Colwell, R.R. "Survival strategies of bacteria in the natural environments". *Microbiol. Rev*. 51 (1987): 365-379).

Sanz-Lazaro, C., Marin, A. "Assessment of finfish aquaculture impact on the benthic communities in the Mediterranean Sea". *In* Russo, R., *Aquaculture I. Dynamic Biochemistry*, Process Biotechnology and Molecular Biology 2; Ed.; Global Science Books: Ikenobe, Japan, 2008. 21–32.

Sanz-Lazaro, C., Navarrete-Mier, F., Marin, A. "Biofilm responses to marine fish farm wastes". *Env. Pollut.* 159 (2011): 825–832.

Sherr, E. B. and Sherr, B. F. "Temporal offset in oceanic production and respiration process implied by seasonal changes in atmospheric oxygen: The role of heterotrophic microbes". *Aquat. Microb. Ecol*. 1 (1996): 91–100.

Snieszko, S.F. "Progress in fish pathology in the century". *Symposium of the zoological society of London* 30 (1986): 1-14.

Staley, J.T., and Konopka, A. "Measurements of in situ activities of non photosynthetic microorganisms in aquatic and terrestrial habitats". *Annu. Rev Microbiol.* 39 (1985): 321-334.

Stankiewicz, B. A., Briggs, D. E. G., Evershed, R. P., Flannery, M. B. and Wuttke, M. "Preservation of chitin in 25-million-yearold fossils". *Science.* 276 (1997): 1541–1543.

Thiyagarajan, V., Soo L., Shin P.K.S. and Qian P.Y. "Spatio-temporal variation in sediment biochemistry alters larval habitat selection and juvenile performance in the polychaete *Capitella* sp". *Marine Ecology Progress Series* 327 (2006): 207–222.

Torsvike V, Sorheim R and Goksoyr J "Total bacterial diversity in soil and sediment communities - *a review". J Ind Microbiol* 17 (1996): 170–178.

Urakawa, H., Tsukamoto K.K. and Ohwada K. "Microbial diversity in marine sediment from Sagami Bay and Tokoyo Bay, Japan, as determined by 16S rRNA gene analysis". *Microbiology* 145 (1999): 3305–3315.

Wietz M, Mansson M, Gotfredsen C.H, Larsen T.O. "Gram L Antibacterial compounds from marine *Vibrionaceae* isolated on a global expedition". *Mar Drugs* 8 (2010a): 2946-2960.

Wietz, M., Gram, L., Jørgensen, B., Schramm, A." Latitudinal patterns in the abundance of major marine bacterioplankton groups". *Aquat Microb Ecol* 61 (2010b): 179-189.

Woese, C.R and Fox, G.E. "Phylogenetic structure of the prokatyotic domain. The primary kingdoms". *Proc. Natl. Acd. Sci.,* USA, 74 (1977): 5088.

Wu, M., Song L.S., Ren J., Kan J. and Qian P.Y. "Assessment of microbial dynamics in the Pearl River Estuary by 16S rRNA m terminal restriction fragment analysis". *Continental Self Research* 24 (2004): 1925–1934.

Yoza, B.A., Harada R.M., Nihous G.C., Li Q.X and Masutani S.M. "Impact of mariculture on microbial diversity in sediments near open ocean farming of *Polydactylus sexfilis". Ecological Indicators* 7 (2007): 108–122.

Zhang, W., Ki J.S., Qian P.Y. "Microbial diversity in polluted harbor sediments: I. Bacterial community assessment based on four clone libraries of 16s rDNA". *Estuarine Coastal and Shelf Science* 76 (2008): 668–681.

Zobell, C.E and Upham, H.C. "A list of marine bacteria including sixty new species". *Bull. Seripps Inst. Oceanogr.* 5 (1944): 239-292.

Zubkov M.V, Fuchs B.M, Tarran G.A, Burkill P.H, Amann R. Mesoscale distribution of dominant bacterioplankton groups in the northern North Sea in early summer. *Aquatic Microb. Ecol* 29 (2002):135–144.

APPENDICES

Appendix I: General acceptable levels of fecal and total Coliforms set by Hongkong, Kenya, Malaysia, Norway, Philippines and the United States.

Country	Faecal coliform		Total coliform	
	Freshwater (count per 100 ml)	**Marine (count per 100 ml)**	**Freshwater (count per 100 ml)**	**Marine (count per 100 ml)**
Hongkong				610
Kenya			30	30
Malaysia	10-100*		100-5,000*	
Norway				100
				70
Philippines		Nil (MPN)	5,000 (MPN)	(MPN)
United States			< 10,000(CFU)	NA

MPN: Most Probable Number

* : Geometric mean

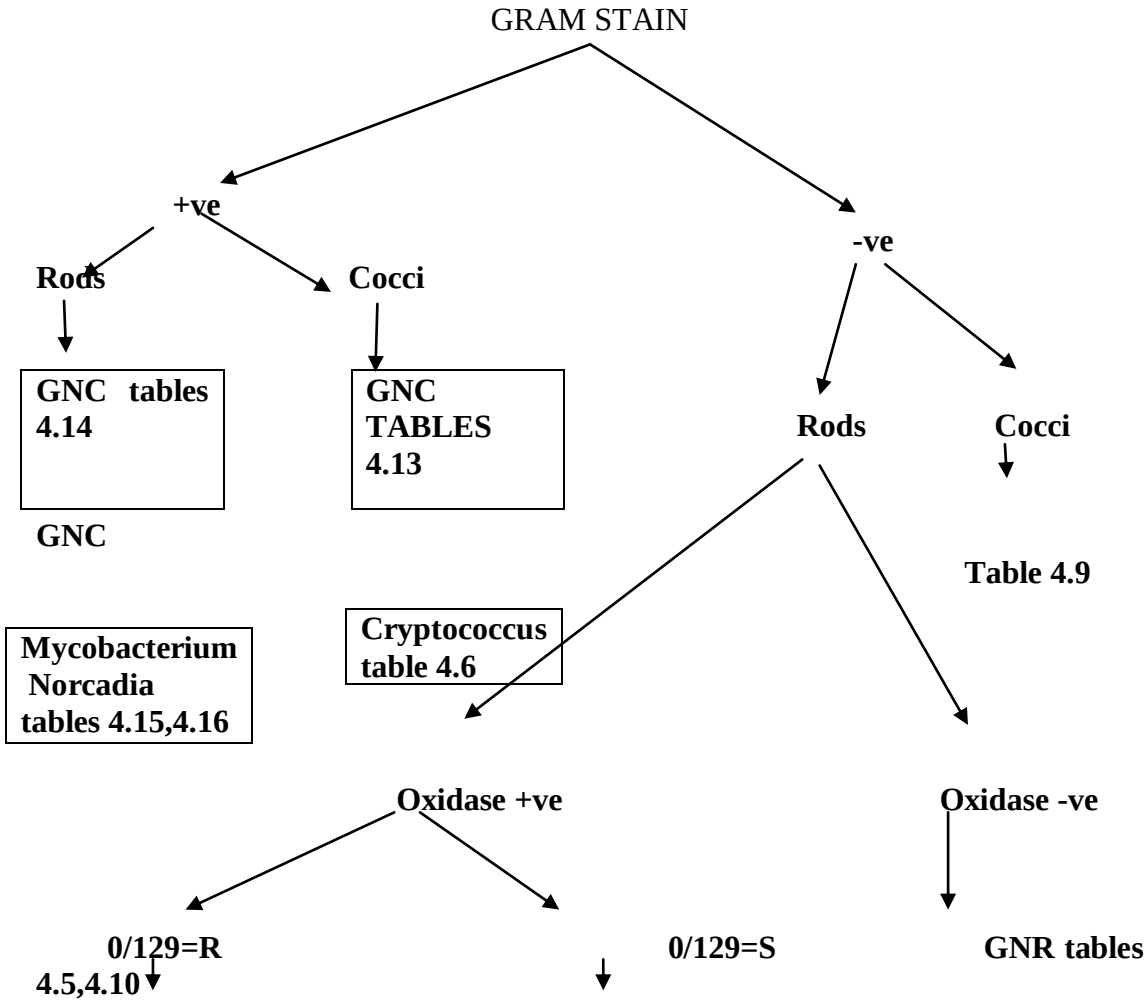
Nil: extremely low concentration, not detected by instrument used

CFU: colony-forming unit

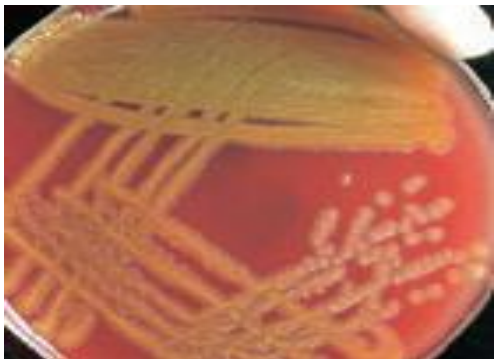
(EPD; EMCR, 2006; SFT; DAO, 1990- 34)

Among the countries, only Malaysia and the Philippines have set a standard exclusively for the presence of fecal coliform, while the rest of the countries set the standard for total coliform. Among these countries, Kenya has the most stringent requirement, i.e. 30 counts per 100 ml for freshwater and marine, followed by the Philippines and Hongkong. The Philippines has a lower required level in the marine waters than in freshwater.

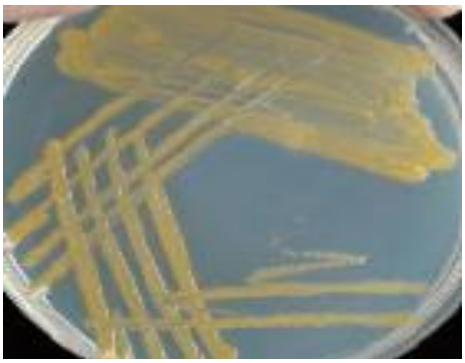
Appendix II: Figure showing Schematic for using the biochemical identification tables.



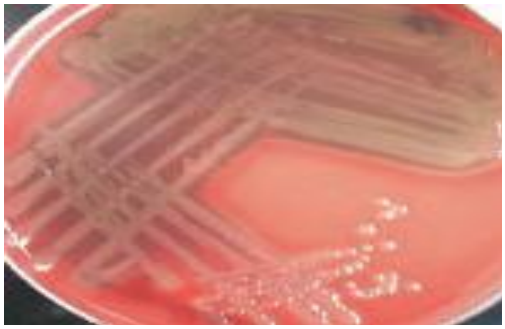
Appendix III: plates showing Photographs of bacteria on various Culture media



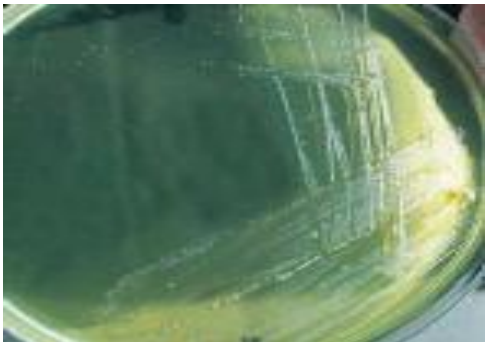
Flavobacterium on BA



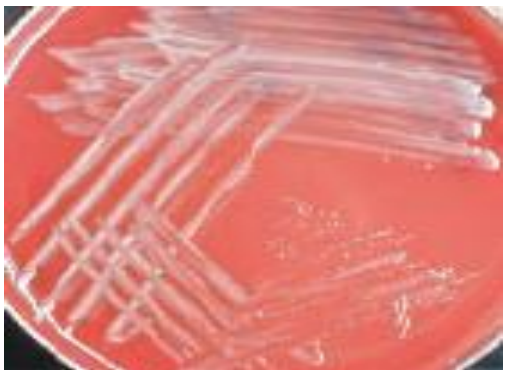
Flavobacterium on NA



Listonella on BA



Listonella on TCBS



Photobacterium on BA

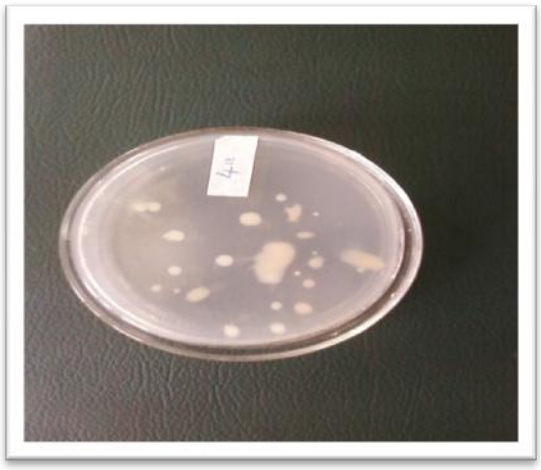
Pseudomonas on BA

Streptococcus on BA

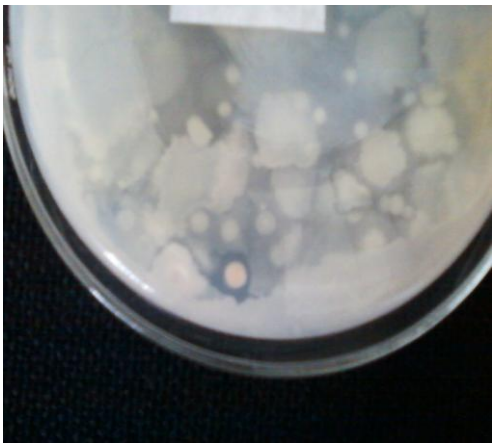
Photobacterium on BA

Vibrio on TCBS

Vibrio on BA



Eschirichia coli on NA



Vibrio inhibiting other bacteria on NA

Actinobacteria inhibiting other bacteria on NA

Indole reaction.
Negative on the left and
Positive on the right.

Citrate test, citrate-positive
(blue), citrate-negative (green)

Methyl red Red reaction.
+ve

Carbohydrate fermentation
Yellow Sucrose positive and
-ve, red .

Appendix IV: Cultural morphological and Biochemical identification of Bacterial Isolates

Bacterial group	**Bacterium**	**Gram reaction**	**Cell morphology**	**βH**	**TCBS**	**Colony characteristics**	**Catalase**	**Oxidase**
Proteobacteria (Gram negative Oxidase positive rods)	Vibrionaceae -Vibrio	Neg	Slightly curved or straight rods	w βH	G	Colonies round smooth 2-3mm greenish grey on TCBS	+	+
	-Enterovibrio	Neg	Cells 0.8×1.0-1.2µm		G	On NA 0.5-2%NaCl colonies are beige, smooth, round, raised at 48h at 28^{o}c	+	+
	-Salinivibrio	Neg	Curved rods 0.5×1.5-3µm	βH		Colonies circular, convex or cream colored in 2 days at 37^{0} c. NA 0.5-20%NaCl.	+	+
	-Listonella	Neg	Short rods, curved or straight, rounded ends, occurring singly and in pairs, pleomorphic 0.5-0.7×1-2µm. rapid motility	βH	Y	At 2 days, colonies are 2mm, glistering cream-colored in young colonies on BA. on NA, colonies are off-white to buff colored, translucent or opaque, circular, shiny 1-2mm.	+	+
	-photobacterium	Neg	Rods 0.5×1-1.5µm. singly or in pairs, rounded ends, straight or curved.	βH, V	NG or G	White colonies on 0.5-2%NaCl, BA	+	+
	Roseobacter	Neg	Rods 0.25×1µm			Growth on 0.5-2%NaCl, 1mm round, non mucoid at 5 days.	w+	+

						Appearance of pink pigment at 7days.		
	Chromobacterium	Neg		NH or βH		Pigmented strains are deep purple, round, slightly raised on BA. Non-pigmented strains show β-haemolysis on BA	+	+
	Actinobacillus	Neg	Pleomorphic rods	-w	N G	Colonies 0.5-2%NaCl 0.75-2mm at 24h, round, smooth, grey.	-	+
	pseudomonas	Neg	rods			Growth on BA, light grey colonies 1.5mm 24h at 25^0 c 3-5 at 48-72h	+	+
	pseudoalteromonas	Neg	Rods 2-4×0.5-0.7µm			On 0.5-2%NaCl, colonies have pink to red pigment.	-	+
	Colwelia	Neg	Curved rods, 0.6-1×2-4µm			0.5-2%NaCl, optimal growth at 15^oc	+	+
Actinobacteria (Gram positive rods)	Actinomyces	pos	Straight or slightly curved rods, some branching.	-		On BA, colonies are 0.5mm, grey, entire and convex at 48h. growth at 37^oc in air with 5% CO_2	-	
	Norcadia	Pos(w)	Coccoid to oval cells, long slender multiseptate rods, branching 5-50µm	N	N G	Less than 1mm, white, cream, rough or with very short dense mycelium. Growth 3-7 days. BA and 0.5-2%NaCl	V, +	
	Rhodococcus	pos	Rods slightly club – shaped, 2-3×0.6µm			Growth on BA in 3-4 days at 25^0 c. No growth at 37^0 c at 8 days colonies slightly domed, round, smooth, dry, deep creamy yellow.		
	Arthrobacter	pos	Cocci in pairs and tetrads 0.8-1.2µm	NH		Colonies on Agar are smooth, matt, entire, with a rose red	+	+

			diameter			pigment that is water insoluble		
	Camobacterium	pos	Rods 0.2×0.5-1.2µm		N G	1-2mm off-white colonies on BA	-	-
	Corynebacterium	pos	Coryneform rods	NH		Shiny, round colonies 1mm on BA at 24h at 28^{o}c	+	
	Clostridium	Pos	3.4–7.5 × 0.7 µm oval, subterminal spores	βH		Semi-opaque to translucent, matt colony, 1–3 mm, irregular lobate margin and raised centre	-	
	Bacillus	Pos	Rods in chains			Rhizoid colonies with counterclockwise filamentous swirling pattern on BA	+	
Cytophaga-Flavobacteriaceae-bacteriodetes group (C-F-B grp) - Gram negative rods)	Aquorivita	Neg	Rods 0.5-20×0.2-0.3µm			On 0.5-2%NaCl, yellow orange colonies, compact, circular, convex with an entire edge, non spreading butyrous consistency.		
	Cryseobacterium	Neg	Non-sporing rods with rounded ends	NH	N G	On 0.5-2%NaCl circular, entire, viscid become mucoid and transparent after 5 days. Bright yellow pigment	+	+
	Flavobacterium	Neg	Long thin rods(4-8µm) filamentous	NH	N G	Bright yellow, flat, dry, rhizoid, slow spreading growth 5days at 20-25^{o}c adheres strongly to Agar.	V	+
	Flexibacter	Neg	Filaments multicellular. At end			0.5-2%NaCl, cobalamine required for growth. Peach colored		

			of each cell is a refractile granule of lipid material			pigment.		
	Pedobacter	Neg	Non-sporing rods, 0.4–0.5 × 0.5–1.0 µm	βH		Slow Growth on 0.5-2%NaCl, 1–3 mm at 48 h, circular, low convex, smooth, opaque. A yellow or creamy white non-fluorescent pigment is produced.	+	+
Gram Negative coccobacilli	Acinetobacter	Neg	Predominantly diplococcal forms 1.0×0.7µm from plate growth	-		Colonies circular, smooth, translucent to slightly opaque, butyrous to mucoid non-pigmented. 0.5-2mm at 24h at 30^{o}c.	+	
Gram negative oxidase negative rods	Hallomonas	Neg	Rods single, pairs or chains			On 0.5-2%NaCl, colonies are smooth, glistering, translucent white, convex, 1-2mm in 24h at20-30^{o}c		-
Gram positive cocci	Staphylococcus	pos	Cocci singly, pairs and clusters	βH		Colonies 1.5-2mm, smooth, round 24h at 37^{0} c	+	-
	Lactococcus	pos	coccobacilli				-	-
	streptococcus	pos	Cocci In small chains	βH		Growth on BA. Colonies 1mm, pale grey at 24h. zone of βH		
	E. coli	Neg						