ECOPHYSIOLOGY OF NONTUBERCULOUS MYCOBACTERIA IN MARINE AQUACULTURE PONDS

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By

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I C A R

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JANUARY 2004

to

the lotus feet of

Sree Vadakkunnathan

DECLARATION

I hereby declare that this thesis entitled **"Ecophysiology of Nontuberculous Mycobacteria in marine aquaculture ponds"** is a record of original and bonafide research carried out by me under the supervision and guidance of **Dr.V.Chandrika**, Principal Scientist, Central Marine Fisheries Research Institute, Kochi-14 and that no part there of has been presented before for any other degree in any university.

Kochi-14

(Latha.M.M)

CERTIFICATE

This is to certify that the thesis entitled **"ECOPHYSIOLOGY OF NONTUBERCULOUS MYCOBACTERIA IN MARINE AQUACULTURE PONDS"** embodies the research of original work conducted by M.M.Latha(Reg. No. 2031) under my guidance and supervision. I further certify that no part of this thesis has previously formed the base of the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.

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Introduction

I INTRODUCTION

The world demand for fish and fishery products is increasing steadily and it is generally accepted that it will not be possible to meet the heavy demand with resources exploited from capture fishery alone. Now aquaculture is well established and fast-developing industry in many countries and is a major focus sector for development. During recent decades, aquaculture has gained momentum, throughout the world especially in developing countries. According to Food and Agricultural Oganisation (FAO, 2000), global aquaculture production was 26.38 tones in 1996 have reached 32.9 million tonnes during 1999. Only marine aquaculture sector has contributed 13.1 million tonnes during 1999.

India is a major fish producing country. About one half of India's brackish water lands are currently being utilized for farming in order to reduce the gap between supply and demand for fish. Aquaculture has become a major source of livelihood for people and its role in integrated rural development, generation of employment and earning foreign exchange, thereby alleviating poverty is being greatly appreciated around the world.

Among the infectious agents, bacteria are becoming the prime causal organisms for diseases in food fishes and other marine animals. Sindermann, (1970) reported that bacterial fish pathogen most commonly found among marine fishes is species of *Pseudomonas, Vibrio* and *Mycobacterium*. These can be categorized into primary pathogens; secondary invaders that may cause systemic disease in immunocompromised hosts; and normal marine flora which are not pathogenic but may occur on body surfaces or even within the tissues of the host. High density of animals in hatchery tanks and ponds is conducive to the spread of pathogen and the aquatic environment with regular application of protein rich feed, is ideal for culturing bacteria. Bacteria, which are normally present in seawater or on the surface of fish, can invade and cause pathological effects in fishes, which are injured or subjected to other environmental stresses.

Mycobacteria except parasites are known as nontuberculosis mycobacteria (NTM), atypical mycobacteria or mycobacteria other than tuberculosis(MOTT). This group of mycobacteria includes opportunistic pathogens and saprophytes. Environmental mycobacteria are ubiquitous in distribution and the sources may include soil, water, warm-blooded as well as cold-blooded animals. Disease caused by environmental mycobacterial strains in susceptible humans (Goslee & Wolinsky, 1976; Grange, 1987), animals and fishes are increasingly attracting attention. Greatest importance of environmental mycobacteria is believed to be their role in immunological priming of humans and animals, thereby modifying their immune responses to subsequent exposure to pathogenic species.

Nontuberculosis mycobacteria are causing slow, chronic and progressive disease in fishes known as Mycobacteriosis or Fish TB and this is one of the few fish diseases which is communicable to humans. This is a common infection of fishes under captivity whether in aquarium or cultured condition and its prevalence may as high as 15% in some fishes (Parisot and Wood, 1970). Infected fish exhibits clinical sign including loss of scales, depigmentation, hyperpigmentation, cachexia, abnormal behaviour such as remaining alone in one corner of the aquarium and lethargy. They may also display slightly concave ventral surface as sequel to lachexia (Noga *et al.*, 1990).

According to Strunjak-Perovic *et al.*, (1995), it is an important bacterial disease in controlled condition including aquaria. Importance of the disease is increasing on considering the fact that this is prevalent in marine, brackish and fresh water environments both in tropical and temperate waters. About 167 species of both fresh water and marine fishes are the hosts for this bacterium. Aronson (1926), Nigrelli & Vogel, (1968), Dulin, (1979), Giavenni *et al.*, (1980) etc. reported this from aquarium fishes and it is clear that fishes maintained in aquaria will show higher incidence of disease than cultured or wild species, due to rather confined or unchanged environment. The incidence of the disease in aquarium environment is varying from 10 to 22% (Santacana *et al.*, 1982). Disease has been reported in food fishes (Chinabut *et al.*, 1990; Hatai *et al.*, 1993), fresh water prawn (Brook *et al.*, 1986) and is widespread in all environments(Lightner, 1996). Mycobacteriosis was widely documented in wild fish stocks (Sutherland, 1992; Mackenzie, 1986) and in intensive fish culturing ponds

(Hatai *et al.*, 1993; Lawhavinit *et al.*, 1988). In wild fishes, the incidence is varying from 10 to 100%. Epizootic was reported by Kusuda *et al.*,(1987) in yellowtail (*Seriola quinquiradiata)* and these sometimes may upset balance of the economy. Kamala *et al.*,(1994), Donoghue *et al.*,(1997) studied about the ecology of environmental mycobacteria.

Knibb *et al.*, (1993) evaluated PCR as a diagnostic tool for mycobacteriosis. Telenti *et al.*, (1993) and Talaat *et al.*, (1997) reported rapid identification method for mycobacteria infecting fish up to the species level by PCR and restriction enzyme analysis. Slowly glowing *Mycobacterium spp.* including *Mycobacterium tuberculosis* are differentiated through gene amplification and Restriction Fragment Length Polymosphism analysis by Ptikaytis *et al.*, (1994). PCR assay based on DNA coding for 1658 RNA for detection and identification of mycobacteria in clinical samples was given by Kox *et al.*, (1995). Belas, (1995) reviewed the potential application of molecular biology to the study of fish mycobacteriosis. Tag *et al.*, (1998) identified similarity of two species of mycobacteria from 1658 RNA sequences. For the identification of *M. chelonei*, Bruno *et al.*, (1998) adopted PCR technique. Thoral *et al.*, (1998) identified *M. bovis* based on DNA fingerprinting system.

The host range of environmental mycobacteria is wide and this includes leopard frog (*Rana pipiens)* (Ramakrishnan *et al.*, 1997), South American wild seal (Romano *et al.*, 1995), Australian fur seal (Woods *et al*, 1995); captive wild fowl (Cromie *et al.*, 1991) etc. Griffith, (1930), Vogel, (1958) and Parisot (1958) reviewed mycobacterial infection in fishes, amphibian and reptiles.

Piscine mycobacteriosis caused by *M. marinum, M. fortuitum* and *M. chelonei* (Nigrelli and Vogel, 1963; Ashburner, 1977; Hedrick *et al.*, 1987; Humphrey *et al.*, 1987; Daoust *et al.*, 1989; Bragg *et al.*, 1990; Shamsudin *et al.*, 1990) has been well documented. These species have been isolated from different samples of water, sediment cultured fishes as well as from external skin ulcers in human. This ulcerative disease is confronting as a threat for fish hobbyists, seal trainers and hatchery operators is known as Fish tank granuloma, Swimming pool granuloma or Fish fancier's finger (Lawler, 1994; Thompson *et al.*, 1993; Little John and Dixon, 1994; Popp and Reichenbach-Klinke, 1982). This transmission, when it takes place can

spread and is very difficult or impossible to eradicate. The hobbyist will get disfigurements in life when it is not aware of the danger and if not treated in time. Nowadays, from African countries, many cases of chronic ulcerative infection called Buruli ulcer has been reported, caused by *M. ulcerans* and the causal organism is not yet been isolated.

Different species of environmental mycobacteria are becoming problem for patients suffering from AIDS causing nasocomial infections making the condition still worse. Reports of this kind are increasing from different hospitals worldwide and most of them are post-surgical in incidence. *M. fortuitum* complex has been reported to cause diffuse pulmonary disease in human (Nigrelli & Vogel, 1963). Persistent colonization in potable waters and hot water taps as well as capability of mycobacteria for forming strong bio film are drawing attention of mycobacteriologists into this area.

Now traditional means of aquaculture has been changed to achieve high yields, by adopting intensive and semi-intensive methods. Water quality deterioration caused by the application of artificial feed in excess of the requirement and the consequent negative impacts within the farms will enhance the proliferation of microbes. Pool management results in abnormal stress and reduction in normal resistance of the host. More sensitive and stressed condition of the host will make them prone to the attack of opportunistic pathogen like mycobacteria.

As an integral part of natural microbial flora, mycobacteria play a major role in pond ecosystem influencing the pH, Eh, carbonate content, oxygen tension, production of organic compounds and thereby stands at the base of the fertility of the aquaculture ponds for connecting the abiotic factors with biotic factors. Saprophytic mycobacteria are capable enough to bio-transform complex molecules like nitropyrene, fluranthene, phenanthrine, polyaromatic hydrocarbons and ground water pollutant mixtures.

Objectives of the Present Study

1. The occurrence and distribution of different species of mycobacteria and also their correlation with respect to environmental variables has been studied for the period of one year from March 1999 to february 2000.

2. Quantitative and qualitative analysis of environmental mycobacteria from brackish aquaculture ponds by adopting standard procedures suggested by clinical mycobacteriology laboratories . An attempt has also been made to find out species-wise seasonal distribution of environmental mycobacteria from different samples of water, sediment and cultured tilapia, *Oreochromis mossambicus*.

3. To find out the best medium for mycobacterial retrieval among the three media used.

4. To identify all the mycobacterial isolates obtained in pure cultures by morphological and biochemical characterisation and to calculate the percentage of occurrence among total bacteria.

5. The phylogenetic relationship among different strains was assessed by RAPD – PCR technique.

6. The influence of different environmental variables on the occurrence and distribution of NTM, species diversity of NTM and heterotrophic bacteria were elucidated statistically.

Studies on ecophysiology of environmental mycobacteria are scarce and the present study is to find out which ecological factor is enhancing the occurrence of mycobacteria at species level in brackish aquaculture ponds. This information will help the aquaculturist to avoid the incidence of mycobacteriosis by controlling environmental factors in stressed cultural conditions and thus controlling of mycobacterial proliferation. The study was also meant to understand the phylogenetic relatioship and thereby to lead a way for the development of species and strain specific markers for diffferent *Mycobacteria* Spp.

Review of literature

II REVIEW OF LITERATURE

Members of the family mycobacteriaceae can be isolated from freshwater, estuarine and marine environment as well as from the intestine of warm blooded animals. Somw species are pathogenic to aquatic animals while some species comprise the ectocommensal flora of finfish, shellfish, frogs, seals etc. and some others participate in rtecycling organic matter(Aronson, 1926).

Isolation of mycobacteria from wild fishes

The presence acid-fast bacteria have been observed by all the investigators who have described the cases of piscine tuberculosis in different fish genera. *M.marinum, M.fortuitum* and *M.chelonei* are the most common among the bacterial species described in connection with mycobacteriosis in fish. Bataillon *et al.,*(1897) were the first to isolate *Mycobacterium sp.* from pond reared carp. Besse (1949) was the first to isolate *M.anbanti* which is more identical with *M.marinum* from infected paradise fish, *Macropodus opercularis.*

In California, Hedrick, MCDowell and Groff (1987) isolated *M.marinum* from striped bass. *M.fortuitum* was isolated from tropical fish, *Hyphessobrycon innesi* by Ross and Brancato (1959) and Beckwith and Malsberger (1980). A new species *M. salmoniphilum* isolated from infected salmonid fishes has been attributed as the causal organism by Ross (1960). *M.chelonei subsp. piscarium* was isolated from five locations in the states of Oregon and Montana by Arakawa and Fryer (1984).

From two lakes in Alberta, *M.chelonei* was isolated from naturally infected yellow perch, *Perca flavescens* by Daoust *et al.,*(1989). Buck (1980) gave a note on acid-alcohol-fast bacteria in mackerel, *Scomber scombrus.*

Murichilano et.al (1986) histopathologically evaluated the gross lesions excised from North Atlantic marine fishes. As it is difficult to characterise the isolated strain up to the species level, the Mycobacterium.sp. has been said to be isolated from yellow tails (Kusuda *et al.,* 1987); naturally infected cod from Danish coastal waters (Dalsgaard *et*

al., 1992); wild caught salmons (Dixon *et al.*,1992); and from European sea bass (Colorni, 1992; Knibb *et al.*,1993). In Japan Hatai *et al.*, (1993) isolated a photochromogenic species of mycobacterium from pejerrey, *Odonthestes bonariensis* with or without saprolegniasis.

Mycobacteria has been isolated from both healthy and infected fishes. It has been isolated successfully from snake heads (Bozzitta *et al.*,1995; Tortoli *et al.*, 1996; Adams *et al.*, 1992) and Siamese fighting fish (Pungkachonboon *et al.*, 1990, 1992; Bozzitta *et al.*, 1995; Adams *et al.*, 1997). Bozzitta *et al.*, (1995) was the first to report *M.gordonae* as the causative agent of fish tuberculosis. Through biochemical methods they identified *M.fortuitum* subsp. *acetamydolyticum* and *M.marinum*.

Mycobacteria from cultured fishes

Workers have isolated mycobacteria from cultured fishes of different genera at any stage during their culture in healthy or in diseased condition.It is reported that from hatchery confined Chinook salmon, *Oncorhynchus tshawytcha* (Ashburner 1977) and from different species of captive fishes (Colorni *et al.*, 1996) *M.marinum* has been isolated successfully. Buckman *et al.*, (1990) noted an outbreak of panophthalmitis in the same species and and the causal organism was either *Mycobacterium.sp.* or *Rhodococcus sp.* In a guppy farm in South Africa, heavy mortalities have been reported for the first time by Bragg *et al.*, (1990) and isolated Mycobacterium.sp. from guppies and Oscars (McCormick *et al.*, 1995).

The etiology of diseased black bream, sampled from marine net cages was explained as *Mycobacterium sp.* through histopathological examination by Zhang-Jonngjea (1991). Smith (1996) gave a detailed report about the causal agents of an outbreak of disease in cultured food fishes. From nodular lesions of cultured snakehead, *M.poriferae* has been isolated by Tortoli *et al.*, (1996). Mbuthia *et al.*, (1996) examined histopathologically and proved the presence of mycobacteria in four different genera like, *Symphysodon sp.*, *Hyphessobrycon innesi*, *Ctenopharyngodon idella* and *Carassius carassius*.

Teska *et al.*,(1997) isolated *M.abscessus* from infected cultured Japanese medaka, *Oryzias latipes* and the mean bacterial count ranged from 6.7X102 to 4.5X108

CFU/gm of the fish. *M.chelonae* has been isolated from two Atlantic salmon rearing farms by Bruno *et al.*, (1998).

Mycobacteria in aquarium fishes

Aronson (1926) was the first to describe the well-established species, *M.marinum* and to isolate it from tropical coral fishes in the Philadelphia aquarium. Giavenni (1982) identified the same species from 41 different species of fishes. Bernstad (1974) isolated *M.borstelense* from infected aquarium fishes. It is reported by Giavenni et.al (1980) that 97 marine topical fishes of the 17 genera were affected in the marine aquarium and isolated mycobacteria from the internal organs of the affected fishes. The factor for the death of 35% of the three spot gouramies , imported from an ornamental fish farm in Columbia was identified as AFB by Santacana e *et al.*, (1982).

Shamsudin *et al.*, (1990) tested several ornamental fishes like gold fish and red eyed tetra, *Moenkauisia sanctaefilomina* for the presence of AFB and isolated the same (Anderson *et al.*, 1987; Dixon *et al.*, 1992; Landsell *et al.*, 1993). Dailloux *et al.*, (1992) reported that *M.marinum, M.kansasii* and *M.fortuitum* are the most frequently found species in the aquaria.

In Thailand acid-fast bacterial isolation from ornamental fishes was isolated by Chen, Adams and Richards (1997) Dixon *et al.*, (1992) used profiles of biochemical growth characteristics to identify the Mycobacterium sp. From salmons and aquarium fishes up to species level.

Isolation of mycobacteria from crustaceans

Mycobacterial infection and its isolation was reported in prawns and crayfishes. Lightner and Redman (1986) reported such a case in white shrimp, *Penaeus vannamei* and from the same species, Mohney *et al.*, (1998) isolated *M.peregrinum*. Brock *et al.*, (1986) and Lightner (1996) isolated *Mycobacterium sp.* Runyon Group II from infected cultured fresh water prawn, *Macrobrachium rosenbergii*. Owens *et al.*, (1992) studied about the pathology of microbial diseases in tropical Australean crustacea and reported the presence of granulomatous lesions in different internal orgns of dying *M.rosenbergii*.

Mycobacterial species has been included as one of the potential bacterilal pathogens of crayfishes, *Austropotamobius pallipes* and the infection was reported by Anderson, Feist and Polydase (1986). Thune (1994) isolated the causal agent from hind gut, haemolymph and exoskeleton of crayfishes.

Occurrence of fish mycobacteriosis

Fish tuberculosis (Fish mycobacteriosis) is an infectious disease caused by organisms of the genus Mycobacterium, resulting usually in the formation of tubercles in various organs. The term Mycobacteriosis was coined by Parisot and Wood, (1960). The disease was reported in carp, *Cyprinus carpio* for the first time (Bataillon *et al.*, 1963).

Parisot (1958) and Vogel (1958) has given reviews on the same disease. The review on mycobacteriosis among fishes was given extensively by Amlacher (1968). Johnstone (1913) and Alexander (1913) found a spontaneous skin infection with acid-fast bacteria in cod. In 1910, Von Betegh reported the disease in marine fish, subsequently it was observed that fishes of fresh, salt and brackish waters, aquarium and hatcheries are susceptible to the disease (Parisot and Wood, 1966).

Many acid-fast bacteria have been recorded as the aetiological agents causing mycobacteriosis in fish; in which *M.marinum*, *M.fortuitum* and *M.chelonei* are the important ones. *M.marinum* was isolated by Aronson (1926) from tropical coral fish in Philadelphia aquarium. It was found to be infecting both marine and freshwater fishes (Giavenni, 1980; Van Duijn, 1981). *M.fortuitum* was another acid-fast bacillus repeatedly found from diseased neon tetra, *Parocheirodon innesi* in 1953, although the taxonomic identification was later described by Ross and Brancato, (1959). *M.chelonei* (Arakawa and Fryer,1984) and its subspecies *M.chlonei* subsp. *chelonei* and *M.chelonei* subsp. *abscessus* also form a major group of causal agents.

Prevalance of mycobacteriosis may seen as high as 15% in some fishes.(Parisot and Wood, 1970) and as high as 100% under extensive culture conditions (Smith, 1996). Fryer and Rohovec, (1984) and Strunjak-Perovic *et al.*, (1995) reviewed the principal bacterial diseases among cultured marine fishes and included mycobacteriosis among them. Fish tuberculosis has been diagnosed both in

fresh water (Conroy, 1966; Majeed, Gopinath and Jolly, 1981; Bragg *et al.*, 1990; Chinabut *et al.*, 1990) and sea water fishes (Kusuda *et al.*,1987; McKenzie,1988). High percentages of infection have been found among fishes in their natural habitats (Hastings *et al.*, 1982).

Fish maintained in aquaria will show a higher incidence of this disease than cultured or wild species, as aquarium fishes are often kept for long periods of time under captivity compared with fish raised for commercial purposes. Aronson, (1926), Besse (1949), Nigrelli and Vogel !968), Giavenni *et al.*, (1980) have reported the disease in a variety of aquarium fishes. The incidence of mycobacteriosis in aquarium fish have been reported to vary from 10 to 22% (Wolke and Stroud, 1978; Santacana *et al.*,1982). The prevalence of infected fish in natural populations vary from 10 to 100% (Abernathy and Lund, 1978; Sakanari *et al.*,1983; Hedrick *et al.*, 1987; Lawhavinit *et al.*,1988; MacKenzie,1988). There appears to be no bias towards the sex of the fish in the prevalence of mycobacteriosis, but the severity of the infection is apparently related to age (Abernathy and Lund, 1978; MacKenzie, 1988).

The occurrence of mycobacteriosis resulted in heavy losses due to mass mortalities (Hedrick *et al.*,1987; Lawhavinit *et al.*, 1988; Bragg *et al.*, 1990). Epizootics have been reported in yellow perch by Kusuda *et al.*, (1987) and Daoust *et al.*, (1989) from two lakes in Alberta. 150 species of marine and fresh water fishes of more than 40 families are found to be infected (Nigrelli and Vogel, 1963).

The first report of the disease in the pond reared carp is by Bataillon *et al.*, (1897) and in cod is by Alexander (1913), Johnstone (1913), later by Dalsgaard *et al.*, (1990) from Danish coastal waters. Besse (1949) and Csaba (1982) recorded a massive outbreak of mycobacteriosis in paradise fish, *Macropodus opercularis*. In three spot gouramies, pearl gouramies and siamese fighting fish, the disease has been reported (Besse, 1952; Reichenbach-Klinke, 1954; Sato, 1962; Nigrelli and Vogel 1963; Conroy and Valdez 1964; and Conroy 1964, 1965).

Wood and Ordal (1958) described tuberculosis in pacific salmon,*Oncorhynchus sp.* and steel head trout, *Salmo gairdneiri*. Parisot and Wood (1960) made a comparative study of the disease among salmonids. Land and Abernathy (1978) reported tubercular lesions in mountain white fish. MacKenzie (1988) recorded presumptive

mycobacteriosis in Atlantic mackerel and a preliminary report on the same was given by Bucke (1980); Hastings *et al.*, (1982) and Marchalano *et al.*, (1986); Aronson (1926). Sakanari *et al.*, (1983) studied about mycobacteriosis in striped bass , *Morone saxatilis* from Cenatral Califiornia and Coosbay. In Eilat, a systemic infection was recorded by Colorni (1992) in the European sea bass, *Dicentrarchus labrax* and the prevalence of infection was 100% in the infected tanks.

In *Sebastes sp.* The description of the disease was given by Moser and Sakanari (1986) and in yellow perch by Kusuda *et al.*, (1987); Daoust *et al.*, (1989). Reports have also come on mycobacteriosis in gold fish and red eyed tetras (Anderson *et al.*, 1987; Shamsudin, 1990). Mycobacterial infection has been recorded in halibut, *Hippoglossus hippoglossus* by Sutherland, (1922); in pejerrey, *Odonthestes bonariensis* (Lawhavinit *et al.*, 1988; Hatai *et al.*, 1993) and in snake head, *Channa striatus* (Chinabut *et al.*, 1990).

Mycobacteriosis is recognised as the cause of mortality in marine and fresh water fishes (Dailloux *et al.*, 1992). Bruno *et al.*, (1988) identified mycobacteriosis as the cause of increasing mortality on two farms rearing Atlantic salmon, *Salmo salar* in the Shetland Isles, Scotland.

Among crustaceans, white shrimp, *Penaeus vannamei* (Lightner and Redman, 1986; Mohney *et al.*, 1998), *Macrobrachium rosenbergii* (Brock *et al.*, 1986; Lightner, 1996) are reported to have mycobacterial infections. Anderson, Feist and Polydare (1986) and Thune (1994) reported the disease in cray fishes, *Austropotamobius pallipes.*

Adams *et al.*, (1994, 1995) developed monoclonal antibody probes for rapid screening and immunodiagnosis of mycobacteriosis. Adams *et al.*, (1996) produced six antibodies against *M.marinum, M.fortuitum* and *M.chelonei* and studied their difference in magnitude of response on mycobacteria. A comparative study of histochemical methods to diagnose mycobacteriosis in swordtail using Avidin-biotin complex and polyclonal and monoclonal antibody was conducted by Gomez *et al.*, (1993) and Gomez *et al.*, (1996). Talaat *et al.*, (1998) developed gold fish, *Carassius auratus* as animal model for studying mycobacterial pathogenesis by injecting them intraperitoneally with doses between 10 super (2) and 10 super (9) CFU of *M.marinum* organisms.

Mycobacteria in biofilms

Schulze –Roebbecke and Fischeder (1989, 1990) were the pioneers to study about mycobacteria in bio films to elucidate their role in bio films as the habitat of aquatic mycobacteria, their growth and inactivation kinetics. They observed the presence of *M.kansasii* and *M.flavescens* in the biofilm of a water distribution system and yielded 2X105CFU/cm super (2) of *M.kansasii* and 7X104CFU/cm super (2) of *M.flavescens.*

Occurrence and distribution of mycobacteria in fresh water and marine environments

Mycobacteria are ubiquitous in nature due to their potentiality to survive in any environmental conditions. Water and sediment are said to be their important sources. They are widely distributed in fresh water, marine or even estuarine habitats both in surface water and shallow sediments.

The presence of mycobacteria has been successfully proved in esturine and oceanic waters (Gruft *et al.*,1979), in river water (Murranzano,1978), in lake water (Hou *et al.,* 1983), in fresh water(Falcao *et al.*,1993). Viallier and Viallier (1982) investigated the modification of mycobacterial flora by the operation of nuclear power plants. Joynson (1979) found the inoculated *M.kansasii* was surviving in water but not in soil and he proposed that water is the natural habitat of the species. Kirchner *et al.,* (1992) indicated the presence of mycobacteria from waters, aerosols and droplets ejected from water in acid and brown water swamps. The circulating hot water systems in hospitals (Von Reyn *et al.,* 1994) and water polluted with industrial and domestic residues (Cordoso and Filho, 1979) are not free from mycobacteria. Kamala *et al.,* (1994) sampled taps, wells and water coolers in different sites of a BCG trial area and strengthened their ubiquitous distribution pattern.

As sediment is very rich with organic and inorganic substances, it forms another important source of mycobacterium. Donnelly *et al.,* (1982) reported the presence of mycobacteria from landfill leachate which drains into the environment, indicating its health hazard. Estuarine sediment (Guerine Jones, 1988), soil from acid and brown water swamps (Kirchner *et al.,* 1992), soil samples (Kamala *et al.,* 1994; Katila e *et al.,*

1995), gravel of culture ponds (Owens *et al.*, 1992) are forming small habitats of the organism. Vanitha *et al.*,(2002) identified of a group of nontuberculous mycobacteria iso-lated from south Indian BCG trial area.

Fresh water and marine fishes in wild, cultured or aquarium conditions are in contact with mycobacteria as they are living in the body of the organisms, and in other important sources like sediment and water. The presence of mycobacteria has been reported from aquaria (Aronson, 1926; Bernstad, 1924; Giavenni, 1980, 1982; Santacana,1982; Shamsudin, 1990; Dixon *et al.*, 1992; Lansdell *et al.*, 1993) from wild fishes (Buck, 1980; Daoust *et al.*, 1989; Dalsgaard *et al.*, 1992; Dixon *et al.*, 1992; Colorni 1992; Knibb *et al.*, 1993; Hatai *et al.*, 1993; Bozzitta *et al.*, 1995; Tortoli *et al.*, 1996; Adams *et al.*, 1997) and from cultured fishes (Ashburner, 1977; Buckman 1990; Csaba *et al.*, 1982; Bragg *et al.*, 1990; McCormick *et al.*, 1995; Zhang Jongjea, 1991; Tortoli *et al.*, 1996). Schulze Roebbecke and Fischeder (1989, 1990) reported the presence and importance in biofilms also. Shrimps (Lightner and Redman 1986, Mohney *et al.*, 1998), crayfishes (Anderson *et al.*, 1986; Thune 1994) and even seaweeds (Kazda *et al.*,1990) are reported to be inhabited by the organism.

Mycobacteria from estuaries and ocean waters

Few attempts have been made to recover mycobacteria from sea water as they already have been isolated from marine animals. Apart from those Viallier (1967) and Viallier and Viallier (1973, 1975, 1977) who had examined 791 samples taken off the coast of France and found mycobacteria in 176 of them. They report all strains except MAIS complex, *M.kansasii* and *M.xenopii*. During their study in 1973, they reported the isolation of *M.marinum*.

Falkinham *et al.*,1978, 1981) recovered MAIS bacilli from coastal waters of the southeastern part of the United States. Gruft *et al.*,1979) tested 38 subsurface and microlayer samples of eatuaries and ocean waters, 16 of which yielded a total of 30 strains. 19 strains were MAIS organisms. *M.gordonae* and *M.terre* were also present and they postulated the sources of *M.intracellulare* and *M.scrophulaceum*.

In United States, Gruft *et al.*, (1981) examined 520 samples from a larger area of the southeastern seaboard and found MAIS bacilli in 128 of them, including

M.gordonae and *M.terre* in191 samples. In New Zealand, Kazda *et al.*, (1990) identified slow growing scotochromogenic mycobacteria from surface waters and sphagnum vegetation. The organism was identified as *M.cookii* sp.nov. From northern Brook waters, mycobacterial strains were isolated by Iivanainen *et al.*, (1997).

Mycobacteria in piped, polluted and treated waters

Report on the presence of acid-fast organisms in slime and scrapings from inner side of water pipes was given by Brem (1909) and Bertzke (1910). Kubica *et al.*, (1961, 1963) and Paull (1969) were trying to find out the possible sources of atypical mycobacteria in environmental material. A variety of scotochromogen and rapidly growing mycobacteria were recovered by Paull (1969) from four of thirty samples of tap water and four of nine colliery shower heads. A variety of fast growing mycobacteria were isolated from a water tank by Stanford and Beck (1969) and were designated as *M.friedmannii.* Bailey *et al.*,(1970) isolated *M.kansasii* from tap water in USA. In an ensuing investigation Bullin *et al.*,(1970) recovered 47 strains scotochromogens and were found randomly distributed in the samples from three hospitals, subsequently it was noted that *M.xenopi* occurred predominantly in the hot water faucets.

McSwiggin and Collins (1974) examined water supplies in one hospital in London and obtained 18 strains of *M.kansasii* and six strains of *M.xenopi* from 65 water taps. However neither organism was recovered from water samples taken from an outside tap connected directly with the mains supply.

In Winnipeg, Manias and Vanbackethout (1976) isolated 17 strains of *M.kansasii* from a hospital water supply. In the same year Dizon et.al found acid-fast bacilli in the tap water. The presence of *M.xenopi* in hot water generators and taps was reported by Gross *et al.*,(1976). Cordoso and Filho (1979) isolated slow growers and potentially pathogenic mycobacteria like *M.avium-intracellulare*, *M.scrophulaceum* and *M.fortuitum* from water polluted with industrial and domestic residues.

Steadham (1980) recovered *M.kansasii* from 8 of 19 representative outlets in a town of Texas. *M.gordonae* was present in all 19 samples and *M.fortuitum* in two. Engel et.al (1980) examined water taps in Rotterdam and found *M.kansasii* in 38 of 78 among them during six samplings in one year. Clostrim *et al.*, (1981) recovered *M.xenopi*

from tap water in new haven and Collins *et al.*, (1981) found scotochromogens and rapidly growing mycobacteria in a tap water of two laboratories.

In a report from Czechoslovakia, Kaustova et.al (1981) referred 510 samples from a municipal water supply, in 18 of which they found *M.kansasii.* They also examined 1589 samples of water from collieries finding scotochromogens and rapidly growing mycobacteria in 233 and *M.kansasii* in 20 samples. 12 of these strains of *M.kansasii* came from one area and all others isolated from pithead shower bath outlets. Park and Brewer (1976) isolated the organisms from pools of Tennesee. Dailloux *et al.*, (1980) found it in swimming pools and also recovered *M.kansasii* and *M.fortuitum* from the same water.

Other mycobacteria have also been found in aquaria. Caroli *et al.*, (1982) isolated 43 strains from 53 samples of aquarium water (19 in households and 36 in pet shops) The most frequent isolate was *M.gordonae* but potential pathogens including MAIS bacilli, Mkansasii, M.chelonei and *M.fortuitum* were found. *M.marinum* was isolated from only one sample. Various species including *M.fortuitum, M.chelonei, M.avium-intracellulare* have been isolated from zoo aquaria (Pattyn *et al.*, 1971; Goslee and Wolinsky 1976). Collins *et al.*, (1984) reviewed the occurrence of mycobacteria in natural, piped and treated waters. Mycobacteria were isolated frequently by Schulze-Roebbecke and Buchholtz (1992) from domestic water supplies. Katila (1995) isolated potentially pathogenic mycobacteria from surface waters in the Finnish environment. They isolated *M.fortuitum* and *M.gordonae* during next year and the findings suggest that the source of mycobacterium is water. From surface and treated waters, the organisms were isolated by Neumann *et al.*, (1997). Kirchner *et al.*, (1992) identified *M.avium, M.intracellulare, M.scrophulaceum* etc. from waters collected from four geographically separate aquatic environments of the south eastern United States.

M.avium strain was isolated by Von-Regn *et al.*, (1994) from circulating hot water systems in two hospitals. Water taps, wells and water coolers at different sites in a BCG trial area has been investigated by Kamala *et al.*, (1994) and found the presence of MAIS complex, *M.diernhoferi, M.vaccae, M.smegmatis* and *M.terrae.*

Isolation of Mycobacteria from sediment

Mycobacteria have been isolated from sediment and soil samples from different locations as these form important sources of non-tuberculous mycobacteria. Donnelly, Scarpino and Brunner (1982) enumerated mycobacteria and streptococci present in landfill leachate to determine the significance of this leachate when it drains into the environment. Propane utilising mycobacteria has been isolated by Hou *et al.*, (1983) from the soil sample in the vicinity of Bayway refinery, Linden. From estuarine sediment, phenanthrene mineralising mycobacterial strain was isolated by Guerin and Jones (1988) and Guerin and Jo (1986). Lee and Lee (1991) reported the presence of mycobacterium among marine heterotrophs during their study about the seasonal distribution in sediment.

Two species of mycobacteria, *M.chelonei* and *M.fortuitum* have been isolated by Owens *et al.*, (1992) from the gravel of culture ponds of *Penaeus esculentus* and *Macrobrachium rosenberghii.* Kirchner *et al.*, (1992) isolated and identified MAIS from soils of some acid and brown water swamps. MAIS complex, *M.aurum, M.chelonei* subsp. *chelonei, M.diernhoferi, M.fortuitum, M.gadium* and *M.thermoresistibile* were isolated by Kamala *et al.*, (1994) from soil samples.

Procedures for mycobacterial isolation

During isolation of mycobacteria, one of the important step is decontamination of the sample which allows the maximum retrieval of mycobacteria from heterotrophs. Cordoso and Filho (1979) treated polluted water samples with 4% NaOH and0.34% benzalkonium chloride. Falkinham's method of decontamination using 1%, 2% and 4% NaOH (Brooks *et al.*, 1984) and Engback's method (Engbaek *et al.*, 1967) using 3% sodium lauryl sulphate and 1% NaOH are adopted by Kamala et.al for isolating mycobacteria from soil and water samples. Gangadharam's method using 1% cetrimide (Joseph *et al.*, 1969); two modifications of Falkinham's method using 2% and 4% NaOH has been employed. For water samples, Falkinham's method with 4% and 8%NaOH (Falkinham *et al.*, 1980); Goslee and Wolinsky's method with NaOH, NaOCl, 4% NaOH and 4% H_2SO_4 (Goslee and Wolinsky, 1976) or Engel's method (Engel *et al.*, 1980) with 3% SLS and 1% NaOH are also used by Kamala *et al.*, (1994).

Decontaminants like 0.7 mol/l NaOH followed by 50 gm/l oxalic acid and 0.9 mol/l H Sub (2) SO Sub (4) combined with 0.5 g/l cycloheximide are used by Iivanainen, Martikainen and Katila (1997) for water samples. Neumann *et al.*, (1997), compared twelve methods to isolate mycobacteria from surface and treated waters. They decontaminated surface waters with cetylpyridinium chloride (CPC) (30 min., 0.05%) firstly then with a cocktail of NaOH, cycloheximide and malachite green after preincubation of the sample in Tryptic soy broth (TSB). They used CPC 0.005%, 0.05% (30min) as decontaminants for treated waters.

Even though the methods employed for isolation of mycobacteria from water and sediment samples can also be used to process fish samples Dalsgaard *et al.*, (1992) and Dixon *et al.*, (1992) specified some methods for successful isolation of the organism. Dalsgaard *et al.*, (1992)used NaOH and oxalic acid as decontaminants but Dixon *et al.*, (1992) used 2%HCl or 4% NaOH.

Teska *et al.*, (1997) followed three isolation procedures from whole fish homogenates and obtained highest isolation rates on submerging whole fish in individual bags of modified broth at 1:10 (weight/volume) dilution for one hour, homogenising and plating on solid media.

Media used for isolation

Workers have used different synthetic media in different combinations during the isolation procedure of mycobacteria and they have their own choice to select the media, ensuring the availability of nutrients for the growth of the organism in required quantity. Most popular among the group of usually using media is Loewenstein Jensen (LJ), an egg based medium and is widely used (Brock *et al.*, 1986, Dalsgaard *et al.*, 1992, Kamala *et al.*, 1994, Neumann *et al.*, 1997). Blood agar plates (BAP) (Brock *et al.*, 1986), Petragnani's medium and Brain Heart Infusion agar (Hedrick *et al.*, 1987) have been used in some cases.

A group of agar based Middlebrook series like 7H10, 7H9, 7H11 etc. have been used widely with or without adding supplements. Kamala *et al.*,(1994) used Middlebrook 7H11 agar and Falkinham's selective medium.

Teska *et al.*, (1997) used modified 7H10 with albumin, dextrose and catalase (ADC) enrichment to isolate mycobacteria from whole fish homogenates. Middlebrook 7H11 with OADC supplement, glycerol egg medium and pyruvate egg medium has been used by Iivanainen, Matrikainen and Katila (1997) to compare the degree of isolation of mycobacteria from Brook waters.Pungkachonboon *et al.*, (1992) isolated rapidly growing photochromogenic mycobacteria from Siamese fighting fish on Ogawa egg medium and its modified forms like Ogawa Egg Yolk medium (OEY) and Ogawa whole egg medium containing afloxacin and ethambutol (OEOE) have been used by Neumann *et al.*, (1997). Chen, Adams and Richards (1997) used Long's medium, Engel's minimal essential medium, Sauton's medium and modified Sauton's medium for producing extra cellular products from *Mycobacterium spp.* Pungkachonboon *et al.*, (1990) mentioned the incubation temperature for the samples as 28°C. Colorni (1992) and Neumann *et al.*, (1997) were done the incubation at 24°C and 37°C respectively.

Antibiotic activity of some natural products against mycobacteria.

The inhibitory activity of the aqueous and alcoholic extracts of *Rhizophora mangle* L. are reported by Rojas- Hernandez and Coto Perez (1978) and found the susceptible nature of mycobacterium strains. They mentioned the minimum inhibitory concentration of the extract for the activity. Organic solvents and hot water extracts of 100 marine microalgae were screened by Miura and Matsunaga (1989) for their antibiotic activity against *M.phlei* using the paper disc method and noticed that organic solvent extracts of 33 strains had activity against the species. Using the same method, Murakami *et al.*, (1984) examined the activity of planktonic organisms. The distinct and widespread activity of the ether extract of *Asterionella japonica* towards *M.smegmatis* was reported by them.

As streptomycetes form an active source of efficient antimicrobials, Chandramohan and nair (1991) isolated them from sediments of andaman and Nicobar islands to study their antagonistic property against mycobacteria. Lohsiri *et al.*, (1994) proved antimicrobial capability of the extract of marine sponges against M.smegmatis. Invitro antibacterial activity of massetolides A-H (1-8) and viscosin , isolated from two species of pseudomonas against *M.tuberculosis* and *M.avium- intracellulare* was reported by Gerand *et al.*, (1997).

Biodegradation of some natural products by mycobacteria

Mycobacteria are well known for their extraordinary capability for degrading natural compounds. Guerin and Jones (1988) isolated mycobacteria capable of utilising phenanthrene. Compounds like 1- nitropyrene from oil contaminated sediments (Heitkamp *et al.,* 1991), PAH in a pristine ecosystem (Heitkamp and Cerniglia, 1989; Spic *et al.,* 1997), ground water pollutant mixtures like acetone, cyclohexane, styrene, benzene, ethylbenzene, propylbenzene, dioxane, 1-2 dichloroethane (Burback and Perry, 1993) are found to be degraded powerfully by different species of mycobacteria including *M.vaccae* (Burback and perry, 1993). Sepic *et al.,* (1997) proposed a floranthene biodegradation pathway. Growth of Mycobacteria on Carbon Monoxide and Methanol was studied by Park *et al.,* 2003.

Molecular studies on Mycobacteria

Knibb *et al.,* (1993) evaluated PCR as a diagnostic tool for mycobacteriosis and found the method as specific and most sensitive. The method is facilitating the screening of samples in the field as well as the new stocks for latent infections. Bruno *et al.,* (1998) adopted PCR technique for the complete identification of *M.chelonei* isolated from moribund atlantic salmons. McCormick, Hughes and McLoughlin (1995) ampilfied 16S rRNA gene sequences through direct gene sequencing of polymerase chain reaction and it is used to identify rapidly growing, acid-fast organism isolated from a cichlid oscar. A 924 bp DNA fragment of 16s rRNA was amplified through PCR technology of Talaat *et al.,* (1997) and rapidly identified *M.marinum, M.fortuitum* and M.chelonei in fish. This report yielded unique restriction patterns for each mycobacterial species infecting fish to the species to the species level. Assessment of genetic diversity is important in epidemiological studies of nontuberculous mycobacteria (NTM), as data from these studies could be used to monitor trends in the occurrence of new strains, identify possible sources of infection, and differentiate individual strains (Tenover *et al.,*1997).Knibb *et al.,* (1992) developed a PCR technique to identify the pathogen of mycobacteriosis in european sea bass without sacrificing the animals. The method is by direct sequencing and analysis of approximately 600 bp of the rDNA. The sensitivity of the method allows the culturist to remove the asymptomatic fishes from the cultural conditions. Knibb et.al (1993) identified *M.marinum* by the same method as before.

Two toluene degrading strains (T103 and T104) were studied by Tay *et al.*, (1998) and identified their similarity with *M.aurum* and *M.komossense* from 16S rDNA *sequences*. Vanitha *et al.*,(2003) reported Large-Restriction-Fragment Polymorphism Analysis of *Mycobacterium chelonae* and *Mycobacterium terrae* Isolates.

Thorel *et al.*, (1998) reported the isolation and identification of *M.bovis* from infected zoo animals by epidemiological study using genetic markers such as IS-6110 based DNA finger printing system and they differentiated *M.bovis* strains, some strains presented a single copy but multiple copies by others. Arakawa and Fryer (1984) adopted biological, physiological genetic and mycolic acid properties for the taxonomic analysis of mycobacteria, isolated from salmonid fishes. From a percent guanine plus cytosine value of 63 plus or minus 1.7 %, they confirmed the isolates as the genus mycobacterium. Shamsudin *et al.*, (1990) also investigated guanine plus cytosine percent value in mycobacteria isolated from infected ornamental fishes; but the strain was not identified upto the species level.

Materials and methods

III MATERIALS AND METHODS

MEASUREMENT OF PHYSICO-CHEMICAL PARAMETERS

Temperature was measured in the sampling station with a high precision mercury thermometer of +/-0.1° C in the early hours of the sampling day. Water pH was measured with digital pHmeter, calibrated with pH buffers. Factors like salinity, dissolved oxygen, phosphate were monitored monthly, following the standard procedure by Strickland and Parsons(1968). Nitrite nitrogen and nitrate nitrogen contents were measured by Azo-dye method(Bendschneider and Robinson, 1952)and method given by Mullin and Rily(1955). Ammonia was determined following the phenol hypochlorite method(Solarzano, 1969) and the standard method of Walkley and Black(1934) waas used to determine the organic carbon content of the sediment.

SELECTIVE MEDIA USED

Both dehydrated and compounded media were used in the study for the isolation and biochemical characterisation of mycobacteria. Peizer TB medium and Dubos broth (base) were brought from Hi-media laboratories (Bombay) in the dehydrated form. All media and chemicals for various tests are from Sigma, Oxoid and Difco company.

1. Peizer TB medium

It is an agar based medium. 29.72gm/l medium is dispersed in to distilled water (1000 ml), sterilised at 15lbs pressure for 15 mts. After cooling to 55°C, egg yolk emulsion was added aseptically; dispensed into sterile tubes in required amounts and made into slopes.

Egg yolk emulsion is prepared in the following manner.

Sterile egg yolks	10 nos.
Normal saline (sterile)	25.0 ml
Dextrose solution,20% (sterile)	1.0 ml
Malachite green solution,1%	13.0 ml

2. Dubos broth

Suspended 1.3 grams of Dubos broth base in 180 ml distilled water containing 10ml glycerol ; boiled to dissolve completely. After sterilising at 15lbs pressure for 15mts, cooled to 50OC and aseptically added 20ml sterile faetal calf serum to each 180ml broth base. Dispensed into sterile tubes in required quantity.

Following are the compounded media prepared in the laboratory with available ingredients.

1. Loewenstein Jensen (LJ) – Egg based medium.

Composition of base.

Monopotassium phosphate	2.4g
Magnesium sulphate	0.24g
Magnesium citrate	0.6g
L-asparagine	3.6g
Potato flour	30.0g
Malachite green	0.4g
Glycerol	12ml
Distilled water	1000ml

pH – 7.0

The base(600ml) , mixed and sterilised at 15lbs was mixed aseptically with 1000ml whole egg emulsion prepared previously, to get a uniform mixture. Distributed in sterile screw-capped tubes in required quantity. After arranging the tubes in a slanted position, coagulated the slopes in a pressure cooker at 85^{O}C for one hour.

2. Nutrient agar with 5% glycerol

Yeast extract	2g
Sodium chloride	5g
Peptone	5g
Agar	15g
Glycerol	50 ml
Distilled water	1000ml

pH – 7.4

The ingredients are dissolved through boiling, sterilised (15lbs) and dispensed aseptically in sterile petri plates.

3. Simmons citrate agar

Magnesium sulphate	0.2g
Ammonium phosphate	1.0g
Dipotassium phosphate	1.0g
Sodium citrate	2.0g
Sodium chloride	5.0g
Agar	15g
Bromothymol blue	0.08g
Distilled water	1000ml
pH (approx.)	6.8 + 0.2

Suspended the ingredients (24.2g/l) and boiled to dissolve. Dispensed in 2ml quantity in screw capped tubes; sterilised at 15lbs for 15mts and made into slants.

4. Tween-80 hydrolysis

Composition of substrate solution is as follows.

0.067M phosphate buffer (pH- 7)	100ml
Neutral red (1% aqueous solution)	2ml
Tween-80	0.5ml

Boiled to get uniform solution; dispensed in 4ml quantity into screw capped tubes; sterilised15lbs or 10lbs for 10mts. (Wayne et.al, 1974).

5. Nitrate reductase activity.

Substrate solution is 0.01M $NaNO_3$ in M/45 phosphate buffer. Composition is as follows.

Sodium nitrate	0.085g
Potassium dihydrogen phosphate	0.117g
Disodium hydrogen phosphate ($12H_2O$)	0.485g
Distilled water	100ml.

Substrate solution was dispensed in 2ml amounts in screw capped bottles and sterilised at 15lbs pressure. (Kubica and David, 1980).

Reagents required for the test are –

Chlorhydric acid (1/1) (vol/vol) in water	
Sulphanilamide	0.2% (in water)

Naphthyl ethylene diamine	0.1% (in water)

Substrate solution and the test reagents should be stored at $4^{O}C$ in darkness.

Preparation of colour standards

Solution I

1. Na_2HPO_4 (anh.)	9.47g/l
2. KH_2PO_4	9.078g/l
3. $Na_3PO_4.12H_2O$	25.47g/l

35ml of 1, 5ml of 2 and 100ml of 3 are mixed.

Solution II

To 10ml of solution I, 0.1ml of 1% ethanolic phenolphthalein and 0.2 ml of 0.01% bromothymol blue are added. A 2ml of solution II in tube1 is 5+ colour standard. Then 2ml solution I is placed into seven additional tubes and 2ml solution II is added to tube 2. A 2ml portion is transferred to next tube and serial dilutions of 2ml are made in the remaining tubes (2ml is discarded from tube 8). Tube 2 corresponds to 4+ colour standard and tubes 3, 5, 6 and 8 correspond to 3+,2+,1+ and +/_ colour standards respectively.

6. Acid- phosphatase activity

Substrate solution

Pyridine salt of phenolphathalein phosphate(Sigma)	100mg
0.2M Acetic acid-sodium acetate buffer (pH – 5.2)	100ml

Buffer is steamed at $100^{O}c$ for 30mts and cooled to RT before phosphate substrate is added. Dispensed in 1ml amounts to screw capped bottles aseptically. (Wayne, 1985).

7. **Pyrazinamidase activity in agar** (Wayne, 1985)

Composition of the test medium.

Dubos broth base	6.5g
Pyrazinamide	100mg
Sodium pyruvate	2g
Agar	15g
Distilled water	100ml

Boiled to get uniform mixture; dispensed in 5ml amounts in screw capped bottles; sterilized at 15lbs pressure for 15mts and cooled in upright position. (Ayers *et al.*,1919).

Reagent required-

Ferrous ammonium sulphate - 1%

8. Acid production from carbohydrates

$(NH_4)_2HPO_4$	1g
KCl	0.2g
$MgSO_4.7H_2O$	0.2g
Agar	15g
Bromocresol purple(0.04% w/v)	15ml
Distilled water	1000ml

pH - 7.0

The inorganic nitrogen agar base is boiled , dispensed in 5ml quantity in screw capped tubes; sterilised by autoclaving at15lbs for 15mts. 10% aqueous solution of carbohydrate, prepared and sterilised previously was added into the base aseptically in half ml amounts into each tube.

9. **Production of urease**

KH_2PO_4	9.18g
Na_2HPO_4	9.5g
Yeast extract	0.1g
Phenol red	0.01g
Distilled water	1000ml

pH - 6.8

10ml of 15%(w/v) solution of urea, sterilised by filtration was combined with 75ml of the autoclaved base prepared as above. The mixture was pipetted aseptically to sterile screw capped tubes in 1.5 ml quantity.

10. **Semiquantitative catalase test**

Reagent mixture

H_2O_2 30% (in water)

Tween-80 10% (in water)

0.5ml of both the solutions are mixed for the test

11. Acid phosphatase activity

Reagent required

Na_2CO_3 10% in water

Suitable colour standards

Stock - 1mg phenolphthalein in 95% ethanol. Required colour standards are prepared from the stock by making phenolphthalein concentrations into 2.5,5 and 10ug/ml in water and are treated with sodium carbonate as in the test.

12. **Aryl sulfatase activity.**

Medium and reagents required

Dubos liquid medium

Phenolphthalein disulfate

(tripotassium) salt (Sigma) - 0.08M

Na_2CO_3 - 1M

Study area

Two perennial aquaculture ponds located in the Vypeen island, along $10^{O}\ 100^{1}$ N and $76^{O}\ 13.5^{1}$ E and $10^{O}\ 103^{1}$ N and $76^{O}\ 12.5^{1}$ E were selected as the areas of the study. One pond was at CMFRI substation,Narakkal and other is at Valappu, under the management of Ajantha co-operative society. Ponds are designated as Station I and Station II respectively. Both the stations are more than 6km apart from each other and hydrographically have typical brackish water environment. Availability of water for cultural purposes is from regular tidal inflow.

Station I and II have dissimilar water and sediment characteristics. Station I is with black sandy sediment and almost clear water with less phytoplankton content. Station II is more productive and actively being cultured throughout the year. Water is turbid with high planktonic growth and sediment is black, fine and clayey in nature.

Sampling

Regular monthly sampling was carried out from both the stations throughout the year (from March 1999 to February 2000) in the morning hours. Water samples were collected aseptically in two sterile 500ml sampling bottles. Fish samples (*Oreochromis mossambicus*) and sediment samples were collected in sterile polythene bags. Collected samples were transported into the laboratory immediately and subjected to analysis.

Estimation of environmental parameters

Environmental parameters of Station I and II were monitored at monthly intervals. Water temperature, pH, dissolved oxygen, salinity, nutrients like nitrite nitrogen, nitrate nitrogen, ammonia, phosphorous and sediment organic carbon were monitored regularly. Water temperature was monitored using using a mercury thermometer during sampling. pH of the water was measured with a glass electrode using pH meter (ELICO digital).

Dissolved oxygen, salinity and nutrients were estimated according to the methods followed by Strickland and Parsons (1968).

Organic carbon content in the sediment samples was determined according to the procedure of Holme and McIntyre (1971) and Ann (1975). In this method, hot chromic acid is used to oxidise any organic carbon content present, and the excess acid not reduced by organic matter is determined volumetrically with ferrous salt and it is expressed as ug atC/gm.

Nitrate nitrogen was determined in 50ml of water sample by Azo-dye method (Bendschneider and Robinson, 1952). It is based on the classical Griess's reaction in which the nitrate ion at the pH of 1.5-2.0 is diazotised with sulphanilamide resulting in a diazo compound which in turn is completed with N-(1-naphthyl)ethylene-diamine to form a highly coloured azo-dye with an absorption maxima at 545nm that is measured colorimetrically and the nitrate content is expressed as ug atNo-N/l.

Estimation of bacteriological parameters.

Fish, water and sediment samples, collected from the stations were pour-plated for enumerating total viable count (TPC) on Nutrient agar. Aged sterilised sea water was used for preparing serial dilutions of the samples as per the standard procedures (Rodina, 1972) and the desired dilutions were used for plating. 500ml water sample was filtered using 0.4um millipore filter and the filter paper was incorporated in 99ml aged, sterilised seawater. One-gram sediment, weighed aseptically, mashed well and incorporated in sea water as above. Samples thus prepared were kept for shaking in an automatic shaker at 150rpm for 30mts, for thorough mixing of the samples.

After shaking, serial dilutions of all the samples were prepared and the desired dilutions were plated in duplicate. Plates were incubated aerobically at RT for 48 hrs. Colony forming units were counted after incubation. Number of colonies appeared per plate was recorded as CFU per unit weight of the sample.

Isolation of mycobacteria on selective media

Mycobacteria were isolated from water, sediment and fish samples like skin, gill, stomach, intestine and liver.

Preparation of sample for mycobacterial isolation

Aged seawater was sterilised in 10ml amounts and one gram each of the samples like sediment, skin, gill, stomach, intestine and liver weighed aseptically were placed in the seawater after thorough mashing. Instead of taking water sample directly, 500ml water sample is filtered and the filter paper is incorporated into 10ml-sterilised seawater for preparing water sample. Prepared samples are kept for shaking at 150rpm for 30mts.

Decontamination procedure.

This procedure is meant for maximum retrieval of mycobacteria on the media by killing the heterotrophs, which may overgrow mycobacterial colonies in culture. 4% NaOH and sterile distilled water were used as decontaminants (Marks and

Thomas,1958) throughout the study. Equal quantity of 4%NaOH was added into each sample, mixed well and left as such for 10mts.

Centrifugation

Decontaminated samples were centrifuged separately at 4000rpm for 20mts; decanted the supernatant and added sterile distilled water. After thorough mixing, centrifuged again in the same speed for the same period.

Inoculation

Centrifuged samples were removed and decanted the supernatant. 2ml amounts of sterile normal saline was added into each centrifuge tube, mixed well and inoculated on fresh Loewenstein Jensen and Peizer TB media slopes, prepared previously. Four slopes of each medium was inoculated with one sample. All the samples except gill, liver and water were inoculated with single drop of the sample on each slope and the rest are inoculated with half ml amounts, as the mycobacterial representation is less in these samples.

Incubation

Inoculated slopes were divided into two sets; one set is incubated at RT and the other is at 37^{O}C for four weeks. From the third day onwards, the slopes were observed for the growth and recorded accordingly. For up to one week, daily observation was carried out and later observed weekly for up to 4weeks.
Contaminated slopes were discarded periodically.

General differentiation of mycobacteria

Colonies appearing on LJ and PTB slopes were differentiated as mycobacteria and heterotrophic bacteria was through Ziehl-Nielson's acid-fast staining method (Silverston and Anderson 1961). Mycobacteria will absorb the pink colour of basic fuchsin in different grades and will resist decolourising action of acid or alcohol. The

colonial morphology of ZN+ve strains will also help in mycobacterial differentiation on the respective media. ZN+ve strains were isolated accordingly.

Enumeration of mycobacteria

Total plate count method was used to compare the mycobacterial count with that of total heterotrophic count. Remaining portion of the decontaminated samples after inoculation on LJ and PTB slopes were decanted into 99ml aged, sterilised sea water for enumerating mycobacteria. Steps were repeated as for heterotrophic TPC and nutrient agar plates were inoculated for mycobacteria. Incubation was at RT for 3days. Number of colonies were counted and recorded as total mycobacterial count.

Preservation of isolates.

Pure cultures isolated from the slopes were preserved on peptone broth with 1% glycerol and Dubos broth at $4^{O}C$ in a refrigerator. Periodical subculturing of the isolates were carried out to prevent decay.

Statistical analysis

The relationship between environmental parameters, bacterial parameters and mycobacterial count of different samples taken was found out by statistical analysis. The correlation coefficient 'r' value of different parameters were estimated using quantitative data collected during the 1 year period.

Classification of mycobacteria.

According to the number of days taken for the appearance of mycobacterial colony on the slope, they have been classified broadly into slow growers (taking more than 7 days) and fast growers (between 3 and 7 days) This classification is helping in the characterisation of the isolates.

During the period of incubation itself, the day of emergence of each colony is noted and the slow as well as the fast growers were separated roughly. Further strict

separation was done through the test of iron uptake (Pattyn and Portaels, 1972). The isolates were streaked on peptone agar (1% Peptone, 2% agar and 2ml glycerol). After attaining full growth on the slopes, 2dropes of 20% ferrous ammonium sulfate was poured on the growth and were incubated for 21 days. Strains giving positive test for iron uptake are classified as fast growers and negative are grouped as slow growers. Both the groups are treated separately through different sets of biochemical tests.

Characterisation of mycobacteria.

Mycobacterial strains isolated from different samples throughout the period were characterised biochemically. The schemes followed were Pattyn and Portaels (1972) and Bergey's manual of determinative bacteriology (Runyon et.al, 1974)

Characterisation of slow growers

Biochemical characteristics like photochromogenicity, capability of grqwth on different temperatures (33,37 &42OC), resistance towards antibiotics like isoniazid, thiophene-2-carboxylic acid hydrazide(TCH), hydroxylamine hydrochloride (HA), para-nitrobenzoic acid (PNB), catalase>45mm, niacin production, nitrate reduction, acid phosphatase, Tween-80 hydrolysis, B-galactosidase activity, growth in 5% sodium chloride, production of urease, nicotinamidase activity, pyrazinamidase activity and growth morphology on Oleic acid albumin agar.

Characterisation of fast growers

Pattyn and Portaels (1972) presented another set of biochemical reactions for characterising fast growers. Test for photochromogenicity, capability of growth on different temperatures (37, 42, 45 &52^{O}C), acid production from sugars like glucose, inositol, mannitol and sucrose, citrate and benzoate utilization, nitrate reduction, acid phosphatase, tween-80 hydrolysis, aryl sulfatase activity, resistance towards hydroxylamine hydrochloride and colonial morphology on OAA and corn meal agar.

1. Pigmentation and photoreactivity

A cell suspension properly diluted to obtain isolated colonies was inoculated on two LJ slopes. One of the tubes was wrapped or placed in any device that ensures total

darkness and both the tubes were incubated at RT until plane growth is visible on the control tube. Immediately after observing the growth, the tubes incubated in the dark were placed at a distance of 20cm from a 60W flourescent lamp for one to two hours after loosening the cap to ensure good aeration for the culture. The cultures are again incubated for 24hours in the same condition. Then the tubes were compared and the cultures were grouped as scotochromogens, photochromogens and nonchromogens (Wayne et.al, 1974)

2. Semiquantitative catalase test

LJ medium was made into butts in screw capped tubes and the surface of the medium was inoculated with 3drops of undiluted suspension of the culture and incubated at RT for flourished growth. On observing good growth on the media, 1ml of freshly prepared reagent mixture was added. After 5mts, the height of the foam column produced was measured keeping the tubes in an upright position. More than 45mm foam is considered as positive and less than 35mm is mea sured as negative (Wayne et.al, 1976).

3. Tween-80 hydrolysis.

The substrate solution containing tween-80 was inoculated with the culture and incubated at $37^{O}C$ for 10days. A change in colour from amber to pink or red in the suspension was recorded as positive after 24hours, 5days and 10days.(Wayne et.al, 1974). Cells will absorb the colour of neutral red and give false positive result (Wayne 1985).

4. Nitrate reductase activity

2ml substrate solution was inoculated with a loopful of cells and incubated at $37^{O}C$ for 2hours. After incubation, one drop of an aqueous chlorhydric acid solution was added, followed by two drops of sulphanilamide solution and later two drops of naphthyl ethylene diamine solution. Colour intensity may range from pale pink to deep red and was compared with colour standards prepared previously, to get the result (Wayne 1985)

3 Acid phosphatase reaction

One ml substrate was inoculated with a loopful of cells and incubated at 37°C for 4hrs. One ml Na_2CO_3 solution was added to stop the reaction and to develop the colour. Intensity of the colour was compared with colour standards prepared previously. Test preparation, colourless or pink which has the intensity less than that of 2.5ug/ml standard was considered as negative and those having intensities correspond to the intensity of 5 or 10ug/ml standard are recorded as positive.

5. Aryl sulfatase activity

Dubos broth containing phenolphthalein disulfate (tripotassium)salt were inoculated and incubated at 37°C for 3 days. After incubation, 0.3 ml of Na_2CO_3 solution was added to develop pink colour, which was recorded as positive reaction.

6. Pyrazinamidase activity

Test medium was inoculated heavily with culture suspension and incubated at RT for 7days. Hydrolysis was detected by adding 1ml of freshly prepared ferrous ammonium sulfate solution. A positive reaction was indicated by the appearance of a pink band in the agar after 4hours.

7. Acid production from carbohydrates.

Agar slopes prepared having sugar were inoculated heavily and incubated at RT for 28days. The slopes were observed for the acid colour change of the indicator added. The result is recorded on 7^{th} and 28^{th} days of incubation.

9. Production of urease

The test tubes were heavily inoculated with actively growing cultures and and the positive reaction was indicated by alkaline colour change of the phenol red after 5,7,14, 21 and 28 days at RT.

10. Resistance to isoniazid, thiophene 2 carboxylic acid hydrazide, hydroxylamine hydrochloride and p- nitrobenzoic acid.

Resistance of isolates to these inhibitory agents were tested by incorporating them in LJ medium in standard concentrations such as isoniazid (10ug/ml), hydroxylamine hydrochloride (250ug/ml for slow growers and 500ug/ml for fast growers), thiophene-2-carboxylic acid hydrazide (1ug/ml) and p-nitrobenzoic acid (500ug/ml). 10^2 and 10^4 dilutions were prepared from standard culture suspension and inoculated into the tubes of LJ with incorporated inhibitory agents. Control tubes were inoculated with 0.2ml of the 10^2 and 10^4 dilutions. When growth was visible on the control tubes, the colonies were counted. If the growth on drug containing medium was less than is less than the growth of the 10^4 dilution control tube, the culture was reported as susceptible. Growth on drug containing medium was equal to the growth of the 10^2 dilution control was reported as resistant. Colonies in the 10^2 dilution control tubes have to be numerous, more than several hundred colonies are confluent cultures to make the interpretation accurate (Wayne 1985; Wayne et.al, 1974 &1976).

RAPD-PCR ANALYSIS

Procedure followed for isolation of mycobacterial DNA

- Inoculated the bacterial strain into 10ml Dubos broth with 1%Tween 80. Incubated for two days.

- Centrifuged culture for 10,000rpm for 10 minutes or until compact pellet forms. Discarded the supernatant.

- Resuspended the pellet in 8ml of TE.

- Added 1ml of 10% SDS and 20µl, 20mg/ml proteinase-K. Mixed thoroughly and incubated at 37^0C for 1hr.

- Added equal volume of chloroform/ isoamyl alcohol mixed thoroughly. A white interface should be visible after centrifugation.

- Extracted the aqueous phase with phenol / chloroform isoamyl alcohol and spin at 10,000rpm for 10 minutes at 4^0C. Transferred the supernatant to a fresh tube. Added 1/10 volume of sodium acetate and 3 volumes of isopropanol to precipitate the nucleic acids.

- Pelleted DNA by centrifuging and transfer the spongy white DNA precipitate to a fresh tube containing 70% ethanol.

- Spinned at 10, 000 rpm for 5 min at room temperature.

- Carefully removed the supernatant and briefly air- dried the pellet.

- Redissolved the pellet in 200µl TE buffer and used for further analysis.

Denaturation of proteins with SDS at room temperature and further extraction with neutral Phenol and Chloroform to augment protein precipitation. The supernatant was re-extracted with neutral Phenol and the DNA was directly precipitated from the resulting aqueous phase. The dried DNA pellet was re-hydrated with sterile deionised water and used for further studies.

The qualitative integrity of DNA sample isolated are checked by 0.8 % agarose gel electrophoresis, detected by subsequent ethidium bromide staining and viewed under UV Transilluminator. The DNA quantification was done by comparing the intensity of the sample DNA with known concentration of molecular weight markers run along with the sample. Based on the quantitative and qualitative analysis of DNA using agarose gel electrophoresis, dilution of the template DNA for RAPD-PCR is decided. Dilution is made in such a way that the final concentration of template DNA for RAPD-PCR is approximately 50 ng/µl.

Polymerase Chain Reaction (RAPD-PCR).

Primers used

A panel of 5 numbers of decamer random primers from M/S Operon Technologies, designated as OPA-02, OPA-07, OPA-18, OPA-20 and L1 were used for PCR amplification of the mycobacterial DNA template.

Preparation of reaction mixture

Standardization of the optimum concentration of different components in PCR was done by varying the concentrations. PCR amplifications were performed in a total volume of 25µl. The PCR mixture consisted of 17.5µl of deionised water, 2.5µl 10x

assay buffer, 2.0µl dNTP's and 0.5µl of *Taq* DNA polymearse per reaction. 1µl of the DNA eluted, was added. The arbitrary primers were added in an amount of 1.5µl.

PCR cycles

Thermal cycling was performed with MJ Research thermocycler (Model PTC200, Massachusetts, USA). Each of the 39 PCR cycles standardized for this work consisted of denaturation of DNA at 94° C for 3 minutes, primer annealing at 37°C for 1 minutes and primer extension at 72°c for 1.5 minutes. All PCR samples were subjected to an initial denaturation step at 94°C for 1 minutes and a final extension at 72°C for 7 minutes. PCR products were stored at -20°C until electrophoresis was performed.

Electrophoresis

The amplified DNA products were resolved through agarose gel electrophoresis. Reagents used were the following.

1) Agarose
2) 1x TEB (pH 8)
   0.89M TRIS Hcl.
   0.02M EDTA
   0.89M Boric acid
3) Loading buffer
   Glycerol 2ml.
   Bromophenol blue (0.5%) 1ml.
   1x TEB
4) Standard DNA marker (λDNA cut with HindIII/Ecorl)
5) Ethidium bromide (1µg/ml.)

Procedure:

An agarose gel of 1.5% strength in 1X TEB was casted. Four microlitres of PCR products were mixed with 2 μl of loading buffer and loaded into the gel. The electrophoresis was carried out at 80 voltages for two hours. The gel was stained in

ethidium bromide for twenty minutes and documented by gel documentation system(model Image master VDS of Amersham Biosciences).

Analysis of RAPD Data

The scorable bands produced by each of the primers, resolved through agarose gel electrophoresis, were scored in a binary matrix. (1/0 ie.1 to represent the presence and 0 to represent the absence of the band at an RAPD locus). These data were analyzed using the POPGENE 1.32 Software according to the procedures suggested by Baranek *et al.* (2001) for the analysis of RAPD data. Various parameters like overall amplicon frequency at each locus, number and percentage of polymorphic loci, Nei's original measures of genetic identity as well as genetic distances; average gene diversity(also known as average heterozygosity – khoo *et,al*; 2002) were estimated using the above software. Dendogram in the phylogram form was also generated using Nei's similarity coefficients by the unweighted pair group method using arithmetic averages (UPGMA Method), modified from NEIGHBOR procedure of PHYLIP Version 3.5. The binary datamatrix was bootstrapped 1000times with Popgene1.32 to test the robustness of each branch of the dendrogram.

Results

IV RESULTS

1. PHYSICO - CHEMICAL PARAMETERS

Table 1. shows the range of physico-chemical parameters observed for the stations studied during the period of study(March 1999-February 2000).

1.1 WATER TEMPERATURE

Station I (Narakkal)

Water temperature was found to be fluctuating between 28°C (May &July) to 34°C (March). During postmonsoon, the lowest temperature (29°C) was recorded in November and January and the highest temperature (31°C) was observed in October. Wide variation in temperature was recorded during pre monsoon which was fluctuating between 28°C and 34°C in May and March respectively. During monsoon , highest temperature was recorded as 32°C in June and September 1999 and the general range recorded was 28-32°C. During the period of study, March was the hottest month reported and there was gradual decrease of temperature upto 28°C in May with sudden rise in temperature to 32°C in June. The temperature decreased to 28°C (July) with gradual rise upto 32°C (September). Post monsoon season showed less fluctuation in temperature compared with other seasons. Figure 1.1 shows the effect of temperature on heterotrophs and nontuberculous mycobacteria (NTM).

Temperature showed statistically significant positive correlations during pre monsoon (Table 4.1) with organic carbon ($P<0.05$) and negatively with dissolved oxygen($P<0.05$) and pH ($P<0.01$). With ammonia, at 1%level ($P<0.01$) during post monsoon(Table 4.2) and with pH ($P<0.05$) during monsoon season. (Table 4.3)

Station II (Valappu)

Annual temperature range observed was 28-34^{0}C in station II during the period of study which was identical with that of station I. The fluctuation in temperature among the three seasons were also similar in both the stations, with wide range during pre monsoon (29-34 °C) and narrow range was recorded during post monsoon (31-32 °C). Among the three seasons, monsoon was observed with low temperature

Table: 1. Physico-chemical parameters recorded from station I and II

	Ranges observed	
Parameters studied	**Station I (Narakkal)**	**Station II (Valappu)**
Water temperature	28 –34°C	28 –34°C
Salinity	3.40 -- 22.52 ‰	1.57 – 24.3 ‰
Dissolved oxygen	1.96 – 5.67 mg/l	1.37 – 7.39 mg/l
Water pH	7.10 – 8.45	7.61 – 9.18
Organic carbon	0.15 – 0.39 mg/gm	0.42 – 1.64 mg/gm
Nitrite nitrogen	0.14 – 0.65µgat/l	0.0 – 4.93µgat/l
Nitrate nitrogen	0.08 – 1.17µgat/l	0.0 – 2.14µgat/l
Ammonia	1.49 –14.90µgat/l	0.12 – 7.35µgat/l
Phosphate	0.77 – 14.20µgat/l	1.03 – 19.8µgat/l

with the range of, 28 °C during September to 30 °C during July(Figure 2.1). Sudden rise in temperature from 31 °C (January) to 34 °C (February) during the change of season from postmonsoon to premonsoon was observed. During December and January water temperature recorded was 31°C and in October & November, temperature was 32°C. The highest water temperature (34 °C) was observed in the month of February 2000(pre monsoon).

Between temperature and salinity, a statistically significant 5% positive correlation existed and during postmonsoon temperature showed significant positive correlation at 1% level with pH(P<0.1) (Table 5.3). Temperature showed a significant negative correlation with ammonia at 5% level, while during monsoon (Table5.2) and post monsoon significant positive correlation was observed between temperature and nitrite. Statistically significant negative correlation was observed between temperature and dissolved oxygen (P<0.1) during premonsoon(Table 5.1).

1.2. SALINITY

Station I (Narakkal)

During monsoon season, the highest salinity observed was 8.58‰(August) and the lowest as 3.4‰(September). Highest salinity values recorded during premonsoon and postmonsoon seasons were 19.19‰ (February) and 22.52‰ (January) and the lowest values were 4.6%(May) and 8.49‰(October) respectively. Observable rise in salinity was recorded during postmonsoon, 8.49‰(October) - 22.52‰(January) and this season recorded highest salinity range during the period of study. But a gradual decrease in the salinity from February to May (premonsoon) from 19.19‰ to 4.6‰ in Narakkal aquaculture pond. During monsoon, gradual rise in salinity was observed for the first three months from 5.6‰ to 8.58‰, whereas lowest salinity (3.4‰) was recorded during September 1999. (Figure 1.1)

During monsoon, salinity showed significant negative and positive correlatios at 1% and %% levels(Table 5.2). During premonsoon , a statistically significant positive correlation existed between salinity and nitrite(P<0.01), nitrate and phosphate(P<0.05) (Table 5.1), with nutrients like nitrate ((P<0.05) and pH(P<0.01) salinity showed significant negative correlation.during postmonsoon(Table 5.3)

Station II (Valappu)

With respect to salinity, Valappu water showed fresh water or brackish water characteristic through the period of study. The range of salinity recorded was comparatively narrow during monsoon (91.57- 5.9‰) among the three seasons. During premonsoon and post monsoon, highest salinity values recorded were 22.52‰(February) and 24.27‰ (January) and the lowest values were 3.14‰ (May) and 1.57‰ (October 1999) respectively (Figure2.1). The highest value for salinity recorded was during January (pre monsoon) as in station I. Similar to station I, a regular pattern of salinity distribution was observed in pre and postmonsoon seasons in station II also. During the period of study, the lowest salinity observed was 1.57‰ recorded in July(monsoon) and October, during the postmonsoon season. There was distinct decrease in salinity from 22.15‰ to 3.14‰ in February, March, April and May. During postmonsoon a gradual decrease was recorded during postmonsoon from 1.57‰ to 24.27‰. During withdrawal of monsoon, sudden decrease in salinity was observed from 5.9‰(September) to 1.57‰ (October).

Salinity showed highly significant positive correlation with ammonia during monsoon at 5% level ($r = 0.991$) and with nitrite ($P<0.1$, $r =0.921$) during premonsoon.

1.3. DISSOLVED OXYGEN

Sation I (Narakkal)

Among the three seasons, premonsoon showed a wide range of dissolved oxygen varying from 1.96mg/l(March) to 5.67mg/l (April). The observed highest values for dissolved oxygen, for monsoon and post monsoon were 3.46 and 3.64mg/l during August and November 1999 respectively. Pre and postmonsoon seasons recorded 2.8mg/l (September) and 2.17 mg/l (October) as the lowest dissolved oxygen values. Specific seasonal distribution pattern was not observed for dissolved oxygen content of water in station I. The overall annual range for dissolved oxygen was small ranging from 1.96mg/l to 5.67mg/l, observed during consecutive months of March and April (premonsoon), but February and May recorded 3.18 and 3.89 mg/l of dissolved oxygen respectively. During monsoon season observed DO values were from 3.39 mg/l (June), 2.98mg/l (July), 3.46mg/l (August) and 2.8 mg/l (September). The lowest value for dissolved oxygen during postmonsoon was 2.17mg/l in October, 2.73 mg/l in December and 2.52% in January.(Fgure 1.1)

Dissolved oxygen showed statistically significant positive correlation with nitrate nitrogen at 5% level during premonsoon(Table 4.1) and negative correlations during monsoon and post monsoon seasons. (Table 4.2,Table 4.3)

Station II (Valappu)

Dissolved oxygen range fluctuated high at Valappu than Narakkal and was observed as 1.37mg/l- 7.39mg/l during months of June (monsoon) and November(postmonsoon) 1999 respectively, whereas a range from 1.37mg/l to 7.12 (July) were recorded during monsoon. Highest values for dissolved oxygen in both the pre and post monsoons were 5.13 and 7.39 mg/l and ranges were comparatively narrow. A gradual rise in dissolved oxygen was observed from 3.34%(March) to 5.13%(May),. During monsoon season a gradual and steady decrease in dissolved oxygen values were observed from July (7.12 mg/l) to September (5.88mg/l). During postmonsoon, the general range of dissolved oxygen was narrow from 5.15 mg/l (October) to 7.39 mg/l (November) and December and January months showed 5.6 mg/l and 6.31mg/l of dissolved oxygen(Figure 2.1).

During premonsoon, dissolved oxygen showed negative correlation at 1% level with nitrite and phosphate(Table5.1)., positive correlation with organic carbon at 5% level and a negative correlation existed between phosphate at 1% level ($P<0.01$)during monsoon(Table5.2).

1.4. WATER pH

Station I (Narakkal)

Hydrogen ion concentration ranged between 7.1 in the month of March (premonsoon) and 8.7 in June (monsoon). A uniform distribution pattern was observed during pre and post monsoons, an increase in pH from march(7.1) to May(7.9) during premonsoon and a decrease from October(8.3) to January(7.4) during postmonsoon was observed. 7.7(July) – 8.7(June) was the general range of pH observed during monsoon. (Figure 1.2) . During monsoon, the highest range was observed in June and lowest in July, the values being 8.7 and 7.7 respectively.

Significant negative correlation was found between organic carbon, nitrate and ($P<0.01$) during monsoon(Table 4.2) and positive correlation with phosphate during

post monsoon(P<0.01) (Table 4.3) and significant negative correlation with ammonia during pre monsoon (P<0.05) (Table 4.1).

Station II (Valappu)

The annual range of water pH fluctuated between 7.6(March) and 9.2(October). The premonsoon and monsoon seasons were showing almost same variation in pH, the range being 7.6 – 8.6 (February) and 7.6(august)-8.4(July) respectively, whereas pH values were recorded high in postmonsoon fluctuating between 8.3 in December and 9.2 in October. No seasonal variation was observed in the data for water pH during the period of study and the distribution was not uniform(Figure 2.2). But during withdrawal of monsoon, sudden rise in pH value was observed from 7.9 (September) to 9.2 (October).

Tables 5.5, 5.2, and 5.3 shows the positive correlation of water pH on ammonia at 1%level during pre monsoon, with nitrite and nitrate during monsoon and 5%level negative correlation with organic carbon during postmonsoon.

1.5. ORGANIC CARBON

Station I (Narakkal)

Organic carbon content fluctuated between 0.15(May & December) and 0.39 mg/gm (July). Seasonal distribution of was uniform with range of 0.19mg/gm(June)-0.39mg/gm(July) during monsoon and 0.15 -0.28mg/gm (February) during postmonsoon. Fluctuation of values between 0.149 to 0.36mg/gm observed during postmonsoon in December and November respectively. During March and April, organic carbon values were similar, the value being 0.25 and 0.24 mg/gm . High organic carbon content was recorded in October (postmonsoon), the value being 0.35, whereas almost similar values of 0.23 and 0.27mg/gm were observed during the months of August and September in monsoon season. (Figure 1.2)

None of the ecological parameters showed statistical correlation with organic carbon during premonsoon, whereas during monsoon(Table 4.2) and postmonsoon(Table 4.3),nitrate, ammonia and phosphate showed significant positive corelations.

Station II (Valappu)

Annual range of organic carbon observed was relatively higher than Narakkal, ie. from 0.42 to 1.64mg/gm. Almost all the months except June(0.42mg/gm) were showing the values nearly 1.00mg/gm or more. During the last three months of monsoon (July – September), the values observed were 1.40, 1.17 and 1.35mg/gm respectively. The range for organic carbon during postmonsoon was 0.93 mg/gm (November)-1.30mg/gm(January). October and December months showed organic carbon values as 1.10mg/gm and 1.23mg/gm respectively(Figure2.2).

During post monsoon (Table 5.3) organic carbon showed positive correlation with nitrate at 1% level and during monsoon(Table 5.2) showed significant negative correlation ($P<0.01$) with phosphate and ammonia.

1.6. NITRITE NITROGEN

Station I(Narakkal)

In Narakkal, during the three seasons, the range of nitrite nitrogen was fluctuating between 0.14μgat/l in months of November and December (postmonsoon) and 0.65μgat/l in the month of February (premonsoon). During monsoon nitrite nitrogen ranged from 0.22 (June) to 0.52μgat/l (August 1999) with gradual rise in the values during the season and a fall was observed from September (0.30 ugat/l) through the postmonsoon season with the lowest value as 0.14 ugat/l. The highest nitrite nitrogen value during post monsoon was recorded as 0.26μgat/l in January and the lowest as 0.18μgat/l during premonsoon, in April 1999. No seasonal distribution was observed in Narakkal, and the ranges for nitrite nitrogen between the seasons were not wide. (Figure 1.3).

During postmonsoon, (Table 4.3) no statistical correlation was observed withnitrate, ammonoia and phosphate. Monsoon season showed a 5%level positive corelation with phosphate(Table 4.2). Statistically significant negative correlations were observedwith nitrate and phosphate(Table 4.1)during premonsoon.

Station II(Valappu)

The general range of nitrite nitrogen recorded at Valappu was varying between 0.09μgat/l (May) and 0.79μgat/l(January) and the extremely high value observed in the month of July(4.93μgat/l) was occassional through the period of study(Figre 2.3). Nitrite nitrogen was absent during the month of June. Uniform order in distribution of nitrite nitrogen was observed in premonsoon, the values decreasing from 0.68 (February) to 0.09 (May) and the value was observed to be zero in June followed by a hike in value as 4.93 (July). Nitrite nitrogen value observed was less (0.80 μgat/l) in August which was found to be increasing through monsoon and post monsoons till the month of November (0.44ugat/l).

Statistically significant positive correlation existed between nitrate during monsoon and post monsoon(Table 5.2 and 5.3), whereas during premonsoon, no stastistical correlation was found with phosphate and , nitrate and ammonia.

1.7. NITRATE NITROGEN

Station I(Narakkal)

Among the three seasons , highest range of nitrate nitrogen was observed during premonsoon which was found to be fluctuating from 0.15μgat/l (February) to 1.17μgat/l(April 1999). Both monsoons and post monsoon seasons were showing high nitrate values as 0.52μgat/l and 0.11 μgat/l in the months of July and October 1999, whereas the lowest nitrate values were recorded as 0.13μgat/l (August) and 0.08μgat/l(November 1999). The annual range of nitrate nitrogen was 0.08ugat/l in November (postmonsoon) and 1.17 in April (premonsoon). The highest value recorded was only in April as low values were recorded throughout the period of study. Distribution was not uniform through seasons and the last three months of postmonsoon showed similar values as 0.08ugat/l except in the month of October (0.11ugat/l). (Figure 1.3)

A statistically significant correlation existed between nitrate, ammonia and phosphate during pre and post monsoon seasons. Monsoon showed negative correlation with nitrate at 1%level(Table 4.2).

Station II(Valappu)

Nitrate nitrogen at Valappu, was recorded high compared to Narakkal during monsoon, which was varying between 0.01µgat/l(September) and 2.14µgat/l(July). Nitrate nitrogen was absent in the month of June (monsoon). The range of nitrate fluctuated between 0.30µgat/l (May) and 1.56µgat/l (March) during premonsoon and 0.06µgat/l (November) and 0.76µgat/l (January) during post monsoon seasons respectively. Except for the high values recorded for the months of March (1.56µgat/l), April (1.06µgat/l) and July (2.14µgat/l), concentration of nitrate nitrogen in valappu pond water was low. Uniform distribution was not observed through the seasons, but from July to September (0.01µgat/l) of monsoon, a decreasing trend was observed and the concentration was found to increase during postmonsoon and the highest value as 0.76µgat/l was recorded in the month of January(Figure2.3).

Highly significant correlation was observed between ammonia and phosphate at 5% level during pre and post monsoon seasons(Table 5.1 and Table 5.3).

1.8. AMMONIA

Station I(Narakkal)

The values for ammonia ranged from 0.88µgat/l (post monsoon) to 14.99µgat/l (monsoon) in the months of November and September 1999 respectively. Generally the values recorded for ammonia were higher than 3.50 µgat/l with occasional lower values as 0.88µgat/l in November, 1.47µgat/l in January(postmonsoon) and 1.52µgat/l in April (monsoon). The lowest value recorded during premonsoon was 1.52µgat/l (April) and monsoon was 4.21µgat/l (June). During premonsoon, high values of 6.90µgat/l was recorded in the month of March. During post monsoon high values for ammonia was recorded as 4.31µgat/l during the month of October 1999. Uniform seasonal distribution was not observed during the period of study. July (monsoon) showed highest ammonia value as 1.68µgat/l, whereas the lowest value, 8.28µgat/l was observed during August. From September through the postmonsoon months, a gradual decrease in ammonia was observed except for the month of November.

Station II(Valappu)

Compared to station I, range of ammonia observed at Valappu was less (0.12 – 7.35µgat/l). During monsoon season 6.42µgat/l (June) ammonia was recorded high and 0.39µgat/l (September) was found as lowest(Figure2.3). The range of ammonia in post monsoon was found fluctuating between 0.98µgat/l (November) to 2.99µgat/l (October), whereas in premonsoon the range was from 0.12 to 7.35µgat/l in the months of March and May 1999. Considerable low values like 0.12µgat/l in March (premonsoon), 0.39µgat/l in September (monsoon) and 0.98µgat/l in November (post monsoon) were observed to be occassional during the period of study. Values observed are highly erratic with no seasonal trend in distribution. During monsoon, July and August showed values of ammonia as 3.23µgat/l and 5.63 µg at/l. February and April months of premonsoon season recorded highest values of 3.09 and 4.31 µg at/l respectively.

Ammonia showed significant positive correlation during post monsoon(Table 5.3) at 5% level and negative correlation during premonsoon (Table 5.1) with phosphate.

1.9. PHOSPHATE

Station I(Narakkal)

The highest value of phosphate was recorded in the month of April as 14.99µgat/l 1999(premonsoon) whereas, for the monsoon and post monsoon, the highest values were 11.86µgat/l(August) and 5µgat/l (October) respectively. The lowest values observed was 0.77 µgat/l in January (post monsoon). In February 2000 (premonsoon) and September 1999(monsoon), 2.99µgat/l and 6.08µgat/l were recorded as lowest phosphate values in Narakkal aquaculture pond. In the distribution of phosphate, occasional low values were observed only during post monsoon, as 1.29µgat/l, 0.83µgat/l and 0.77µgat/l from November to January respectively and a general decreasing trend was observed in the season. During premonsoon and monsoon, occassional high values were recorded as 14.19 µgat/l in April and 11.86µgat/l in August. The range of phosphate during premonsoon was from 2.99 µgat/l to 4.53µgat/l and during monsoon was from 6.08µgat/l to 7.68µgat/l respectively.(Figure 1.2)

Station II(Valappu)

Phosphate ranged from 1.03µgat/l in the month of November 1999 (postmonsoon) to 19.81 µgat/l in the month of June (monsoon). The highest value for postmonsoon (January 2000) and the lowest value for monsoon (September 1999) were recorded as 2.01µgat/l and 1.55 µgat/l respectively. The concentration of phosphate fluctuated between 4.18 µgat/l in the month of May and 10.07µgat/l in the month of March 1999(Figure 2.2).

The recorded values for phosphate was low during post monsoon, the range was observed as 1.03µgat/l(November) – 2.01µgat/l (January). The lowest value recorded for the period of study was occasional during monsoon, whereas July and August months showed high values as 9.24µgat/l and 7.69µgat/l respectively. Likewise, 10.07µgat/l was the occasional highest value during premonsoon and the general range was 4.18µgat/l – 5.98µgat/l (February). Considerable rise in phosphate content was observed on commencement of dry season at Valappu aquaculture pond.

Cultured fish (*Oreochromis mossambicus*) and environmental samples like water and sediment from two aquaculture ponds (Station I, Narakkal and station II, Valappu) were examined for a period of one year from March 1999 to February 2000 to assess the occurrence, distribution and activity of heterotrophic bacteria and environmental mycobacteria (Nontuberculous Mycobacteria, NTM) by taking the Total plate count (TPC) for heterotrophic bacteria and Total mycobacterial count (TMC) for Nontuberculous mycobacteria from samples of cultured fish like skin, gill, stomach, intestine and liver as well as pond surface water and sediment.

2. QUANTITATIVE ANALYSIS

2.1. HETEROTROPHIC BACTERIA

Station I(Narakkal)

The seasonal distribution of heterotrophic bacteria from fish samples. Skin showed highest mean TPC (179 x 10^3) during premonsoon was obtained from skin followed by intestine (118.8x103) during post monsoon, whereas gill was giving maximum mean TPC (67.25 x 10^3) during monsoon. In both premonsoon and monsoon periods liver harboured lowest mean count i.e., 5.75 x 10^3 and 2.13 x 10^3 respectively.

Table: 2.1. Mean TPC distribution among different samples in Station I

Mean Total Plate Count ($x10^3$)

Samples	Skin	Gill	Stomach	Intestine	Liver	Sediment	Water
Seasons							
Pre monsoon	179	22.2	30.35	54	5.75	36.9	178.75
Monsoon	64.4	67.25	30.4	34.9	2.13	18.79	36.5
Post monsoon	97.5	12.48	39.5	118.8	53.9	27.5	33.5

Table: 2.2. Mean TPC distribution among different samples in Station II

Mean Total Plate Count ($x10^3$)

Samples	Skin	Gill	Stomach	Intestine	Liver	Sediment	Water
Seasons							
Pre monsoon	127.6	85.7	59.58	139.1	11.8	25.65	15.38
Monsoon	101.4	18.5	101.43	38.5	6.28	33.56	100.5
Post monsoon	150.75	6.61	63.0	61.48	10.95	11.26	118.5

Table: 2.3. Mean TMC distribution among different samples in Station I

Mean Total Mycobacterial Count ($x10^3$)

Samples	Skin	Gill	Stomach	Intestine	Liver	Sediment	Water
Seasons							
Pre monsoon	25	6.36	3.38	1.75	0.73	8.73	7.85
Monsoon	16.74	9.13	7.66	25.9	2.58	4.41	110.95
Post monsoon	11.33	0.26	1.74	1.74	23.66	1.48	0.025

Table: 2.4. Mean TMC distribution among different samples in Station I

Mean Total Mycobacterial Count ($x10^3$)

Samples	Skin	Gill	Stomach	Intestine	Liver	Sediment	Water
Seasons							
Pre monsoon	3	16.53	22.85	2.25	275	3.82	9.48
Monsoon	90.73	16.09	63.3	11.15	0.25	22.59	8.58
Post monsoon	2.13	2.96	51.8	4.67	101.31	10.49	2.78

But liver tissue gave highest count (53.9 x 10^3) during post monsoon, which was higher than that from gill (12.48 x 10^3) in the same season. TPC observed from stomach during premonsoon and monsoon were of equal density i.e., mean count being 30.4 x 10^3. Intestine gave lowest mean TPC during monsoon (34.9 x 10^3) whereas during post monsoon, 54 x 10^3 could be retrieved from intestine. 97.5 x 10^3 and 64.4 x 10^3 were the mean TPCs obtained from skin from monsoon and post monsoon seasons respectively.(Table. 2.1.)

Among the environmental samples like water and sediment, highest mean TPC was obtained from water (178.75 x $10^{3)}$ during premonsoon, whereas the values for monsoon and post monsoon were 36.5 x 10^3 and 33.5 x 10^3 from sediment and the lowest (18.79 x $10^{3)}$ and highest (36.9 x $10^{3)}$ mean counts for heterotrophs were retrieved during monsoon and premonsoon season respectively. (Table. 2.1.)

Surface water gave the highest counts for heterotrophs, 314 x 10^3CFU/500ml and 318 x 10^3CFU/ 500ml, among the fish and environmental samples studied, in March and May 1999 respectively with the annual mean TPC as 82.9 x10^3. Highest annual mean value for heterotrophs was obtained from skin of the cultured fish (113.6 x $10^{3)}$ and the lowest from liver (20.6 x 10^3). But liver showed highest TPC of 180 x 10^3CFU/gm in October . Even though the annual mean TPC was 69.283 x 10^3, the sample could harbour maximum heterotrophs during August, November and January, the values being 46.5 x 10^3CFU/gm, 121 x 10^3CFU/gm and 312 x 10^3CFU/gm respectively. High counts of heterotrophs from skin were recovered as 80 x 10^3CFU/gm (April), 203.1 x 10^3CFU/gm (July) and 297 x 10^3CFU/gm (February). Gill harboured maximum heterotrophs of 192 x 10^3CFU/gm in September and sediment recorded 71 x 10^3CFU/gm during December.

Heterotrophs from various samples studied were showing interrelationships among each other. Heterotrophic bacteria from intestine and liver showed significant positive correlation at 1% level (r =0.992) during premonsoon season. Heterotrophs from gill tissue showed significant positive correlation ($P<0.01$; r= 0.720) with that from stomach. The saprophytic heterotrophs from intestine and liver showed a statistically significant positive correlation at 5% level ($P<0.05$; r= -0.626).

Station II(Valappu)

At Valappu, among all the fish samples, highest mean TPC values were observed in skin and stomach (101.4 x 10^3) during monsoon. Intestine (139.1 x 10^3) and skin recorded high values (150.75 x 10^3) during premonsoon and post monsoon respectively. The liver tissue harboured lowest mean counts like 11.8 x 10^3(premonsoon), 6.28 x 10^3(monsoon) and 10.95x10^3 (postmonsoon). Extremely highest mean count 85.7 x 10^3 was observed in gill during premonsoon compared with monsoon (18.5 x10^3) and post monsoon (6.61 x 10^3). Mean TPC of stomach and intestine during post monsoon were 63 x $10^3$and 61.48 x10^3 respectively, whereas skin could harbour 27.6 x 10^3 heterotrophs during premonsoon season. (Table 2.2.)

Overall mean TPC values showed that maximum values were from skin (126.58 x 10^3) followed by intestine (79.69 x 10^3) and lowest were recordedfrom liver (9.68 x 10^3). In environmental samples like water and sediment, water gave higher annual mean TPC (78.13 x 10^3) than sediment (23.49 x 10^3). Monsoon and post monsoon seasons recorded maximum mean counts like 100.5 x 10^3 and 118.5 x 10^3 respectively. The lowest mean TPC in water and sediment were 15.38 x 10^3 and 11.26 x 10^3 during pre and post monsoons respectively.(Table.2.2.)

Like station I, skin harboured highest TPC during months like April (315.2 x 10^3CFU/gm), June (321.6 x 10^3 CFU/gm), November (239 x 10^3CFU/gm), January (321 X 10^3CFU/gm) and February (79 x 10^3CFU/gm) among all the samples during the period of study, whereas stomach gave maximum counts in July (290 x10^3CFU/gm) and September (61.7 x 10^3CFU/gm) and lowest (5.55 x 10^3 CFU/gm) during December. The intestine was found harbouring the highest number of heterotrophs, the counts being 374x10^3 CFU/gm and 195x10^3 CFU/gm during May and October respectively. Water was found to have highest number of heterotrophs during months of August (312 x 10^3CFU/gm) and December (313 x 10^3CFU/gm). Whereas, highest TPC was recorded (322 x 10^3CFU/gm) from the gills during March.

Throughout the period of study skin gave the lowest TPC as 1.25 x 10^3CFU/gm in August whereas gills and stomach gave 1.8 x 10^3CFU/gm (April) and 5.55 x 10^3CFU/gm (December) respectively. 2 x 10^3CFU/gm was the lowest count observed from the liver in August and in intestine in February. From water and

Fig. 1.1 Effect of environmental parameters on TPC and TMC - station I

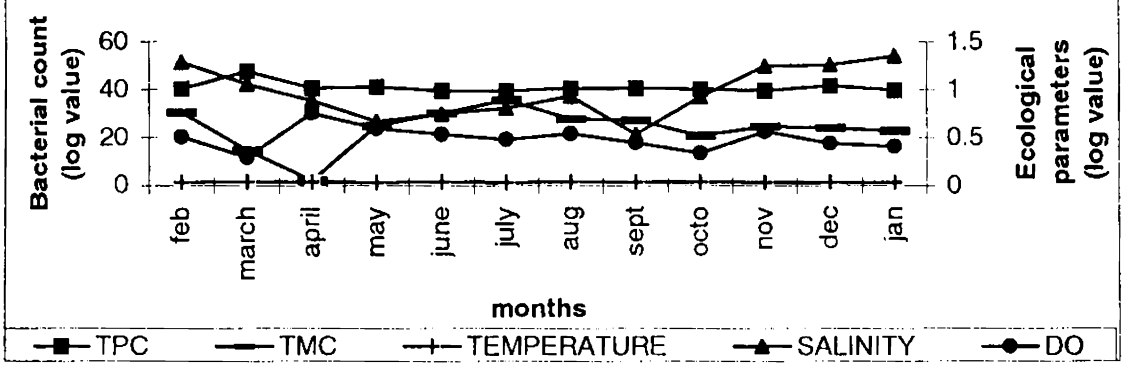


Fig. 1.2 Effect of environmental parameters on TPC and TMC - station I

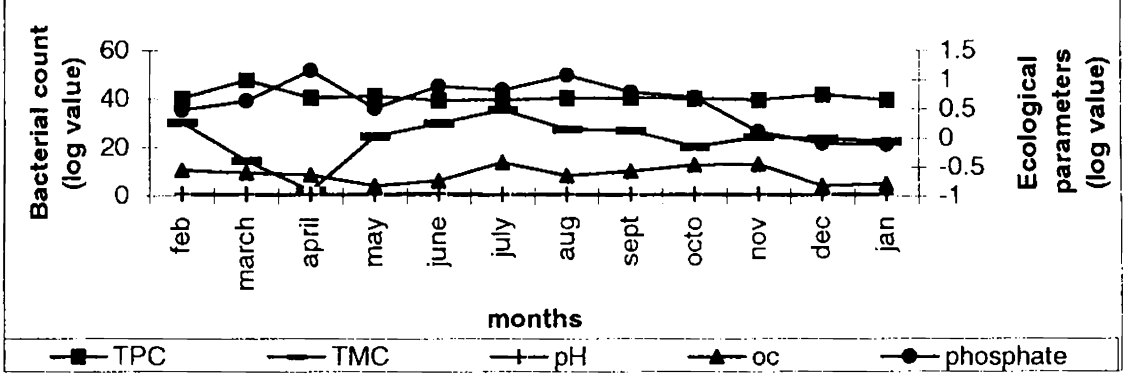


Fig. 1.3 Effect of environmental parameters on TPC and TMC - station I

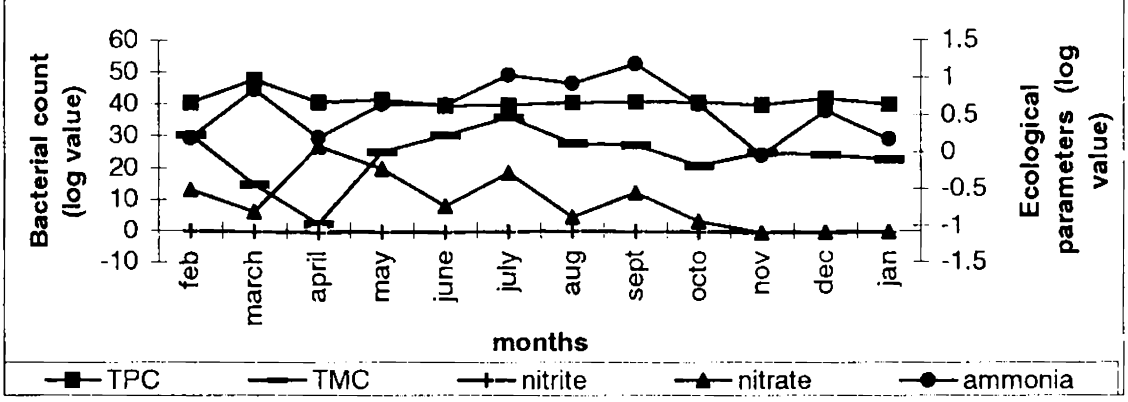


Fig. 2.1 Effect of environmental parameters on TPC and TMC - station II

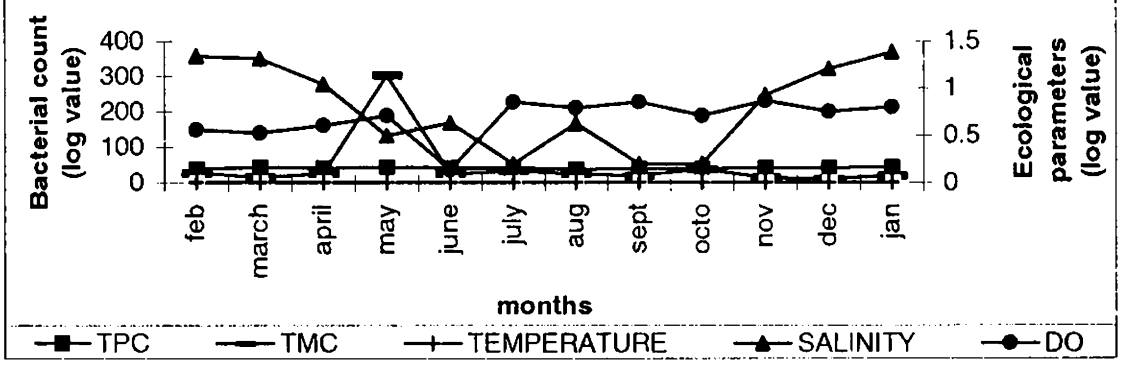


Fig. 2.2 Effect of environmental parameters on TPC and station II

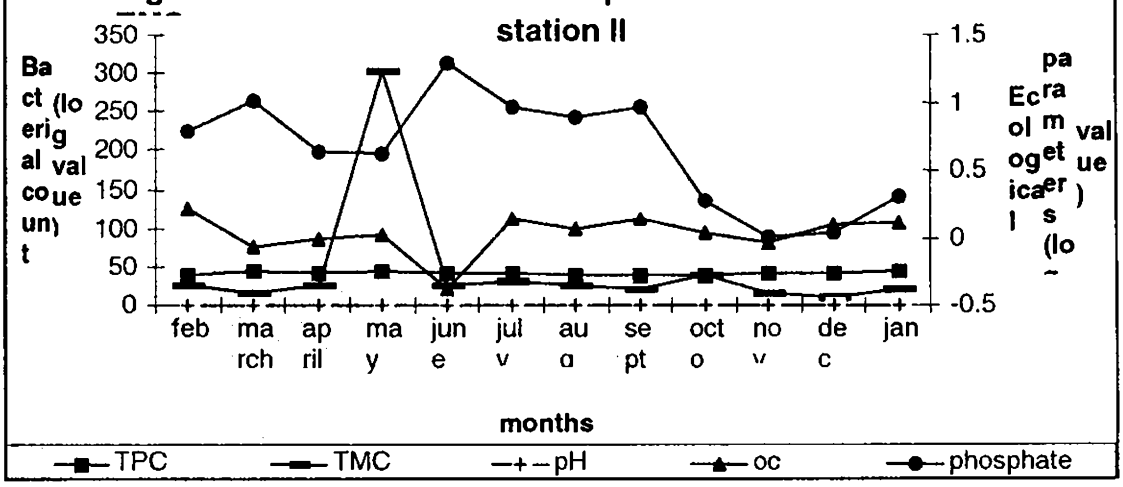
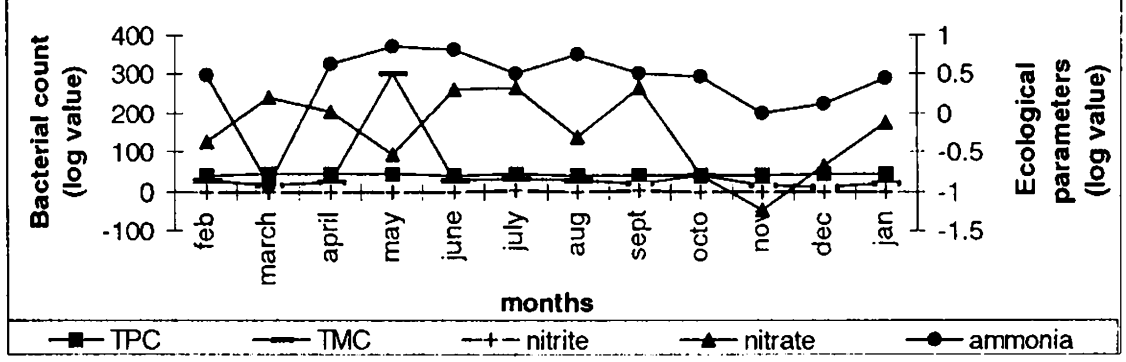


Fig. 2.3 Effect of environmental parameters on TPC and TMC - station II



sediment, minimum number of heterotrophs were retrieved, the count being 1.83 x 10^3CFU/gm (December) and 5 x 10^3CFU/gm (July) respectively.

Correlation coefficient between heterotrophs from skin and intestine showed a statistically significant postitive correlation at 5% level($P<0.05$; $r=0.594$).

2.2. NON TUBERCULOUS MYCOBACTERIA (NTM)

Station I(Narakkal)

NTM count obtained from fish showed that skin gave the highest mean count (17.69 X 10^3) and the lowest was recorded from gill (5.25 X 10^3). Liver harboured fairly high number of NTM giving the count as 8.99 X 10^3,whereas among all the samples, water gave maximum mean number of NTM (39.6 x 10^3). Sediment was observed with lowest count of 4.87 x 10^3. The mean NTM counts of different seasons showed that skin harboured maximum (25 x 10^3) during premonsoon whereas, intestine and liver gave highest count in monsoon (25.9 x 10^3) and post monsoon (23.66 x 10^3) respectively. But during monsoon extremely high mean NTM count was recorded from surface water, i.e., 110.95 x 10^3CFU/gm, whereas the pre and post monsoon were showing low values like 7.85 x 10^3 and 0.025 x 10^3 respectively. Sediment gave minimum counts during all the seasons with the lowest in post monsoon (1.48 x 10^3) and the highest in premonsoon (8.73 x 10^3) as given in Table. 2.3..

Premonsoon recorded the lowest mean TMC (0.73x 10^3) from liver. The pre and postmonsoon recorded same intensity of TMC from intestine (1.75 x 10^3). Gill showed fairly high NTM counts during premonsoon (6.36x 10^3) and monsoon seasons (9.13x 10^3CFU/gm) whereas post monsson recorded low count (0.26 x 10^3).Stomach retrieved maximum mean count during post monsoon (9.9x 10^3) whereas NTM count during premonsoon and monsoon seasons were minimum, the counts being 3.38 x 10^3 and 7.66 x 10^3 respectively. (Table. 2.3.)

NTM was retrieved from skin tissue in all the twelve months except in September1999 whereas water sample gave no representation in April, October, November and December 1999. Sediment and intestine showed presence of NTM during all the months of investigation, whereas NTM was not retrieved in March, June and September from liver. It is observed that from the gill NTM was not recorded in

April 1999 and January2000. No NTM was recorded from stomach during March and May 1999.

The lowest TMC observed for water and sediment was 0.1x 10^3CFU/gm in April and January respectively, but the highest TMC was recorded as 16.1 x 10^3CFU/gm (March). The range of TMC for intestine was, 0.001 x 10^3CFU/gm (May) – 46x10^3CFU/gm (July) and for stomach it was 2 x 10^3CFU/gm (April) –18x 10^3CFU/gm (January). 2x10^3CFU/gm was the maximum count obtained during April from stomach and intestine. During months of May, August and December, maximum TMC were retrieved from skin (25 x 10^3CFU/gm, 31.5 x 10^3CFU/gm and 41.4x 10^3CFU/gm) respectively. Liver harboured maximum TMC in November i.e., 86 x 10^3CFU/gm. In January (18x10^3CFU/gm) and February (11.5x 10^3CFU/gm), maximum TMC was observed from stomach. During September extremely high count of NTM as 110x 10^3CFU/gm recorded from water. Lowest TMC was observed from skin (0.87x 10^3CFU/gm) in June. Sediment recorded lowest count, 0.076x10^3CFU/gm in July. During September (0.037x10^3CFU/gm) October (0.003x10^3CFU/gm), November (0.086x10^3CFU/gm), December (0.94x10^3CFU/gm) and February (0.4x 10^3CFU/gm) lowest TMC were observed from stomach samples. Intestinal tissue harboured the minimum number of NTM during January, the count being 0.005x 10^3CFU/gm.

Station II (Valappu)

Table.2.4 is self-explanatory and shows that, the annual mean TMC from liver was highest (125.52x 10^3) and sediment retrieved maximum NTM (12.3x 10^3) in the study period. Among all the samples, intestine showed minimum mean TMC , the count being 6.02x 10^3. In premonsoon and post monsoon seasons liver tissue recorded highest TMC, 275x 10^3 and 101.31x 10^3 respectively and the count during monsoon was the lowest (0.25 x 10^3). Skin harboured maximum NTM count 90.73 x 10^3 during monsoon period whereas, in the same season, skin gave minimum mean TMC value (2.13 x 10^3CFU/gm) (Table- 2.4)

The range of TMC in all the samples during the period of study was fluctuating with the lowest count as 0.2 x 10^3CFU/gm (December) in skin and the highest as 66 x 10^3CFU/gm (March) in gill. In stomach and intestine, the range was recorded as 1.25 x

10^3CFU/gm (September)- 200 x 10^3CFU/gm (January) and 0.88 x 10^3CFU/gm (November) – 38.18 x 10^3CFU/gm (July) respectively. TMC fluctuated between 0.29 x 10^3CFU/gm (October) – 67.5 x 10^3CFU/gm (July) in sediment between 0.1 x 10^3CFU/ml(December) - 32 x 10^3CFU/500ml (July) in water.

TMC was completely absent in all samples during May except in sediment (1.29 x 10^3CFU/gm) indicating the scarcity of NTM during this month. In June and July, liver has not recorded any NTM. Gill and intestine in April was also devoid of NTM. NTM was not recorded in stomach during August and December and in water during January.

The highest TMC was recorded in gill in October (10.8 x 10^3CFU/gm) and lowest in January and February (0.1 x 10^3CFU/gm), whereas lowest counts were recorded during March (2 x 10^3CFU/gm) and September (1.25 x 10^3CFU/gm), and the highest counts of June (130 x 10^3CFU/gm), July (122 x 10^3CFU/gm) and January (200 x 10^3CFU/gm) from stomach. In November lowest count was obtained from intestine (0.88 x 10^3CFU/gm) and highest from liver (400 x 10^3CFU/gm). During September, liver recorded the minimum and sediment recorded the maximum TMC, the values being 0.1 x 10^3CFU/gm and 18 x 10^3CFU/gm respectively . Liver harboured maximum TMC during February (100 x 10^3CFU/gm) and March (300 x 10^3CFU/gm), whereas, minimum number of NTM were retrieved from sediment during December (3.46 x 10^3CFU/gm), October (0.29 x 10^3CFU/gm) and April (1 x 10^3CFU/gm). The lowest TMC was recorded from water during December (0.1 x 10^3CFU/500ml) and August (0.9 x 10^3CFU/500ml).

NTM from gill and sediment showed a significant correlation at 5%level ($P<0.05$), whereas NTM from intestine and water were found to have significant correlation at 1% level ($P<0.001$).

Table: 4.1. Correlation matrix showing effect of Physico-chemical parameters on TMC (premonsoon) – station I

	Mean TMC	Temp.	Salinity	DO	pH	Org.Carbon	Nitrite	Nitrate	Ammonia	Phosphate
Mean TMC	1									
Temp.	-0.46904	1								
Salinity	0.2846	0.45699	1							
DO	-0.37461	-0.5796Ψ	-0.43243	1						
pH	0.307776	-0.88514*	-0.16461	0.76288*	1					
Org.Carbon	-0.11913	0.65965Ψ	0.91784*	-0.28225	-0.28667	1				
Nitrite	0.770436*	0.167174	0.77034*	-0.7217Ψ	-0.16614	0.47556	1			
Nitrate	-0.44456	-0.5544	-0.54127	0.99018*	0.69416Ψ	-0.36697	-0.8074	1		
Ammonia	0.071756	0.328088	-0.33054	-0.7028Ψ	-0.7299Ψ	-0.38336	0.100568	-0.6012Ψ	1	
Phosphate	-0.94489*	0.269695	-0.22031	0.61575Ψ	-0.0084	0.166861	-0.78798*	0.65181Ψ	-0.38646	1

Ψ =(P<0.05) *=(P<0.01)

Table: 4.2. Correlation matrix showing effect of Physico-chemical parameters on TMC (monsoon) – station I

	Mean TMC	Temp.	Salinity	DO	pH	Org.Carbon	Nitrite	Nitrate	Ammonia	Phosphate
Mean TMC	1									
Temp.	-0.89862*	1								
Salinity	0.267699	-0.38789	1							
DO	-0.15532	0.216212	0.7778*	1						
pH	-0.75928*	0.96562*	-0.3475	0.308922	1					
Org.Carbon	0.66849Ψ	-0.84923*	-0.06607	-0.6713Ψ	-0.91212*	1				
Nitrite	-0.23333	-0.18562	0.5704Ψ	0.234467	-0.37526	0.178097	1			
Nitrate	0.77061*	-0.7287Ψ	-0.3366	-0.7485Ψ	-0.6893Ψ	0.86655*	-0.33639	1		
Ammonia	-0.04603	-0.26353	-0.427	-0.81196*	-0.47153	0.70870Ψ	0.374418	0.475476	1	
Phosphate	-0.3356	0.150118	0.8176*	0.84400*	0.102359	-0.45122	0.7099Ψ	-0.78391*	-0.37359	1

Ψ =(P<0.05) *=(P<0.01)

Table: 4.3. Correlation matrix showing effect of Physico-chemical parameters on TMC (post monsoon) – station I

	Mean TMC	Temp.	Salinity	DO	pH	Org.Carbon	Nitrite	Nitrate	Ammonia	Phosphate
Mean TMC	1									
Temp.	-0.7230Ψ	1								
Salinity	0.7437Ψ	-0.8895*	1							
DO	0.89796*	-0.7078Ψ	0.5239Ψ	1						
pH	-0.5338Ψ	0.7774*	-0.9573*	-0.25633	1					
Org.Carbon	-0.24423	0.242343	-0.6479Ψ	0.189371	0.78993*	1				
Nitrite	-0.5581Ψ	-0.1218	0.11574	-0.5799Ψ	-0.34116	-0.25597	1			
Nitrate	-0.9371*	0.8602*	-0.9302*	-0.7656Ψ	0.79297*	0.473741	0.246541	1		
Ammonia	-0.6207Ψ	0.9101*	-0.6239Ψ	-0.7913*	0.452133	-0.18117	-0.02538	0.6647Ψ	1	
Phosphate	-0.7603Ψ	0.7842*	-0.9781*	-0.47241	0.94577*	0.75237*	-0.01734	0.9283*	0.471285	1

Ψ =(P<0.05) *=(P<0.01)

Table: 5.1. Correlation matrix showing effect of Physico-chemical parameters on TMC (pre monsoon) – station II

	Mean TMC	Temp.	Salinity	DO	pH	Org.Carbon	Nitrite	Nitrate	Ammonia	Phosphate
Mean TMC	1									
Temp.	-0.9310*	1								
Salinity	-0.9409*	0.9886*	1							
DO	0.9172*	-0.9308*	-0.9752*	1						
pH	0.074421	-0.09226	-0.22835	0.417946	1					
Org.Carbon	-0.09073	0.378315	0.25179	-0.05476	0.525896	1				
Nitrite	-0.7876*	0.95688*	0.9208*	-0.8292*	-0.0316	0.6033Ψ	1			
Nitrate	-0.7294Ψ	0.492683	0.592378	-0.7151Ψ	-0.47468	-0.611Ψ	0.231587	1		
Ammonia	0.520089	-0.48924	-0.6128Ψ	0.7686Ψ	0.8895*	0.442271	-0.3618	-0.7606Ψ	1	
Phosphate	-0.5355	0.6003Ψ	0.7011Ψ	-0.8194*	-0.8507*	-0.19402	0.540149	0.61056Ψ	-0.9631*	1

Ψ =(P<0.05) *=(P<0.01)

Table: 5.2. Correlation matrix showing effect of Physico-chemical parameters on TMC (monsoon) – station II

	Mean TMC	Temp.	Salinity	DO	pH	Org.Carbon	Nitrite	Nitrate	Ammonia	Phosphate
Mean TMC	1									
Temp.	0.043403	1								
Salinity	-0.04334	-0.9998*	1							
DO	0.025452	0.6424Ψ	-0.6549Ψ	1						
pH	0.02891	0.6080Ψ	-0.5949Ψ	-0.21774	1					
Org.Carbon	0.027678	0.6905Ψ	-0.7023Ψ	0.997914*	-0.15428	1				
Nitrite	0.04055	0.902842*	0.8956*	0.25054	0.8903*	0.312516	1			
Nitrate	0.029076	0.6120Ψ	-0.599Ψ	-0.21275	0.9997*	-0.14923	0.8926*	1		
Ammonia	-0.04245	-0.9889*	0.9912*	-0.748Ψ	-0.48377	-0.7900*	-0.8292*	-0.48823	1	
Phosphate	-0.01433	-0.39736	0.412354	-0.9585*	0.48692	-0.9381*	0.035815	0.482454	0.528881	1

Ψ =(P<0.05) *=(P<0.01)

Table: 5.3. Correlation matrix showing effect of Physico-chemical parameters on TMC (post monsoon) – station II

	Mean TMC	Temp.	Salinity	DO	pH	Org.Carbon	Nitrite	Nitrate	Ammonia	Phosphate
Mean TMC	1									
Temp.	0.5439Ψ	1								
Salinity	-0.8424*	-0.8102*	1							
DO	-0.5632Ψ	0.136691	0.467854	1						
pH	0.7586Ψ	0.945287*	-0.8557*	0.001167	1					
Org.Carbon	-0.05198	-0.865v	0.449448	-0.52206	-0.67853	1				
Nitrite	-0.25025	-0.5323	0.694Ψ	0.428923	-0.36543	0.451604	1			
Nitrate	0.022918	-0.78128*	0.48695	-0.3066	-0.53552	0.9302*	0.7144Ψ	1		
Ammonia	0.74138Ψ	-0.12895	-0.28432	-0.6129Ψ	0.192061	0.5858Ψ	0.299358	0.6878Ψ	1	
Phosphate	0.7233Ψ	-0.07967	-0.2381	-0.45176	0.249397	0.509664	0.422094	0.6788Ψ	0.9813*	1

Ψ =(P<0.05) *=(P<0.01)

1	1												
2	0.464702	1											
3	0.52931	**0.720214**	1										
4	0.227529	**-0.62627**	-0.21024	1									
5	-0.1515	**-0.66594**	**-0.63324**	0.421504	1								
6	0.289055	-0.16432	-0.23196	0.239753	0.489163	1							
7	0.231508	-0.27915	0.093339	0.37339	0.396206	0.295765	1						
8	0.136199	0.133334	0.030354	-0.12628	0.215614	0.426338	0.27753	1					
9	-0.06175	0.548699	0.430743	**-0.70409**	**-0.60471**	-0.09	-0.51212	0.004606	1				
10	**-0.56611**	-0.24963	-0.13664	0.106365	-0.32304	-0.30144	-0.30133	-0.33309	0.161392	1			
11	-0.24079	0.479534	0.196345	-0.46344	-0.43047	-0.11044	-0.53784	0.335755	0.535869	0.228853	1		
12	-0.45951	-0.42282	-0.19164	0.10974	0.147519	-0.30301	0.149684	-0.097	0.03936	0.298263	0.11484	1	
13	-0.19898	0.130239	-0.1376	-0.40397	-0.48157	0.023166	-0.51359	-0.24068	0.64443	0.493459	0.192035	-0.02674	
14	0.51484	0.535857	0.446399	-0.2511	-0.31758	0.493809	0.083833	0.466405	0.331886	-0.22875	0.093474	**-0.73411**	

Columns 1. TPC - Skin
2. Gill
3. Stomach
4. Intestine
5. Liver
6. Sediment
7. Water
8. TMC - Skin
9. Gill
10. Stomach
11. Intestine
12. Liver
13. Sediment
14. Water

2	**0.270386**	1											
3	-0.00765	-0.24905	1										
4	**0.594039**	0.141059	0.214576	1									
5	0.440436	-0.14837	-0.26674	0.064283	1								
6	0.164687	-0.03204	0.390697	0.025856	0.084434	1							
7	0.013695	-0.08327	-0.1155	0.310379	-0.44238	**-0.70074**	1						
8	-0.17862	-0.5902	0.17035	-0.27122	0.032177	-0.01929	0.247144	1					
9	0.070795	0.250474	0.004327	0.498608	-0.11944	-0.56695	0.573455	-0.28426	1				
10	-0.03079	0.21409	**0.635343**	-0.1331	-0.07423	0.51888	-0.48125	0.232703	-0.29495	1			
11	-0.396	0.208089	-0.44219	-0.52157	0.144238	0.026975	-0.33012	0.105069	-0.22706	0.158176	1		
12	-0.24869	-0.40103	0.089239	-0.07016	-0.13807	0.508791	-0.26693	-0.0428	-0.25705	-0.14357	0.035012	1	
13	0.282216	-0.05413	0.165972	0.067154	0.342394	**0.690949**	**-0.72244**	-0.10721	**-0.64377**	0.371441	0.100456	0.438454	1
14	**-0.6897**	0.273055	-0.37201	**-0.69673**	-0.24674	0.03363	-0.35863	-0.12617	-0.33568	0.135922	**0.780502**	0.081464	0.00784

Columns 1. TPC - Skin
2. Gill
3. Stomach
4. Intestine
5. Liver
6. Sediment
7. Water
8. TMC - Skin
9. Gill
10. Stomach
11. Intestine
12. Liver
13. Sediment
14. Water

2.3. STATISTICAL CORRELATIONS

2.3.1. CORRELATIONS BETWEEN NTM AND PHYSICO- CHEMICAL PARAMETERS

In Stations I and II, premonsoon, monsoon and postmonsoon seasons were observed with high influence on the occurrence anddistribution of NTM as shown in Tables 4.1-4.3 and 5.1-5.3.

In station I, during pre monsoon, nitrite and phosphate content in water($P<0.01$) were observed as the factors affecting distribution of NTM, whereas in station II during the same season, temperature, salinity, pH, dissolved oxygen, nitrite ($P<0.01$) and nitrate ($P<0.05$) were observed as influencing factors.

Tempetrature, nitrate ($P<0.01$) and organic carbon($P<0.05$) content showed distinct 1% and5%level correlations with TMC in Station I during monsoon season. During post monsoon, the occurrence of TMC in Station II was affected by all factors studied except organic carbon, and in Station II, salinity($P<0.01$), dissolved oxygen, pH, ammonia and phosphate ($P<0.05$) were observed as influencing factors.

2.3.2. CORRELATIONS BETWEEN HETEROTRO BACTERIA AND NONTUBERCULOUS MYCOBACTERIA

In station I, only TPC of skin tissue was observed to be correlated negatively($P<0.05$) with TMC of stomach(Table 6.1). In Station II, distinct 5% level positive and negative correlations were found with TPC from skin, stomach,intestine, sediment and water with TMC from water, stomach and sediment samples(Table 6.2).

2.4. PERCENTAGE OF NTM OCCURRENCE

Percentage of occurrence of mycobacteria for the one year period of study at Narakkal was 21. The incidence of highest percentage of mycobacteria was in the stomach (28.4%) and the lowest was observed in liver sample(12.90%) as shown in figure 3.1. Of the three seasons studied , during pre monsoon 32.8% of the heterotrophs was NTM which was the highest percentage observed during postmonsoon the

Table: 7. Percentage of NTM observed in stations I and II

Station I (Narakkal)

	Samples studied							
Seasons	**Skin**	**Gill**	**Stomach**	**Intestine**	**Liver**	**Sediment**	**Water**	**Total**
Pre monsoon	10.3	19.9	28.2	23.6	10.2	37.1	2.5	20.3
Monsoon	35.9	31.5	34.7	39.2	15.8	26.0	46.7	32.8
Postmonsoon	14.4	2.0	30.3	14.8	12.9	12.5	0.25	12.4
Total	20.6	17.8	28.4	25.3	25.2	25.2	16.5	21.0

Station II (Valappu)

	Samples studied							
Seasons	**Skin**	**Gill**	**Stomach**	**Intestine**	**Liver**	**Sediment**	**Water**	**Total**
Pre monsoon	1.7	4.9	24.6	13.7	4.1	11.4	35.4	13.7
Monsoon	35.9	19.5	20.4	32.2	8.1	27.9	22.7	24.3
Postmonsoon	10.4	21.1	11.1	12.0	30.5	22.3	2.9	15.7
Total	17.3	15.1	18.7	19.3	15.0	20.5	20.3	18.0

percentage was only 12.4, the lowest recorded. With the incidence of 0.25% of NTM, water sample during post monsoon was found to be harbouring lowest count of mycobacteria , whereas water during monsoon showed highest mycobacterial occurrence(46.7%), highest percentage of all the samples in three seasons. During premonsoon and postmonsoon seasons, occurrence of NTM showed its maximum percentage in sediment (37.1%) and stomach(30.3%) samples respectively(Table 7).

In Valappu, throughout the period of study 18% of NTM were retrieved with highest and lowest percentages observed in sediment and liver samples, even though the high NTM counts in Liver were only occasional (Figure 3.2.). During monsoon, pre monsoon and postmonsoon seasons, the percentage of occurrence of NTM were 24.3, 13.7 and 15.7 respectively. During pre monsoon, highest percentage of mycobacteria (35.4) was retrieved from water sample and in post monsoon it was from liver with 30.5% of the total heterotrophs. Skin sample was giving highest mycobacterial representation with 35.9% during monsoon season.(Table 7).

2.5. FREQUENCY AND DISTRIBUTION OF NTM

Station I (Narakkal)

Of 84 samples studied,344 NTM isolates were recovered from 73 samples (86.9%) in the station which formed 49.9% of the total (n=689). NTM was yielded by100% (N=12/12) of the intestinal and stomach tissues as well as sediment, whereas 58.3 % of water and 91.7% of the skin and gill samples showed mycobacterial incidence. NTM were recovered from 8 samples(66.7%) of liver out of total 12 examined during the study.

The pattern of recovery of NTM from fish and environmental samples were irregular in this area of study.. Maximum of 84 strains (24.4%) were retrieved from intestinal tissues and minimum of 18 isolates (5.2%) from water sample. Figure 4.1. shows the distribution pattern of the NTM among 7 different samples (from skin to water). Retrieval of NTM was also lowest from liver tissues which was only 5.5% (N=19/344) of the total . Among the three seasons, post monsoon season harboured maximum number of NTM in the station, ie. 39.2% (N=135/344) of the total. Premonsoon season showed low prevalence of NTM only with 96 (27.9%) isolates.

Fig. 3.1 Percentage of NTM - Valappu

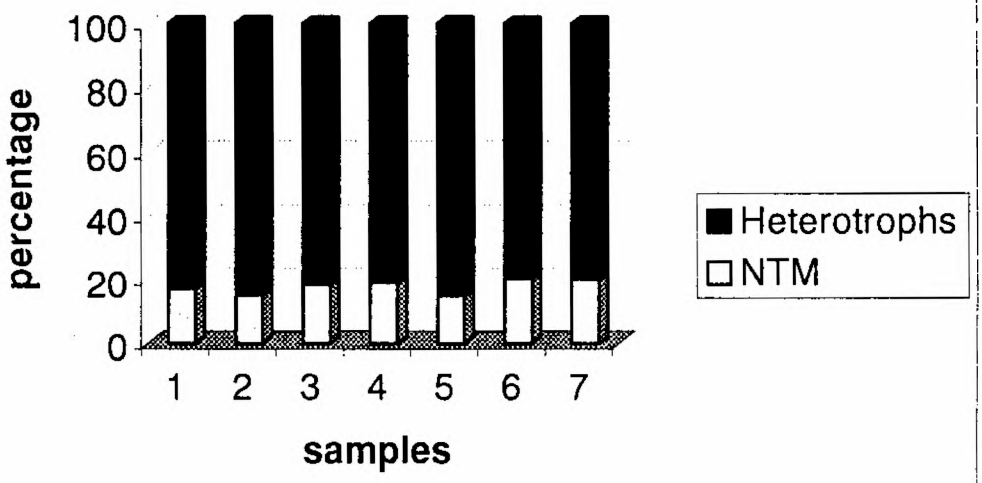
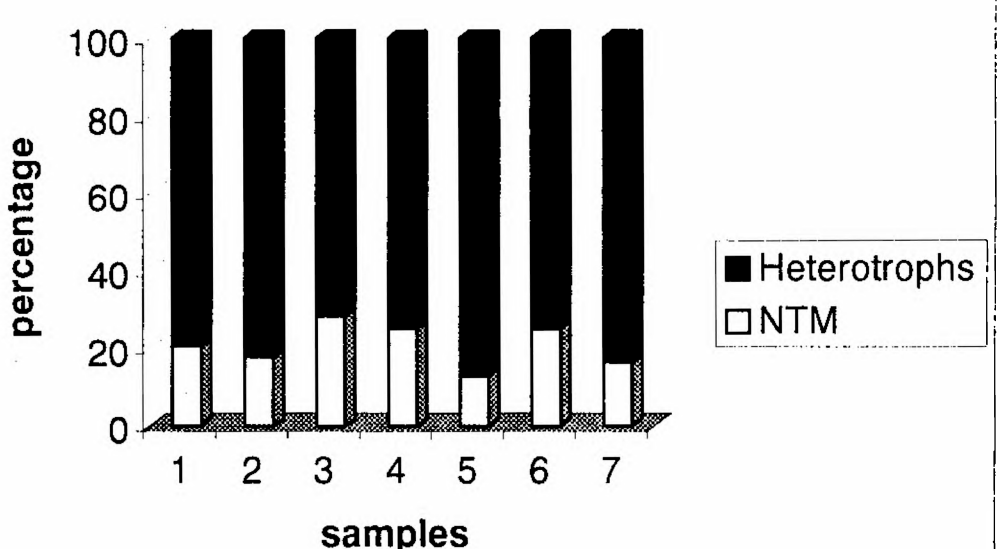


Fig. 3.2 Percentage of NTM- Narakkal



Station II (Valappu)

Among the 84 samples examined, 92.9%(n=78) of NTM were recovered from skin, stomach and intestinal tissues whereas sediment and water yielded NTM during the whole months of examination. But 83.3%(N=10/12) of gill tissue and 66.7% (N=8/12) of liver tissue showed mycobacterial recovery in the study.

From fish and environmental samples of Valappu aquaculture pond, a total of 345 strains of NTM were obtained. Maximum number of 85 (24.6%) isolates were recovered from sediment and minimum, forming 4.9%(n=17) from liver tissue . As Figure 4.2. shows from stomach and intestinal tissues, 65(18.8%) and 68(19.7%) of NTM were isolated respectively whereas water sample yielded only 48 strains of mycobacteria.

Highest incidence of NTM with 120 strains(34.8%) was observed during monsoon and lowest during premonsoon(n=108) . Frequency of occurrence of NTM was found to be high during Postmonsoon, yielding 33.9% (N=117/345) of the total.

3. QUANLITATIVE ANALYSIS

3.1. CLASSIFICATION OF NTM

After three days of incubation at RT and37°C, all the colonies developed on Nutrient agar, PTB and LJ media were acid fast stained and enumerated. TMC and TPC were counted from NA plates incubated at RT. Plates and slants ,incubated at RT were seen to develop maximum number of strains than at 37 °C. Periodical observation of incubated plates and slants were carried out up to 3 weeks for NA medium and up to 5-6 weeks for PTB and LJ media as prolonged incubation was impossible due to some technical difficulty. The number of colonies developed on PTB and LJ media were ranging from one to hundreds.

The acid fastness of the strains were varying from 10 and 100% and the cells were morphologically coccoid, plumpy or long rods. It was interesting to note that the degree of acid-fastness was related with the source of mycobacteria, isolates recovered

from environmental samples showed less than 50% of acid fastness and the degree was higher in those from fish samples.

NA showed varied as well as innumerable colony morphologies for acid fast strains. Isolation as well as sub culturing of the strains were found to be easy from agar based media, NA and PTB. Pigmentation was highly variable among isolates which were greenish yellow, light yellow, bright yellow , light orange , dark orange , rosy pink or rarely red.

Isolates found to be acid fast were isolated from the media and sub cultured either on Peptone Agar with 2%glycerol or LJ media slants. Some strains developed on PTB and LJ media failed to multiply on these simple media but gradually observed to adapt on these. Maintenance of the cultures for long periods was done at 4°C under refrigeration with periodical sub culturing.

3.1.1. Classification of NTM as slow and fast growers

In the present study, strains giving positive result for Iron uptake test(Wayne and Doubek, 1968) were roughly grouped and accounted as fast growers and negative as slow growers. NTM strains were broadly classified as slow and fast growers according to the results of iron uptake positive for fast growers and negative for slow growers. Due to unexpected fungal contamination, inability of growth of isolates during sub culturing as well as difficulty in getting pure isolate form mixed bacterial flora, only 83.3%(N=574/689) of the recovered strains were purely isolated, classified and identified up to species level. Tables 8.1. and 8.2. indicate the number of slow and fast growers isolated and identified from station I and II and the prevalence of fast growers in these study areas. Of the 310 isolates identified in station I, 49% (n=152) were slow growers and 51.0 % (n=158) were fast growers, whereas 126 slow growers and 167 fast growers in station II were identified up to species level (Tables 8.1 and 8.2). Fig. 4.1 and 4.2 shows the distribution fo NTM among seven different samples studied in stations I and II respectively.

Table: 8.1. Distribution of pigmented and non pigmented slow and fast growers - Station I

	Skin	Gill	Stomach	Intestine	Liver	Sediment	Water	Total
				Number of slow growing NTM				
				Samples				
Pigmented	6	4	15	13	5	11	4	58
Non pigmented	12	7	18	29	7	17	4	94(32.1)
Total	18	11	33	42	12	28	8	152
				Number of fast growing NTM				
Pigmented	5	6	11	7	4	10	2	45
Non pigmented	19	11	20	19	2	19	6	96(32.8)
Total	24	17	31	26	6	29	8	141

Table: 8.2. Distribution of pigmented and non pigmented slow and fast growers - Station II

	Skin	Gill	Stomach	Intestine	Liver	Sediment	Water	Total
				Number of slow growing NTM				
				Samples				
Pigmented	7	3	11	12	5	11	5	54
Non pigmented	8	5	14	16	3	16	10	72
Total	15	8	25	28	8	27	15	126
				Number of fast growing NTM				
Pigmented	5	5	9	13	6	13	10	61
Non pigmented	12	8	19	17	1	20	17	94
Total	17	13	28	30	7	33	27	155

Fig.4.1 Distribution of NTM in Narakkal among different samples

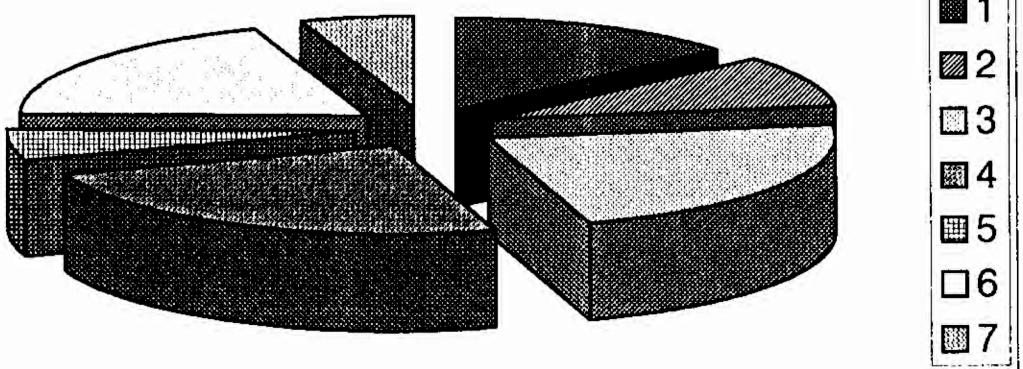
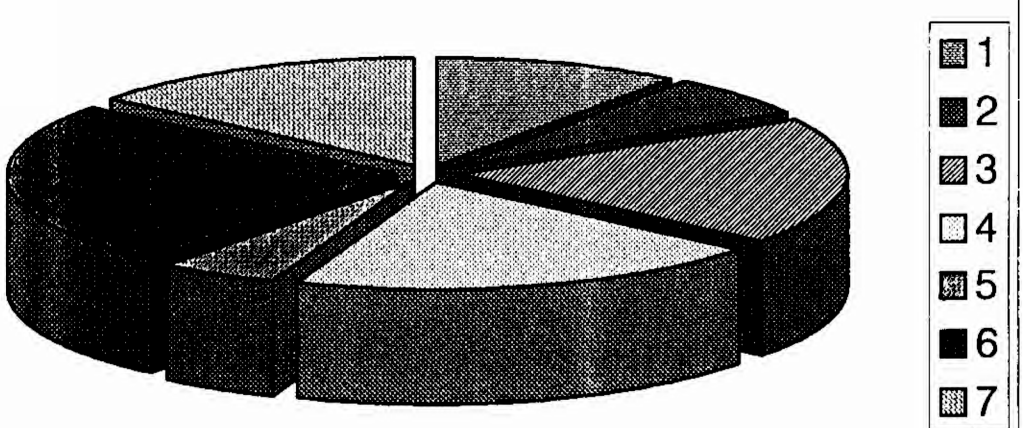


Fig.4.2 Distribution of NTM among different samples- Valappu



Station I(Narakkal)

Table 8.1. shows the uneven distribution of slow and fast growing NTM among different samples examined. Of the 152 slow growers identified in station I, intestinal tissue of fish yielded maximum number of 42 strains (27.6%) whereas only 33 strains were retrieved from stomach. NTM in skin and gill tissue of fish as well as water samples recorded to be with frequency of NTM as 11.8% (n=18), 7.9% (n=12) and 5 .3% (n=8) respectively(Table 8.1). Stomach was found to harbour maximum number of fast growers(36 strains). Liver tissue (n=6) of fish and sediment with 33isolates of NTM as well as water (n=9) was with minimum mycobacterial incidence in station I. The intensity of NTM was high in skin and intestine of fish with 27 and 28 numbers of fast growers.

Seasonal distribution of identified slow and fast growing NTM in station I was uniform in three seasons. Highest retrieval of slow growers (n=67; 44.1 %) and fast growers (n=59; 37.3 %) was from Post monsoon season of station I, indicating the maximum intensity of mycobacterial flora during the season. Premonsoon yielded the minimum number (n=40) of slow and fast growers (n=47) during the period of study (Figure 5.1)

Station II(Valappu)

The frequency of occurrence of slow growing NTM was observed to be maximum in the intestine of fish (n=28) followed by sediment (n=27) and stomach(n=25). As per Table 9.2, the incidence of slow growers in gill and liver was found as equal with 8 strains each. Mycobacterial intensity was same for skin and water (15 strains). Maximum number (33 strains) of fast growers were isolated from sediment, whereas stomach and intestine yielded only 28 and 30 isolates respectively. Minimum number of 7 strains was recorded from liver tissue of fish. Table 9.2 indicated that there is abundance of fast growing NTM in water with maximum of 27 strains retrieved.

Monsoon and pre monsoon seasons were observed with highest abundance of fast growing (n=57) and slow growing NTM (n=50) in station II. As Figure 5.2. shows, the distribution of fast growers in pre and Postmonsoon were almost equal . Monsoon season was recorded with lowest slow growing mycobacterial abundance in the station I.

Fig. 5.1 Seasonal distribution of NTM-Station I

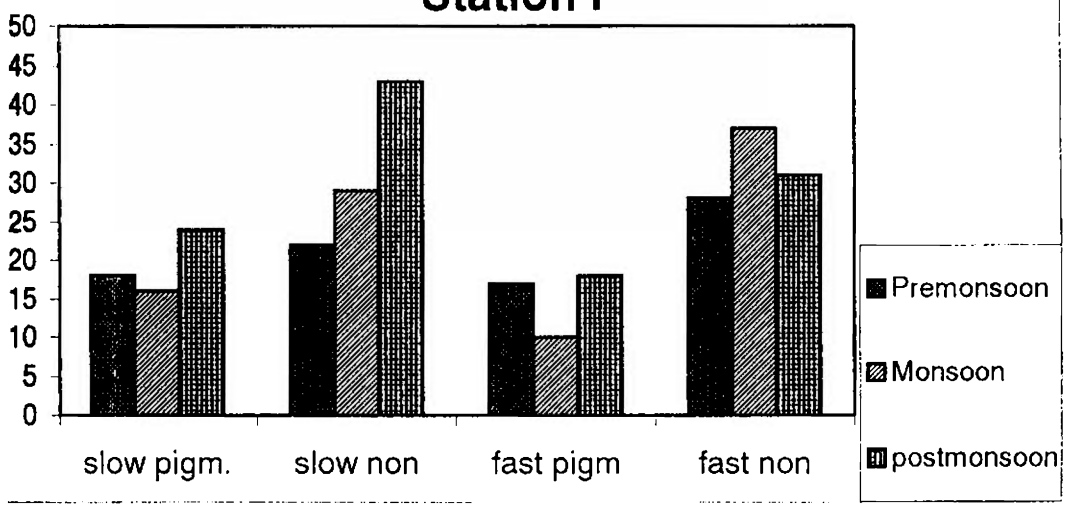
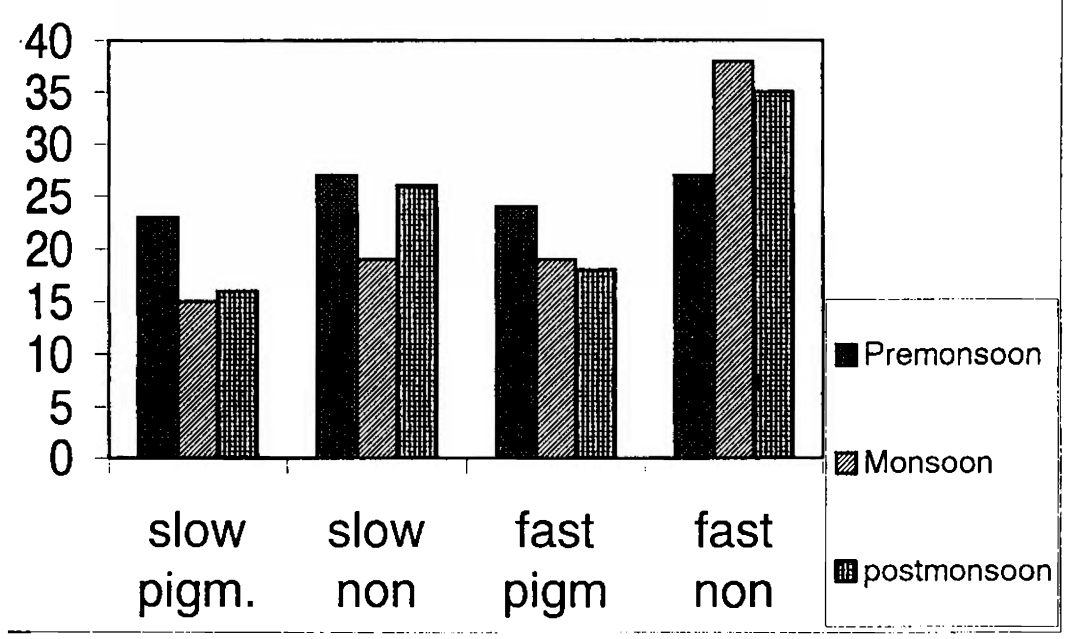


Fig. 5.2.Seasonal distribution of NTM-Station II



3.1.2. Classification of NTM as pigmented and nonpigmented slow and fast growers

Identified slow and fast growing NTM in station I and II were classified as pigment producers and nonpigment producers. Pigmentation of the strains was observed to be varying from yellow, dark orange or rarely dark red in different intensities according to cultural conditions prevailing. Among the pigmented slow and fast growers, some strains showed pigmentation property as a result of an affinity toward light termed as photochromogens and those which gave pigment in darkness are grouped in scotochromogen.

Station I(Narakkal)

Among the slow and fast growers, nonpigmented strains were more forming 32.1% and 32.8% respectively. Within samples studied, pattern of occurrence was not uniform for the four groups of NTM as in Table 8.1. Pigmented slow growers were retrieved maximum from stomach (N=15) followed by intestinal tissue (N=13). Intestine gave maximum number of 29 isolates of nonpigmented NTM whereas gill and liver tissue as well as water samples were observed with minimum occurrence of both pigmented and nonpigmented NTM as Table 8.1. shows, whereas, among 45 pigmented fast growing NTM stomach and sediment were recorded with high NTM prevalence with 11 and 10 strains respectively. Water recorded 2 isolates of pigmented fast growers. Stomach tissue gave 20 strains of the total 96 nonpigmented fast growers in station I followed by skin, intestine and sediment samples with 19 isolates each. Liver tissue showed the least abundance of nonpigmented fast growers.

With maximum retrieval of 24 pigmented and 43 nonpigmented slow growers, Postmonsoon was found to be with highest frequency of these NTM, whereas monsoon was with lowest occurrence of pigmented slow growers (N=16). Frequency of fast growing pigmented NTM were minimum (N=10) and of fast growing nonpigmented NTM were maximum (N=37) during monsoon (Figure 5.1). Highest number of fast growing pigmented NTM (N-18) were recorded in postmonsoon and premonsoon recorded only lowest number (N=28) of fast growing nonpigmented strains.

Station II(Valappu)

Table 8.2. represents the distribution of nonpigmented and pigmented fast and slow growing NTM in different samples in station II. Totally 12 pigmented and 16 nonpigmented NTM were recovered, intestine recorded maximum intensity of slow growers. Gill and liver tissues gave 3 strains of pigmented and nonpigmented slow growers respectively, which showed the lowest mycobacterial abundance in these tissues. Occassional high intensity of nonpigmented slow growing and pigmented fast growing NTM was recorded from water retrieving 10 strains each. (Table 8.2.). Intestine and sediment showed maximum intensity for pigmented fast growing NTM occurrence with 13 strains, whereas skin, gill and liver were showing minimum abundance of pigmented fast growers. Only a sigle strain of nonpigmented fast growers were recorded in liver tissue indicating the negligible frequency of the group of NTM in the tissue.

Abundance of pigmented and nonpigmented slow growers, as well as pigmented fast growers were observed maximum during premonsoon with 23, 27 and 24 strains respectively (Figure 5.2.), whereas, the monsoon was with minimum frequency of occurrence of pigmentd (N=15) and nonpigmented (N=19) slow growers. Among 94 nonpigmentd fast growers, 38 strains weree isolated from monsoon and minimum number of 27 from premonsoon.

3.2. IDENTIFICATION OF NONTUBERCULOUS MYCOBACTERIA

Biochemical identification method published by Pattyn and Portaels (1972) was the basic scheme followed, gives separate sets of biochemical tests for rapid and slow growers as shown in Tables 9.1, 9.2, 9.3. Strains negative for iron uptake test were further tested for growth in 5% NaCl, positive for this test were treated as both slow and fast growers biochemically to understand exact specific identities because some of the species are intermediate in their respective growth rates.

33 species of NTM were identified from 574 isolates tested from stations I and II in varying frequencies as shown below.

Table: 9.1. Identification of rapidly growing mycobacteria (modified from Pattyn and [illegible])

	Pigment production			Temperature range (°C)				Carbohydrate fermentation				Citrate utilization	Benzoate utilization	Nitrate reduction	Acid phosphatase	Tween 80 hydrolysis	Aryl sulphatase	Putrescine oxidase	Resistance to hydroxylamine	Iron up take	Colonial morphology	
	otochromic	Scotochromic	Nonchromic	37	42	45	52	Glucose	Inositol	Mannitol	Sucrose										OAA	CMA
M. fortuitum	-	-	+	+	+	-	-	+	-	-	-	+	-	+	+	M	+	+	+	+	SmF	Smf/Rf
M .chelonei	-	-	+	+	-	-	-	+	-	-	-	M	-	-	+	F	+	+	+	-	R/SmSa	R/SmSa
M. smegmatis	-	-	+	+	+	+	-	+	+	+	-	+	+	+	-	+	-	+	-	+	R	R
M. phei	-	+	-	+	+	+	+	+	-	+	-	+	-	+	+	+	-	+	-	+	R	R
M. vaccae	+	-	-	+	+	-	-	+	+	+	+	+	+	M	-	+	-	M	-	+	R/SmK	R/SmK
M. parafortuitum	F	+	-	+	+	-	-	+	M	+	M	+	-	+	-	+	-	+	-	M	R/SmK	R/SmK
M. chitae	-	-	+	+	-	-	-	+	+	+	-	-	+	+	+	+	-	+	+	-		
M. duvalii	-	+	-	+	+	-	-	+	-	+	-	-	+	+	-	+	-	+	-	-		
M. gilvum	-	+	-	+	-	-	-	+	+	+	-	+	-	+		+	+	+	-	+		
M. gadium	-	+	-	+	-	-	-	+	+	-	-	-	-	+	+	-	-	+	-	-		
M. arurum	-	+	-	+	-	-	-	+	+	+	M	+	-	F	-	+	-	+	-	+		
M. neoaurum	-	+	-	+	-	-	-	+	+	+	-	+	-	M	-	+	-	+	M	+		
M. marinum	+	-	-	F	-	-	-	M	-	-	-	-	-	-	+	+	-	+	+	-	R/SmK	R/SmK
M. flavescens	-	+	-	+	M	-	-	+	-	M	-	-	F	+	-	+	+	-	-	-	**SmS/SmK**	**SmS/Sm**
M. thermoresistibile	-	+	-	+	+	+	+	-	-	F	-	-	-	+	-	+	-	-	-	-	**SmG/R**	**K SmG**

Key: + = > 85% of strains positive; - = < 15% of strains positive; M = 50 to 85% of strains positive; F= 15 to 49% of strains positive; Blank spaces = few or no data available; OAA = oleic acid albumin agar; CMA= cornmeal agar: SmF: Smooth Fortuitum: Smf = Smooth with filamentous centre: R= Rough; Rf = Rough filamentous; SmSa = Smooth scotochromogen surrounded by an apron; SmK= Smooth Kansasii: SmG = Smooth Kansasii: SmG; Smooth Granular; SmS = Smooth scotochromogenic

	Pigment production			Temperature range (°C)			Resistance to														Colonial morphology
	Photochromic	Scotochromic	Nonchromic	33	37	42	INH	TCH	IIA	PNB	Catalase 45 mm	Niacin production	Nitrate reduction	Acid phosphatase	Tween 80 hydrolysis	b-Galactosidase	Growth in 5% (w/v) NaCl	Urease	Nicotinamidase	Pyrazinamidase	OAA
M. tuberculosis	-	-	+	+	+	+	-	+	-	-	-	+	+	-	F	+	-	+	+	+	R
M. bivis BCG	-	-	+	+	+	-	-	-	-	-	-	F	-	+	-	-	-	+	-		R
M .africanum	-	-	+	+	+	F	-	-	-	-	-	F	-	-	-	-	-	+	F	F	R
M. bovis	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	R
M. microti	-	-	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+	Oc
M. ulcerans	-	F	M	M	F	-	M	+	F	-	-	-	-	-	-	-	-	F	F	F	R
M. kansasii	+	-	-	+	+	+	-	+	+	+	+	-	+	+	+	-	-	+	+	-	SmK
M. marinum	+	-	-	M	F	-	-	+	+	+	-	-	-	+	+	-	-	+	M	F	SmK/R
M. simiae	M	-	F	+	+	F	F	+	+	+	+	M	-	-	F	F	-	+	-	-	SmS
M. asiaticum	M	-	F	+	+	-	+	+	+	+	+	-	-	+	-	-	-	-	F	F	SmS
M. scrofulaceum	-	+	-	+	+	+	+	+	+	+	M	-	-	-	-	-	-	+	+	+	SmK/ SmS/ SmG
M. gordonae	-	+	-	+	+	-	-	+	+	+	M	-	-	+	+	F	-	+	-	-	SmK/ SmS/ SmG
M. flavescens	-	+	-	+	+	M	-	+	-	-	M	-	+	+	M	-	F	+	+	+	SmK/ SmS/ SmG
M. thermoresistibile	-	+	-	+	+	+	-	+	-	-		-	+	+	+	-	-	+	+	+	SmK/ SmS/ SmG

Key: + = > 85% of strains positive:, - = < 15% of strains positive: M = 50 to 85% of strains positive:, F= 15 to 49% of strains positive: Blank spaces = few or no data available: INH = isoniazid (10 mg ml^{-1}); TCH= thiphene-2-carboxylic acid hydrazide (1 mg ml^{-1}) IIA = Hydroxylamine hydrochloride (250 mg ml^{-1}); PNB = p-nitorbenzoic acid ((500 mg ml^{-1}) OAA : oleic acid albumin agar: R= Rough: SmK= Smooth Kansasii: SmG = Smooth Kansasii: SmG: Smooth Granular: SmS = Smooth scotochromogenic SmT= Smooth transparent: Xf= Xenopi filamentous

	Pigment production			Temperature range (°C)			Resistance to														Colonial morphology
	Photochromic	Scotochromic	Nonchromic	33		42	INH	TCH	HA	PNB	Catalase >45 mm	Niacin production	Nitrate reduction	Acid phosphatase	Tween 80 hydrolysis	β-Galactosidase	Growth in 5% (w/v) NaCl	Urease	Nicotinamidase	Pyrazinamidase	OAA
M. szulgai	-	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	+	-	-	SmK
M. avium	-	-	+	+	+	+	M	+	+	+	-	-	-	-	-	-	-	-	+	+	SmS/SmT
M .intracellulare	F	-	M	+	+	M	-	+	+	M	F	-	-	-	-	-	-	F	+	+	SmS/SmT
M. xenopl	-	+	-	+	+	+	-	+	M	+	-	-	-	-	-	F	-	-	+	+	Xf
M. gastri	-	-	+	+	+	-	-	+	M	-	-	-	-	-	-	F	F	+	+	-	SmK/R
M. terrae	-	-	+	+	+	-	+	+	+	+	+	-	+	+	+	+	-	-	-	-	SmK/R
M. nonchromo -genicum	-	-	+	+	+	F	+	+	+	F	+	-	-	+	+	+	F	-	-	M	SmK/R
M. triviale	-	-	+	+	+	F	+	+	+	+	+	-	+	+	+	-	+	-	-	-	
M. paraturberculosis	-	-	+	+	+	+	+	+	+	-	-	-	-	-	M	-	-	-	+	+	R
M. malmoense	-	-	+	+	+	-	+	+	M	M	-	-	-	-	-	-	-	-	+	+	

Key: + = > 85% of strains positive; - = < 15% of strains positive; M = 50 to 85% of strains positive; F= 15 to 49% of strains positive; Blank spaces = few or no data available; INH = isoniazid (10μg ml^{-1}); TCH= thiophene-2-carboxylic acid hydrazide (1 μg ml^{-1}); HA = hydroxylamine hydrochloride (250μg ml^{-1}); PNB = ρ-nitrobenzoic acid ((500μg ml^{-1}); OAA : oleic acid albumin agar; R= Rough; SmK= Smooth Kansasii: SmG = Smooth Kansasii; SmG = Smooth Granular ; SmS = Smooth scotochromogenic; SmT= Smooth transparent: Xf = Xenopi filamentous.

	Strain no.(%)	
Slow growing pigmented NTM	**Station I**	**Station II**
*1. M. marinum**	*19(6.5)*	*23(8.2)*
*2. M. xenopi**	*3(1.0)*	-----
*3. M .asiaticum**	*24(8.2)*	*14(5.0)*
*4. M. scrofulaceum**	*10(3.4)*	*10(3.6)*
*5. M. kansasii**	*2(0.7)*	*5(1.8)*
*6. M. szulgai**	----	*2(0.7)*
Slow growing non pigmented NTM		
7. M. gastri	*32(10.9)*	*18(6.4)*
8. M. avium complex*	*2(0.7)*	*13(4.6)*
*9. M. shimoidei**	*3(1.0)*	*2(0.7)*
*10. M. malmoense**	*12(4.1)*	*14(5.0)*
11. M. triviale	*13(4.4)*	*10(3.6)*
12. M. terrae	*13(4.4)*	*7(2.5)*
13. M. nonchromogenicum	*19(6.5)*	*8(2.8)*
Fast growing pigmented NTM		
14. M. parafortuitum	*4(1.4)*	---
15. M. obuense	*4(1.4)*	*7(2.5)*
16. M. aichiense	*5(1.7)*	---
17. M. flavescens	*1(0.3)*	*3(1.1)*
18. M. gadium	*17(5.8)*	*22(7.8)*
19. M. thermoresistibile	*2(0.7)*	*2(0.7)*
20. M. poriferae	*4(1.4)*	*6(2.1)*
21. M. komossense	*1(0.3)*	*6(2.1)*
22. M. sphagni	*6(2.0)*	*2(0.7)*
23. M. vaccae	*1(0.3)*	----
24. M. phlei	----	*2(0.7)*
25. M. gilvum	----	*4(1.4)*
26. M. aurum	----	*7(2.5)*

ast growing non pigmented NTM		
7. *M. chelonae**	*29(10.0)*	*27(9.6)*
8. *M. chitae*	*23(7.8)*	*23(8.2)*
9. *M. diernhoferi*	*8(2.7)*	*4(1.4)*
0. *M. abscessus**	*19(6.5)*	*23(8.2)*
1. *M. fortuitum**	*9(3.1)*	*7(2.5)*
2. *M. peregrinum**	*6(2.0)*	*11(3.9)*
3. *M. smegmatis*	*2(0.7)*	*1(0.4)*

* shows pathogenic species identified

During isolation, specific colony morphology on LJ and PTB helped in presumptive identification for selecting appropriate biochemical methods, whereas on NA, morphological differentiation of the colonies was impossible due to high variability.

For almost all the strains of *M. marinum*, distinct cross barring was observed for long rods. Cream coloured , smooth, hemispheric, multilobate colony morphology on LJ and beaded appearance of the cells indicated the presumptive identification of some strains as *M. fortuitum*. *M. gadium* colonies on LJ were very , minute , dark orange , smooth and granulated but on ageing observed to become dry and rough.

The tests were performed by the standardized procedures recommended by IWGMT(Wayne *et,al.* 1974, 1976). Tests like pigment production, resistance to Hydroxyl ammonium chloride and tests for Nitrate reduction, Acid phosphatase activity and Tween 80 hydrolysis were common for both rapid and slow growers. Care has been taken for high degree of reproducibility for all the tests. Among the biochemical tests for slow growing mycobacteria, Niacin test was not conducted.

The battery of the test results in the percentages has been given in Tables10.1 and 10.2. for all the species identified. The biochemical activities of species such as *M.obuense, M.aichiense, M. poriferae, M.komossense, M.sphagni, M.diernhoferi* and *M. abscessus* were referenced from Bergey's Manual of Systematic Bacteriology (1986), Jenkins *et, al* (1981), Padgitt and Moshier (1987), Kuzunoki and Ezaki (1992). For these species, identification was carried out on specific activities listed , but the results of all the activities shown by the strains as per the basic scheme followed; were entered

	Pigment production			Temperature range (°C)				Carbohydrate fermentation												Colonial morphology	
	Photochromic	Scotochromic	Nonchromic	37	42	45	52	Glucose	Inositol	Mannitol	Sucrose	Citrate utilization	Benzoate utilization	Nitrate reduction	Acid phosphatase	Tween 80 hydrolysis	Arylsulphatase	Resistance to hydroxylamine	Iron up take	OAA	CMA
M. fortuitum	0	0	100	100	100	13	0	100	6	0	13	94	0	100	88	6	81	63	+	SmK/SmS	SmK/Rf/SmG
M.chelonei	0	0	100	100	11	0	0	96	0	7	9	100	0	0	98	13	73	100	-	Rarb/SmSa	Rf/SmSa/SmK
M. smegmatis	0	0	100	100	100	100	0	100	100	100	33	100	100	100	0	100	0	33	+	Rf/R	Rf/R
M. phlei	0	100	0	100	100	100	100	100	50	100	50	100	0	100	100	100	0	0	+	Rarb	ND
M. vaccae	100	0	0	100	100	0	0	100	100	100	100	100	100	0	0	100	0	0	+	SmK	SmSy
M. parafortuitum	50	50	0	100	100	75	0	100	50	100	100	100	0	100	25	100	100	75		SmK	SmK
M. chitae	0	0	100	100	7	4	0	98	65	100	0	0	50	91	98	70	0	100	-	Rarb/ SmSy	Rarb/ SmSy
M.gilvum	0	100	0	100	25	0	0	100	100	100	0	100	50	25	100	100	100	75	+	SmSa/SmSy	SmK/ SmT
M. gadium	0	100	0	92	3	0	0	97	92	36	8	3	0	77	97	0	97	5	-	Rarb/ SmG	Rarb/ SmG
M. arurum	0	100	0	100	14	0	0	100	71	100	100	86	0	29	86	100	14	100	+	SmK/ SmKw	SmSy
M. flavescens	0	75	25	100	75	0	0	100	0	50	0	0	50	100	100	100	100	25	-	SmSa/SmG	SmSa/SmG
M. thermoresistible	0	100	0	100	100	100	100	0	0	25	0	0	0	75	0	100	25	0	-	SmSy/ SmDy	SmSy/ Rf
M. abscessus	0	0	100	100	8	0	0	15	8	0	0	0	0	3	98	0	88	75		SmSy/ Rf	SmSy/ SmK
M. peregrinum	0	0	100	100	100	12	0	100	6	12	0	53	59	94	88	100	94	88		R/ SmK	SmK/SmSy
M. diernhoferi	0	0	100	100	8	0	0	92	100	100	16	92	0	100	100	75	25	0		ND	SmS
M. poriferae	0	100	0	100	100	100	20	100	90	10	100	90	0	90	40	90	10	100		SmSy/SmG	SmKt/ SmKw
M. komossense	0	100	0	100	86	14	0	86	14	100	100	100	0	0	86	0	0	43		SmK/SmKt	Rarb/ SmSy
M. sphagni	0	100	0	100	38	13	0	88	100	100	100	63	ND	100	88	0	100	38		SmSy	SmS
M. aichense	0	100	0	100	100	0	0	100	0	80	0	0	0	0	100	0	100	80		SmK	SmK

Key: + = > 85% of strains positive; - = < 15% of strains positive; M = 50 to 85% of strains positive; F= 15 to 49% of strains positive; Blank spaces = few or no data available; OAA = oleic acid albumin agar; CMA= commeal agar: SmF: Smooth Fortuitum: Smf = Smooth with filamentous centre: R= Rough; Rf = Rough filamentous; SmSa = Smooth scotochromogen surrounded by an apron; SmK= Smooth Kansasii: SmG = Smooth Kansasii: SmG; Smooth Granular; SmS = Smooth scotochromogenic

Table: 10.2. Biochemical and enzymatic activities of identified slowly growing NTM strains (in percentages) from stations I and II

	Pigment production			Temperature range (°C)			Resistance to											Colonial morphology
	Photochromic	Scotochromic	Nonchromic	33	37	42	INH	TCH	HA	PNB	Catalase>45 mm	Nitrate reduction	Acid phosphatase	Tween 80 hydrolysis	Growth in 5% (w/v) NaCl	Urease	Pyrazinamidase	OAA
M. szulgai	0	100	0	100	100	0	50	100	100	100	100	100	0	100	0	100	0	SmK/SmG
M. avium complex	0	0	100	93	100	87	80	100	53	87	7	13	100	93	20	27	100	SmS/SmKt
M. xenopi	0	100	0	67	100	100	67	100	100	100	0	0	33	33	0	0	100	Xf
M. gastri	0	0	100	100	100	0	0	0	98	4	2	10	4	0	0	94	100	SmKt/SmS/SmG
M. terrae	0	0	100	100	100	5	95	90	70	100	100	85	90	95	5	100	90	SmS/SmK/Rarb
M. nonchromogenicum	0	0	100	100	100	74	96	89	100	30	74	7	70	93	74	100	89	SmKt/ SmS/ SmSy
M. triviale	0	0	100	100	100	78	83	91	97	100	91	70	87	78	97	9	0	SmK/SmS/R
M. malmoense	0	0	100	96	100	92	100	100	72	88	8	0	0	0	12	4	0	SmK/SmSy
M. shimoidei	0	0	100	20	100	20	100	100	100	ND	0	0	100	100	0	0	ND	SmS/SmK
M. kansasii	29	71	0	100	100	14	57	100	86	100	71	86	100	100	14	86	100	SmSy/SmK
M. marinum	98	2	0	81	23	0	0	71	69	100	2	7	95	98	12	100	100	SmK/Xf
M. asiaticum	26	74	0	100	100	0	97	95	79	100	92	3	97	8	100	74	39	SmF/ SmSa/ SmSy
M. scrofulaceum	0	95	5	100	95	85	100	95	90	100	65	5	100	85	90	100	100	SmDy/ SmG/ SmS

Key: + = > 85% of strains positive; - = < 15% of strains positive; M = 50 to 85% of strains positive; F= 15 to 49% of strains positive; Blank spaces = few or no data available; INH = isoniazid (10µg ml^{-1}); TCH= thiophene-2-carboxylic acid hydrazide (1 µg ml^{-1}); HA = hydroxylamine hydrochloride (250µg ml^{-1}); PNB = ρ-nitrobenzoic acid ((500µg ml^{-1}); OAA : oleic acid albumin agar; R= Rough; SmK= Smooth Kansasii: SmG = Smooth Kansasii; SmG = Smooth Granular ; SmS = Smooth scotochromogenic; SmT= Smooth transparent: Xf = Xenopi filamentous

	Nutrient Agar							PTB medium							LJ medium							Total(%)
	S.	G.	St.	Int.	Li.	Sed.	Wat.	S.	G.	St.	Int.	Li.	Sed.	Wat.	S.	G.	St.	Int.	Li.	Sed.	Wat.	
M. marinum	3		1	2		2				1	3				1	1	2	1		1	1	19(6.5)
M. xenopi			1		1	1																3 (1.0)
M. asiaticum		2	3	4	4	6	3	1		1												24(8.2)
M. scrofulaceum			3					1		2	1						1	1		1		10(3.4)
M. kansasii		1									1											2 (0.7)
Total	3	3	8	6	5	9	3	2		4	5				1	1	3	2		2	1	58

Table: 11.2. Distribution of non-pigmented slow growing NTM – Station I

	Nutrient Agar							PTB medium							LJ medium							Total(%)
	S.	G.	St.	Int.	Li.	Sed.	Wat.	S.	G.	St.	Int.	Li.	Sed.	Wat.	S.	G.	St.	Int.	Li.	Sed.	Wat.	
M. gastri	2	1		1	2	1	1	3	1	2	7	1	2	1			1	2	1	3		32(10.9)
M. avium complex						1					1											2(0.7)
M. shimoidei	1						1	1														3 (1.0)
M. malmoense	1	1	3	1	1	2		1										1		1		12(4.1)
M. triviale	1								1	1	5		1			1	1	2				13(4.4)
M. terrae			1		1	2				2					2		2	2	1			13(4.4)
M. nonchromogenicum		1	3	2		3	1		1	2	4		1					1				19(6.5)
Total	5	3	7	4	4	9	3	5	3	7	17	1	4	1	2	1	4	8	2	4		94

	Nutrient Agar							PTB medium							LJ medium							Total(%)
	S.	G.	St.	Int.	Li.	Sed.	Wat.	S.	G.	St.	Int.	Li.	Sed.	Wat.	S.	G.	St.	Int.	Li.	Sed.	Wat.	
M. flavescens									1													1(0.3)
M. parafortuitum				1						1			1				1					4(1.4)
M. obuense			2		2																	4 (1.4)
M. aichiense									1	1	1					1				1		5(1.7)
M. gadium	1	1	2		1	3	1	1		2					2		1			1		17(5.8)
M. thermoresi-stibile											2		1									2(0.7)
M. poriferae		1		2		1																4(1.4)
M. komossense																				1		1(0.3)
M. sphagni		1	1	1	1	1	1															6(0.3)
M vaccae								1														1(0.3)
Total	1	3	5	4	4	5	2	2	2	4	3		2		2	1	2			3		45

Table: 11.4. Distribution of non-pigmented fast growers – Station I

	Nutrient Agar							PTB medium							LJ medium							Total(%)
	S.	G.	St.	Int.	Li.	Sed.	Wat.	S.	G.	St.	Int.	Li.	Sed.	Wat.	S.	G.	St.	Int.	Li.	Sed.	Wat.	
M. chelonei	4	2	3	4		1	3	1	2	5	2		1				1					27(9.6)
M. chitae	2	1	2	1		3	1	1	1	3	1		2		1		1	1		2		23(8.2)
M. diernhoferi	1	1	1	2		2	1															4(1.4)
M. abscessus	3	2	1		1	3	1	3					1		1			3				23(8.2)
M .fortuitum			1	1		1				1			1		1	1	1	1				7(2.5)
M. peregrinum	1	1		2	1	1																11(3.9)
M smegmatis											1		1									1(0.4)
Total	11	7	8	10	2	11	6	5	3	9	4		6		3	1	3	5		2		96

	Nutrient Agar							PTB medium							LJ medium							Total(%)
	S.	G.	St.	Int.	Li.	Sed.	Wat.	S.	G.	St.	Int.	Li.	Sed.	Wat.	S.	G.	St.	Int.	Li.	Sed.	Wat.	
M. marinum		1	2	1	1	2	3	3		2	4		1			1			1	1		23(8.2)
M. szulgai					1		1															14(5.0)
M. asiaticum		1	3	1	2	3	1				1		1							1		10 (3.6)
M. scrofulaceum	1		1	1				2		2	2									1		5(1.8)
M. kansasii										1	1				1			1		1		2(0.7)
Total	1	2	6	3	4	5	5	5		5	8		2		1	1		1	1	4		72

Table: 12.2. Distribution of Non pigmented slow growing NTM – Station II

	Nutrient Agar							PTB medium							LJ medium							Total(%)
	S.	G.	St.	Int.	Li.	Sed.	Wat.	S.	G.	St.	Int.	Li.	Sed.	Wat.	S.	G.	St.	Int.	Li.	Sed.	Wat.	
M. gastri		1	2	1		2	4						3		1					2		18(6.4)
M. avium complex	1	2		2		4	2			2	1											13(4.6)
M. shimoidei							1															2(0.7)
M. malmoense			1			1	1	3	1	2	3							1		1		14(5.0)
M. triviale					1		1	2	1	2							1		1	1		10(3.6)
M. terrae	1									3									1	1	1	7(2.5)
M. nonchromo-genicum				3		1				1	2							1				8(2.8)
Total	2	3	3	6	1	8	9	5	2	10	8		3		1		1	2	2	5	1	61

	Nutrient Agar							PTB medium							LJ medium							Total(%)
	S.	G.	St.	Int.	Li.	Sed.	Wat.	S.	G.	St.	Int.	Li.	Sed.	Wat.	S.	G.	St.	Int.	Li.	Sed.	Wat.	
M. flavescens			1		1	1																3(1.1)
M. phlei				1			1															2(0.7)
M. obuense	1		1	2			1												1	1		7(2.5
M. gadium		1	3	6	3	4	1				2		1			1						22(7.8)
M. thermore-sistibile			1											1								2(0.7)
M. gilvum					1	2	1															4(1.4)
M. poriferae	1	1	1	1		1	1															6(2.1)
M. komossense			1			1	1				1					1				1		6(2.1)
M. sphagni						1	1															2(0.7)
M. aurum	1	1					2	2		1												7(2.5)
Total	3	3	8	10	5	10	9	2		1	3		1	1		2			1	2		61

Table: 12.4. Distribution of Non-pigmented fast growing NTM – Station II

	Nutrient Agar							PTB medium							LJ medium							Total(%)
	S.	G.	St.	Int.	Li.	Sed.	Wat.	S.	G.	St.	Int.	Li.	Sed.	Wat.	S.	G.	St.	Int.	Li.	Sed.	Wat.	
M. chelonei	1	2	2	3	1	4	2			1	4				1		4	2				27(9.6)
M. chitae	4	2	4	2		5	2	1			1			1							1	23(8.2)
M. diernhoferi						1	3															4 (1.4)
M. abscessus	1		1	1		3	3	2		2							3	1		2	2	23(8.2)
M. fortuitum		1	1	2		1									1	1						7(2.5)
M. peregrinum	1	2	1	1		4	2															11(3.9)
M. smegmatis													1									1(1.40
Total	7	7	9	9	1	17	13	3		3	5		1	1	2	1	7	3		2	3	94

in the results. Following Bergey's Manual of Systematic Bacteriology (1986), the strains exbhibiting some of the biochemical and enzymatic characteristics of *M. intracellulare* and *M.avium* were treated as *M.avium* complex as the specific characters for differentiating the two were absent.

Precise identification was not possible for some and were left unidentified. Among the strains of each species, variation were found to be existed morphologically and biochemically which strongly indicated subspecific variations among the strains.

Acquainted some difficulty in interpreting the photochromogenicity of few strains due to lack of accountable chromic nature of the strains during dark and on light exposure. 18% of the strains produced pink or coral pigment after conducting test for photochromogenicity. Tween 80 hydrolysis(Plate 1)and Acid phosphatase test(Plate 2) were carried out for all the strains and obtained satisfactory and reproducible results. Care has been taken to ensure the exact concentration of antituberculous drugs on LJ slants and to inoculate viable culture in required dilution. For nitrate reduction test(Plate 3), the results were made to improve by adding Dubos broth base (1%) to the reaction mixture.

Purple coloration , obtained for the test result of aryl sulfatase activity for rapid growers was optimum, but showed clear differentiation between positive and negative results(Plate 4). For testing the susceptibility of the strains for various growth temperatures, it has been tried maximum to maintain accurate test temperatures. Most of the strains were recorded with high temperature resistance.

Strains of *M.fortuitum*, *M.asiaticum*, *M.kansasii* and *M. szulgai* showed variations on both photo and scotochromic activities. The degree of temperature tolerance was also high for strains identified. Most of the slow growers were resistant to antituberculous drugs, whereas strains of *M. poriferae*, *M.chelonei*, *M.chitae* and *M.peregrinum* showed 100% tolerance towards HA (500 µgat/l). Even though considerable strain variations observed for each identified species, they were assigned to distinct species, on comparison with majority of the test results. Variability was recorded for acid production capacity of fast growers among the strains. But the

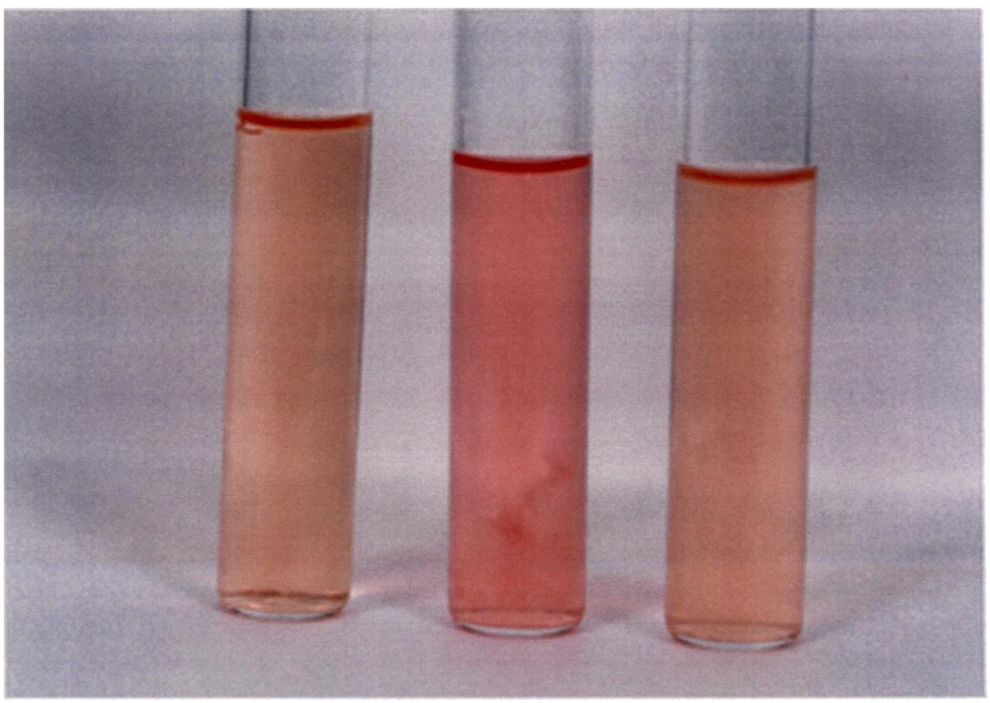


Plate No. 1: Tween 80 hydrolysis

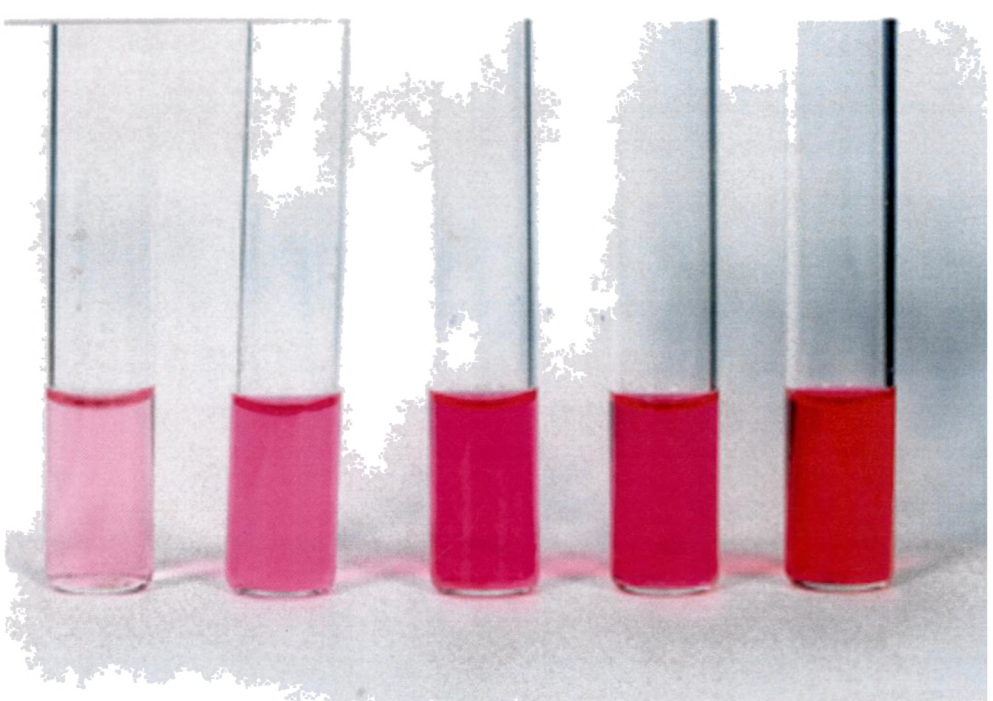


Plate No. 2: Acid phosphatase test

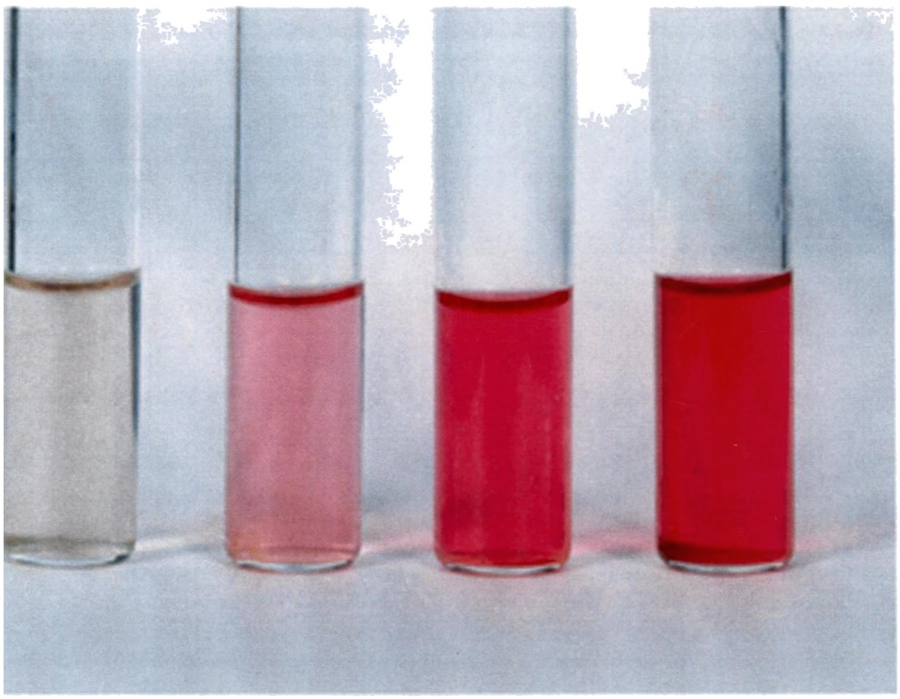


Plate No. 3: Nitrate reduction test

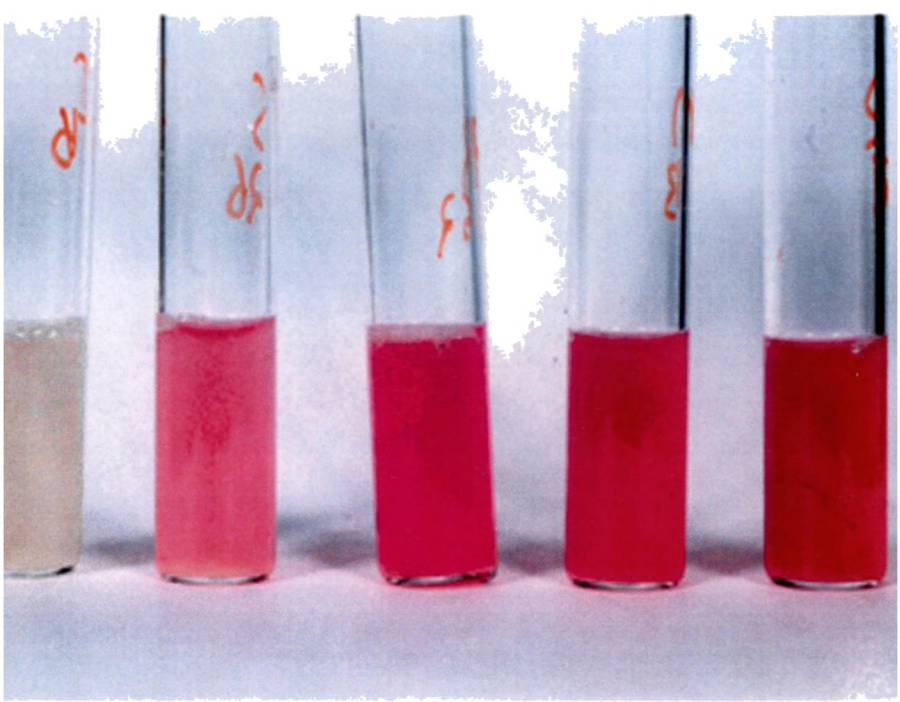


Plate No. 4: Aryl sulfatase activity

reproducibility of important tests like Nitrate reduction, Acid phosphatase acivity, Aryl sulfatase activity, and Tween 80 hydrolysis were high.

All the strains of *M. aurum* were pleasant yellow pigment producers . The strains of *M. thermoresistibile* showing rough morphology on CMA , showed greenish yellow pigment on LJ. And some strains of *M. poriferae* showed characteristic bright yellow pigmentation. Brown coloured pigment was observed to be spreading on LJ from *M. komossense* colonies. Most of the colonies of *M. chelonei* were characteristically brittle and easily removable from peptone agar slopes and were strongly citrate positive.Production of pink or coral pigment was observed common among strains of *M. komossense* , *M. abscessus* and *M., chitae.* Golden yellow pigment was observed for *M. marinum* and were changing to brownish yellow on ageing. Comparative flourishing of *M. asiaticum* were recorded at RT than at 37 °C. peptone agar was found to be brownish in co lour on growth of some strains of *M.triviale* and *M.gastri.*

The colonial morphology of other strains on OAA and CMA were quiet varied and were confusing to interpret specifically the intraspecific strain variation among environmental mycobacteria. Colonial morphologies observed for some NTM species on NA medium were shown in Plates 5-10. Plates 11,12,13 and14 shows colony morphologies of *M.kansasii, M.chitae, M.abscessus, M.triviale, M.scrofulaceum* and *M.chelonei* on PTB mediumcolony morphologies of important fish pathogenic NTM like *M.chelonei, M.fortuitum,* and *M.kansasii* has been shown in Plate 15, 16 and 17.

The species of NTM identified among four groups, namely pigmented slow growers, nonpigmented slow growers, pigmented fast growers, nonpigmented fast growers, their frequency of occurrence and distribution among fish and environmental samples on different media(Tables 11.1, 11.2, 11.3, 11,4, 12.1, 12.2, 12.3 and 12.4).

3.3. FREQUENCY DISTRIBUTION OF DIFFERENT NTM SPECIES

The individual count for different species of NTM identified were varying with the general range observed from 100cfu to too numerable to count as given in Table 16.

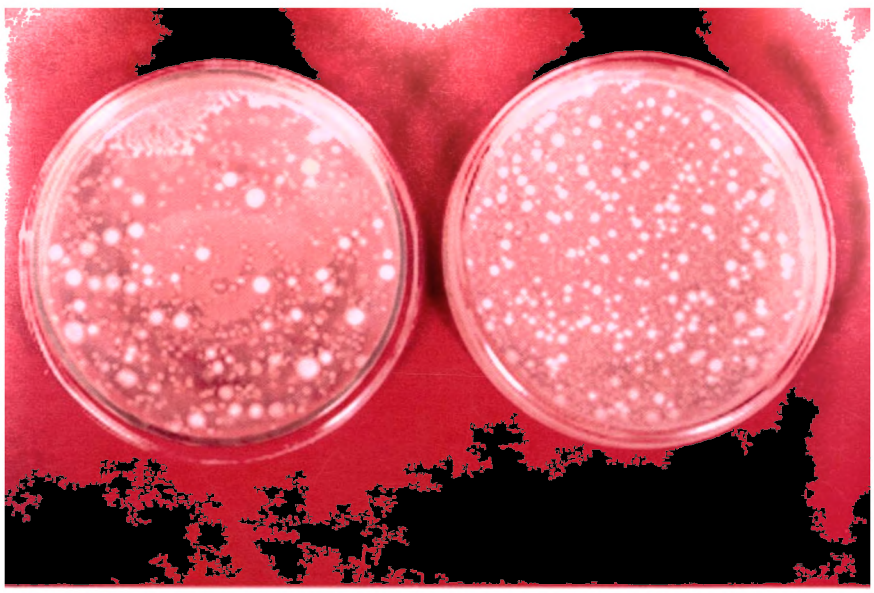
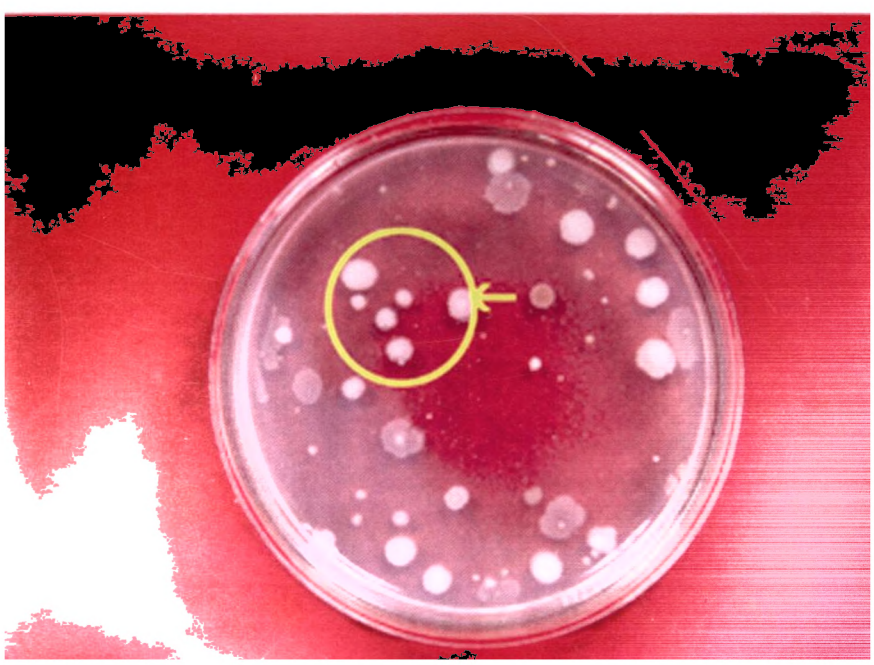


Plate No. 5: Colonies of *Mycobacterium fortuitum*



COLONIAL MORPHOLOGY OF NTM ON NUTRIENT AGAR

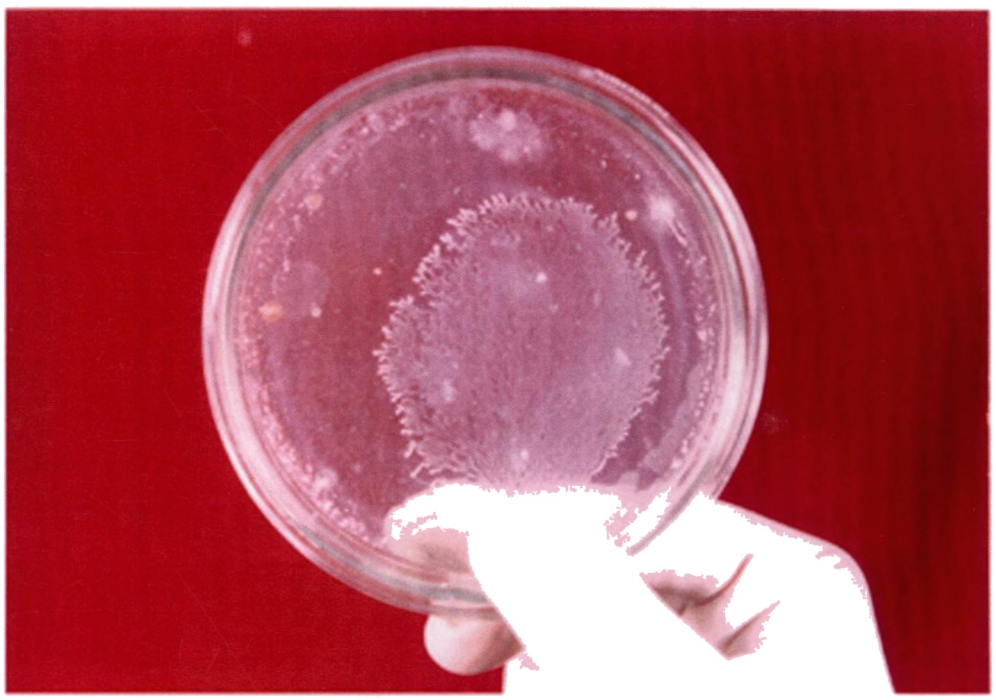


Plate No. 7: Single large colony of *Mycobacterium chelonei*

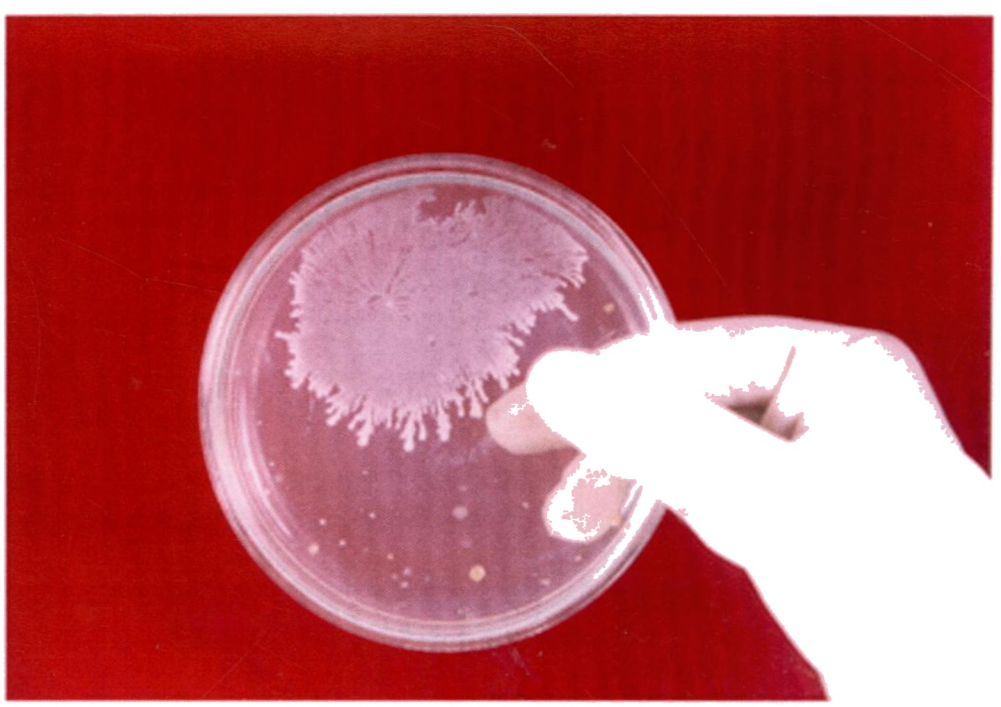


Plate No. 8: Single large colony of *Mycobacterium asiaticum*

ONIAL MORPHOLOGY OF NTM ON NUTRIENT AGAR

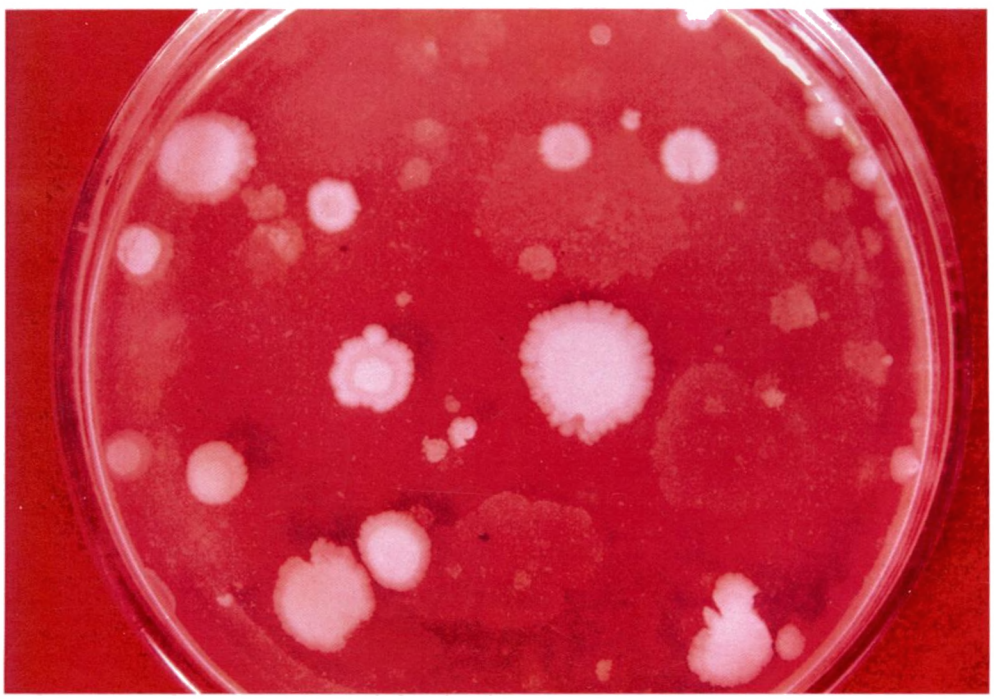


Plate No. 9: Colonies of *Mycobacterium chelonei*

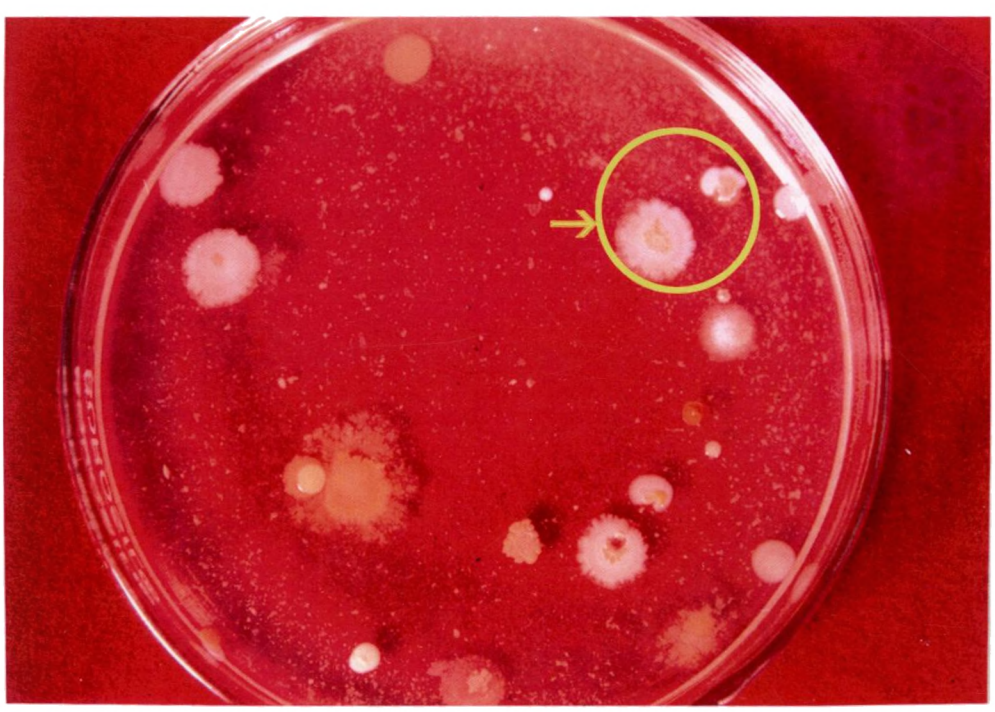


Plate No. 10: Colonies of *Mycobacterium abscessus*

COLONIAL MORPHOLOGY OF NTM ON PEIZER TB MEDIUM

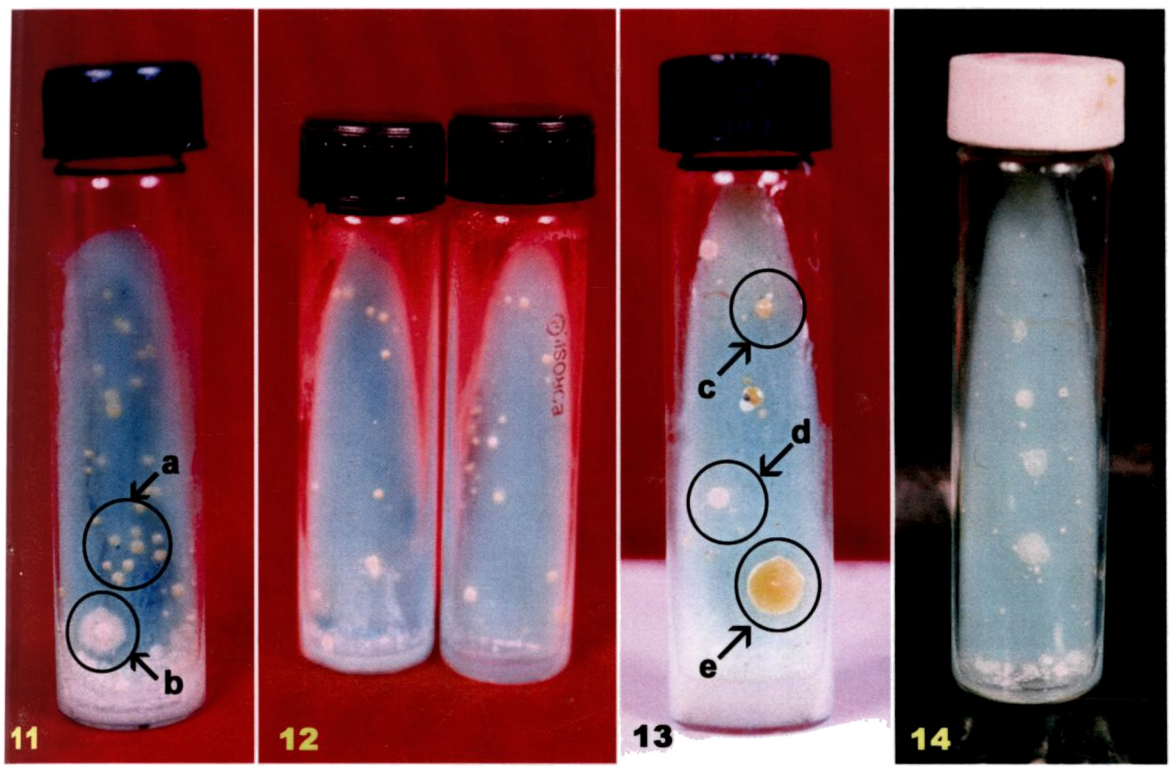


Plate No. 11: Colonies of (a) *Mycobacterium kansasii* and (b) *Mycobacterium chitae*

Plate No. 12: Colonies of *Mycobacterium kansasii*

Plate No. 13: Colonies of (c) *Mycobacterium abscessus*, (d) *Mycobacterium triviale* and (e) *Mycobacterium scrofulaceum*

Plate No. 14: Colonies of *Mycobacterium chelonei*

COLONIAL MORPHOLOGY OF NTM ON LOWENSTEIN JENSEN MEDIUM

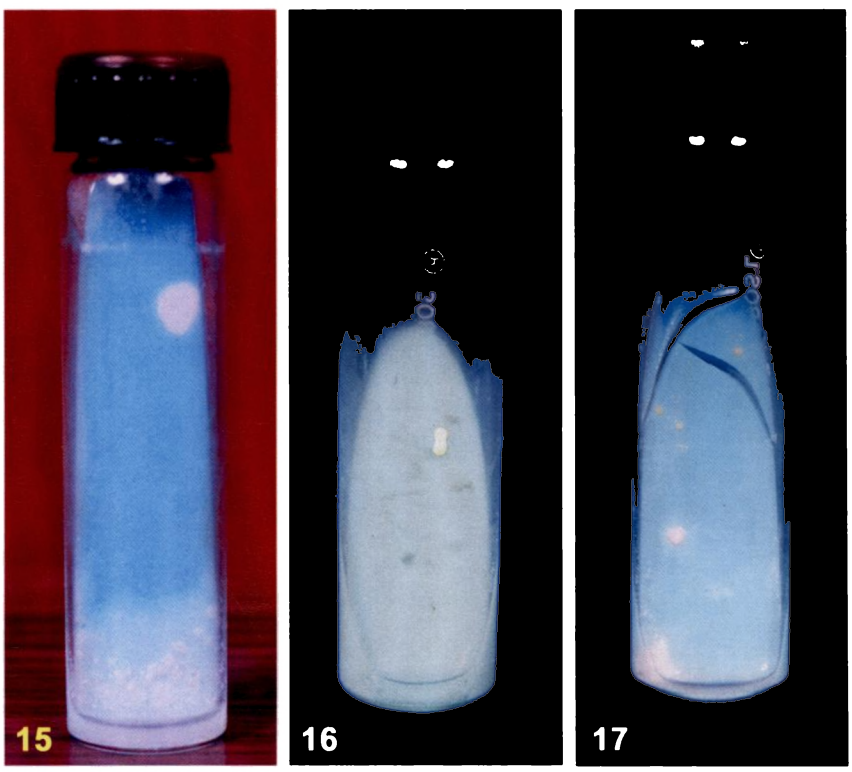


Plate No. 15: Colonies of (a) *Mycobacterium chelonei* and (b) *Mycobacterium terrae*

Plate No. 16: Colony of *Mycobacterium fortuitum*

Plate No. 17: Colonies of (c) *Mycobacterium kansasii* and (d) *Mycobacterium fortuitum*

Species like, *M. gadium, M.terrae, M.phlei, M.chitae, M.diernhoferi, M.peregrinum* were found in numerous colony forming units during examination, whereas *M.scrofulaceum, M.shimoidei* were accounted in a range of 100 - 10,000cfu's. Range of number of colony forming units for *M.kansasii was* from 100cfu to 20,000cfu. Colonies of species like *M.aichiense* and *M.smegmatis* were not developed on Nutrient agar. For *M.shimoidei* and *M.triviale,* observed number of colony forming units ranged from 100 cfu to 1000cfu.

3.3.1. Pigmented slow growers

In this group of NTM, five species were encountered and four species were commonly observed in Stations I and II while the sporadic occurrences of *M. xenopi* in Station I and *M. szulgai* in station II with representation of two strains each was showing the geographical similarity in the species diversity of NTM in the stations.

The most abundant species of pigmented slow growing NTM in Narakkal aquaculture pond(Table 11.1) was *M.asiaticum* with 24 strains (44.4%) while Valappu was observed with highest intensity of *M. marinum*(N=23/54) forming 42.6% of the pigmented slow growers (Table 12.1). The intensity of occurrence of *M. marinum* in station I was observed as 32.8%(N=19/58) and of *M.asiaticum* in station II was25.9 % (N= 14/54). *M. kansasii*, the most important fish pathogen was of less prevalence in the stations, with 2 strains(3.4%) in station I and 5 strains (9.3%) in station II.

3.3.2. Nonpigmented slow growers

Seven species of nonpigmented slow growers were identified. The species were similar in both station I and II indicates these as parts of natural flora of NTM. Sporadic occurrence of *M. avium* complex was observed in station I with 2 strains , but recorded frequently in station II with 13 strains. With 34% (N=32/94) in station I and 25% (N= 18/72) in station II, M.gastri was represented as the most abundant species among the nonpigmented slow growers identified. In Narakkal, prevalence of *M. nonchromogenicum* was more with 19 strains forming 20.2% of the total than from Valappu(11.1%). Occurrence of *M. terrae* was similar in both the stations with 13 strains each. *M.shimoidei* was identified sporadically in the stations., while *M.*

malmoense and *M. triviale* were represented by 14 and 10 strains respectively from Valappu aquaculture pond(Tables 11.2 and 12.2).

3.3.3. Pigmented fast growers

Of the 13 species of NTM identified among the group, seven were observed commonly in both the stations of study as shown in Table 11.3 and 12.3, whereas species like *M. parafortuitum*(n=4), *M.aichiense*(n=5) and *M. vaccae* (1isolate)were obtained only from Narakkal. With 2 ,4 and 7 strains of *M.phlei*, *M. gilvum* and *M. obuense*, station II showed the sporadic occurrence for these species. Predominance was recorded for *M. gadium* in both the stations with 37.8%(N= 17/45) in station I and 36.1%(N=22/61) in station II. As the Tables 11.3 and 12.3 indicate, sporadic distribution was recorded for other identified species in the stations.

3.3.4. Nonpigmented fast growers

The seven species of this group of NTM identified and were observed as same from the stations, indicating that these are parts of indigenous microbial flora in the aquaculture systems. Except the sporadic occurrence of *M. smegmatis* (2 strains in station I and single strain in station II), identified species were with high intensities in the systems. *M. chelonei* was with maximum abundance in the stations with 29 strains(30.2%) and 27 strains(28.7%) in stations I and II respectively, followed by *M. abscessus* (19.8%in station I and 22.3%in station II). Occurrence of species like *M. diernhoferi, M. fortuitum* and *M. peregrinum* were recorded in different intensities as indicated.(Tables 11.4 and 12.4).

3.4. EFFICIENCY OF SELECTIVE MEDIA ON ISOLATION OF NTM

The data showed that , in station I and II, the maximum retrieval of NTM were reported from NA with 5% glycerol and the minimum from LJ medium. Of the total, 689 strains observed was 53.8%(N=185/344) and 61.2%(N=211/345) of NTM were recovered from NA medium only, from stations I and II respectively(Table13), whereas the percentages of NTM recorded from PTB was 27%(N=155/574) and from LJ was 16.70%(N=96/574). Among the total 278 low growers (152 in station I and 126 in station II), a number of 130 (46.76%) was retrieved from NA along with

Table: 13. Media wise distribution of NTM in station I and II

	Distribution of NTM No.(%) Stations		
Media used	**Narakkal**	**Valappu**	**Total**
Nutrient agar	185(53.8)	211 (61.2)	396(57.5)
Peizer TB medium	102(29.7)	82(23.8)	184(26.7)
LJ medium	57(16.6)	52(15.1)	109(15.8)
Total	344(49.9)	345(50.1)	689(100)

Table: 14.1 Overall positivity rate on PTB version LJ - Station I

Sample	Total tube	PTB medium positivity		Total tubes	LJ medium positivity		Ratio % PTB/ % LJ
		No. of tubes	Rate (%)		No. of tubes	Rate (%)	
Skin	48	18	38	48	9	19	2*
Gill	48	5	10	48	2	4	2.5*
Stomach	48	26	54	48	16	33	1.6*
Int.	48	26	54	48	17	35	1.7
Liver	48	2	4	48	3	6	0.7
Sediment	48	11	23	48	15	31	0.7
water	48	3	6	48	1	2	3

(* P<0.05)

Table: 14.2. Overall positivity rate on PTB version LJ - Station II

Sample	Total tubes	PTB medium positivity		Total tubes	LJ medium positivity		Ratio % PTB/ % LJ
		No. of tubes	Rate (%)		No. of tubes	Rate (%)	
Skin	48	13	27	48	9	19	1.4*
Gill	48	4	8	48	5	10	0.8
Stomach	48	20	41	48	10	21	2*
Int.	48	24	50	48	10	21	2.4*
Liver	48	4	8	48	1	2	4
Sediment	48	11	23	48	13	27	0.9*
water	48	3	6	48	4	8	0.8

(* P<0.05)

64.18(N=190/296) of fast growers from both the stations. From Figures 6.1 and 6.2, the highest number of pigmented and nonpigmented slow and fast growers were observed to be recovered from NA and minimum from LJ. Even though PTB and LJ media contributed from both the stations, the study revealed the extraordinary capability of NA for NTM retrieval from fish and environmental samples. 47.37%(N=72/152) and 46.03%(N=58/126) of slow growers and 56.02%(N=79/141) and 71.6%(N=111/155) of fast growers were yielded by NA medium. The percentage of retrieval of slow growers in station I and II from PTB was 33.8%(N=94/278) and from LJ was 18.3%, whereas 20.6 and 14.6 were the percentages of fast growers from PTB and LJ media (Figures 6.1 and 6.2) .

Out of the 33 species identified from both the stations, 23 from station I and 27 from station II were recorded from NA and number of strains was higher than that from PTB and LJ media. From PTB and LJ in station I, 22 and 15 number of species were reported to be identified, while 17 and 16 number of species from the media in station II.

The overall positivity rates of LJ and PTB for seven samples were compared in stations I and II(Table 14.1 and 14.2) and highly significant correlation at 5% level were observed on positivity rates among the two media in both the stations.

Species like *M.xenopi, M.obuense, m. poriferae, M.sphagni, m.diernhoferi, m.peregrinum, m.szulgai, M.phlei* and *M. shimoidei* were recovered only from NA. The frequency of isolation of *M. thermoresistibile* was found to be high in PTB than in NA.3 strains of *M. smegmatis* and single strain of *M. vaccae* recovered from stations were obtained from PTB medium, whereas *M. aichiense* was from both PTB and LJ. The intensity of occurrence of species like *M. kansasii*, was observed as high in PTB and LJ than in NA medium(Tables 11.1 and 12.1).

3.5. SEASONAL DISTRIBUTION OF NTM SPECIES

The number of species identified from the three seasons of the stations studied were varying with ,maximum of 28 species from post monsoon of station I and 23 from monsoon of station II and the strains observed also varied considerably. Monsoon

Fig. 6.1 Retrieval of NTM from selective media in Station I

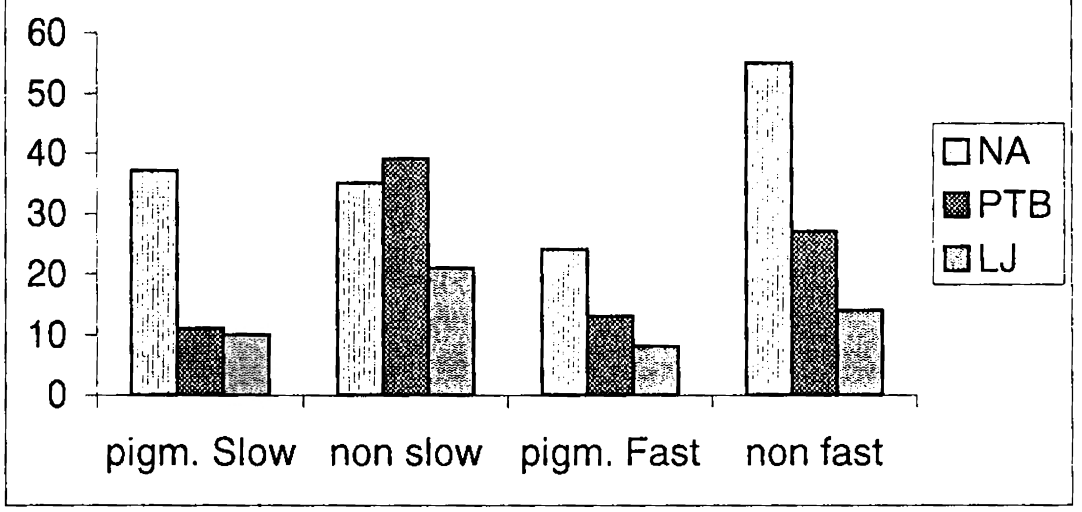
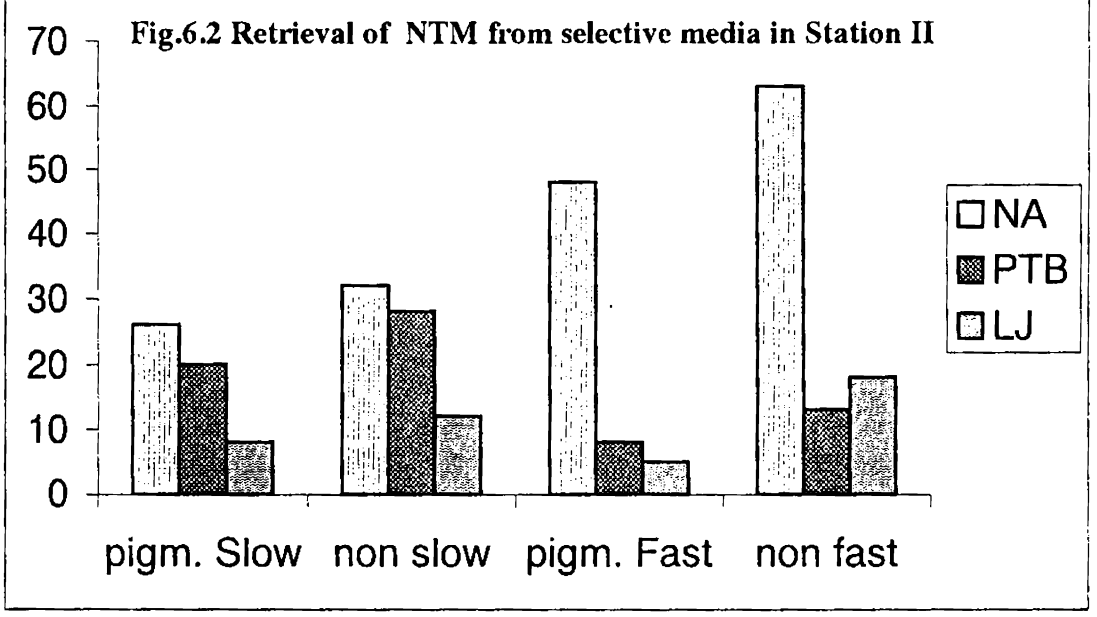


Fig.6.2 Retrieval of NTM from selective media in Station II



was the season with lowest number of 7 species of NTM in Narakkal and 21 and 22 species were retrieved from post and pre monsoon seasons of Valappu. Tables 15.1 and 15.2 show the diverse seasonal distribution of different species of slow and fast growers in the areas of study. Species *like M.marinum, M.asiaticum, M.gastri, M.malmoense, M.chelonae, M.chitae, M.abscessus , M.fortuitum* were recorded among all the seasons in both the stations, even though the number of strains of each species were different seasonally. *M. xenopi* and single strain of *M.flavescens* in station I as well as *M.szulgai* of station II were represented only during monsoon season, whereas, some sporadic species like *M.scrofulaceum, M.kansasii, M.shimoidei, M.parafortuitum, M.obuense, M.poriferae, M. sphagni* and *M.thermoresistibile* were absent during some seasons of the period (Tables 15.1 and 15.2).

3.6. SAMPLE WISE DISTRIBUTION OF NTM SPECIES

The number of species isolated from seven samples examined were varying with maximum of 23 species in station I from sediment and of 24 species each from sediment and water samples of station II respectively shows the highest affinity of NTM towards environmental samples for colonisation. Among the fish samples, liver tissue was with lowest species diversity of NTM (10 species identified) and intestine of station I and stomach of station II were with highest species abundance(22 species). Sixteen species of NTM were retrieved and identified from skin sample.

Distribution slow growing NTM species, identified has given in Tables 11.1, 11.2, 12.1, 12.2 and of fast growing in Tables 11.3, 11.4, 12.3, 12.4. *M. asiaticum* and *M.gastri* from station I and *M.marinum* and *M.chelonei* from station II were found to be harboured in fish and environmental samples studied and the other species identified were observed to be colonized differently among the samples.. the number of strains of the same species differed among samples in station I and station II.

3.7. EFFECT OF PHYSICO-CHEMICAL FACTORS ON THE DISTRIBUTION OF NTM SPECIES

The occurrence and distribution of different mycobacterium species were found to be influenced differently by all physico-chemical parameters monitored.(Table 16).

Table: 15.1. Seasonal distribution of slow growing NTM species in stations I and II

Pigmented slow growers

	Seasons					
	Premonsoon		Monsoon		Postmonsoon	
	Stations					
Species of NTM	**I**	**II**	**I**	**II**	**I**	**II**
M. marinum	7	9	9	8	3	6
M. xenopi	-		-		3	
M .asiaticum	10	3	5	5	9	6
M. scrofulaceum	-	8	2	-	8	2
M. kansasii	1	3	-	2	1	-
M. szulgai		-		-		2

Nonpigmented slow growers

	Seasons					
	Premonsoon		Monsoon		Postmonsoon	
	Stations					
Species of NTM	**I**	**II**	**I**	**II**	**I**	**II**
M. gastri	6	10	14	2	12	12
M. avium complex	2	6	-	7	-	-
M. shimoidei	2	1	-	1	1	1
M. malmoense	6	5	1	1	5	5
M. triviale	3	1	1	1	9	9
M. terrae	1	2	2	5	10	10

Table: 15.2. Seasonal distribution of fast growing NTM species in stations I and II

Pigmented fast growers

	Seasons					
	Premonsoon		Monsoon		Postmonsoon	
	Stations					
Species of NTM	**I**	**II**	**I**	**II**	**I**	**II**
M. parafortuitum	-		1		3	
M. obuense	4	3	-	1	-	3
M. aichiense	1		-		4	
M. flavescens	-	-	-	-	1	3
M. gadium	9	12	2	5	6	5
M. thermoresistibile	1	-	-	-	1	2
M. poriferae	-	-	3	6	1	-
M. komossense	1	2	-	3	-	1
M. sphagni	-	-	4	1	2	1
M. vaccae	1		-		-	
M. phlei		-		2		-
M. gilvum		-		-		4
M. aurum		5		1		1

Nonpigmented fast growers

	Seasons					
	Premonsoon		Monsoon		Postmonsoon	
	Stations					
Species of NTM	**I**	**II**	**I**	**II**	**I**	**II**
M. chelonei	10	4	15	8	4	15
M. chitae	3	4	10	14	10	5
M. diernhoferi	-	1	6	3	2	-
M. abscessus	11	5	2	4	6	12
M. fortuitum	3	2	4	4	2	1
M. peregrinum	1	4	-	5	5	2
M. smegmatis	-	1	-	-	2	-

Due to the scarcity of enough data to conduct statistical analysis, it was impossible to derive the relationship between ecological factors and the occurrence of species such as *M.xenopi, M.aichiense,* and *M.smegmatis.* Temperature was observed to effect positively on the occurrence and distribution of *M.triviale, M.parafortuitum, M.fortuitum,* whereas species like *M.scrofulaceum, M.aurum* and *M.abscessus* were influenced by variations in water pH. Factors like nitrite nitrogen, nitrate nitrogen, ammonia and phosphate were found to be affecting the predominance of *M.asiaticum, M.gastri, M.shimoidei, M.malmoense , M.obuense , M.sphagni, M.gilvum, M.diernhoferi* and the most important fish pathogen *M.marinum* . Abundance of *M.marinum* was also controlled by dissolved oxygen content in culture pond system(Table 16). ANOVA tables showing the effect of ecological factors on different Mycobacterium Spp. in Station Iwere given in Appendix1(Appendix1.1-1.10) and in Station II were given in Appendix 2(Appendix 2.1-2.17)

4. RANDOMLY AMPLIFIED POLYMORPHIC DNA ANALYSIS

Thirty mycobacterial isolates were randomly selected for the study by examining the species diversity as well as morphological variations existed among the strains of the same species after biochemical confirmation of their identity. The counts of the isolates at the time of DNA extraction ranged approximately from 10^5-10^8cfu/ml. The isolates analyzed through RAPD-PCR were designated serially from M1 to M30

Isolation of the total DNA of NTM isolates was done, following the method developed by Murray and Thompson, (1980) with some modifications. Quantitative analysis of the isolated DNA to check the yield and integrity by agarose gel electrophoresis revealed that DNA remained intact, without any shearing and was in sufficient quantities for the envisaged work.

Molecular genetic profiles of the 30 isolates of mycobacteria were carried out using RAPD-PCR. The PCR conditions were optimized from a number of standardization trials with varying concentration of ingredients as well as thermal cycles. The annealing condition in the PCR cycle was optimized at 37°C. Identity and number of amplicon bands decreased rapidly as the annealing temperature was

Table: 16. Range of count and factors affected of different NTM spp.

Species of NTM	Range of observed count($x10^3$)	
M. marinum	*0.9 –100*	Dissolved oxygen* (r= 0.8943) Nitrate nitrogen*(r= 0.7856) Ammonia*(r= 0.9021)
M. xenopi	*10- 100*	
M .asiaticum	*0.1- 270*	Nitrite nitrogen*(r= 0.8943) Phosphate*(r= 0.7980)
M. scrofulaceum	*0.1 - 10*	Water pH*(r= 8765)
M. kansasii	*1- 20*	
M. szulgai	*0.1- 0.5*	
M. gastri	0.1- 100	Ammonia*(r= 0.8423)
M. avium complex	*1- 20*	
M. shimoidei	*0.1- 1*	Phosphate*(r= 0.7650)
M. malmoense	*0.1- 100*	Ammonia*(r= 0.7711)
M. triviale	*0.1- 1*	Temperature @ (r= 0.9867, --0.7546*) Dissolved oxygen@(r= 0.9903)
M. terrae	*1-TNTC*	
M. nonchromogenicum	*0.2- 200*	
M. parafortuitum	*1- 250*	
M. obuense	*1- 296*	*Nitrite nitrogen**(r= --0.8879)
M. aichiense		

M. flavescens	*0.3- 20*	
M. gadium	*0.1- TNTC*	
M. thermoresistibile	*1- 100*	
M. pori	*0.1- 100*	Dissolved oxygen*(r= 0.9121)
ferae		
M. komossense	*0.1- 100*	Salinity*(r= 0.7781)
M. sphagni	*0.1- 20*	Ammonia*(r= 0.8650)
M. vaccae		
M. phlei	*120-TNTC*	
M. gilvum	*1-20*	*Ammonia**(r= 0.8676)
M. aurum	*1-10*	*Water pII**(r= 0.8346)
M. chelonei	*0.1- 300*	
M. chitae	*0.1- TNTC*	
M. diernhoferi	*0.3- TNTC*	*Phosphate**(r= 0.8091)
M. abscessus	*0.1-100*	*Water pII**(r= 0.9201)
M. fortuitum	*0.4-100*	*Temperature**(r= 0.9011) *Dissolved oxygen**(r= 0.9286) *Organic carbon**(r= - 0.7988)
M. peregrinum	*0.6- TNTC*	
M. smegmatis		

*(P<0.05), @(P<0.01)

increased to 55 °C. The initial screening of the PCR amplification using a panel of 25 primers (OPA 01-20 and OPAC 01-05)gave amplification by all of them. Among these, OPA-02 and OPA-18 gave sharp, highly reproducible and strain specific bands. The total number of amplicons were also few with these primers so that comparison of scoring and pattern were not confusing. Hence these two primers were shortlisted for final detailed screening of the isolates. Altogether 15 samples of each isolate were analysed.

The amplification of the DNA from each of the 30 isolates with the above two primers produced a total of 39 amplicons, which were consistent and appeared as distinct bands on agarose gel after electrophoresis. The molecular weight of the amplicons ranged from 2.2kb on the higher side to approximately 0.2kb on the lower side as determined from relative mobility of marker DNA viz. λDNA cut with HindIII/EcoRI. The RAPD pattern generated by both the primers is presented below in detail.

OPA-02

All the isolates were screened with OPA-02. This primer produced 21 amplicons, which were scorable as distinct bands in the gel and the fingerprints generated for each of the isolates using OPA-02 are presented in Plate 18.1 and 18.2. Polymorphism of the isolates was observed rarely. Eighteen fragments showed variation between isolates and three were amplified in almost all the isolates. The amplicons shared by all the isolates were 0.2kb and 1.2kb size. These are genus specific amplicons and can serve as molecular markers for NTM. DNA fragments of 0.8kb, 09kb, 1.0kb and 1.2kb were shared by some of the isolates i.e., 16, 20, 16, and 17 isolates respectively. 1.3kb and 2.0kb fragments were found in 6 and 8 isolates, whereas, 1.4kb fragments were observed in 11 isolates. The least shared fragments were 0.25kb, 0.28kb and 2.1kb having amplified only in 2, 1 and 1 isolates respectively.

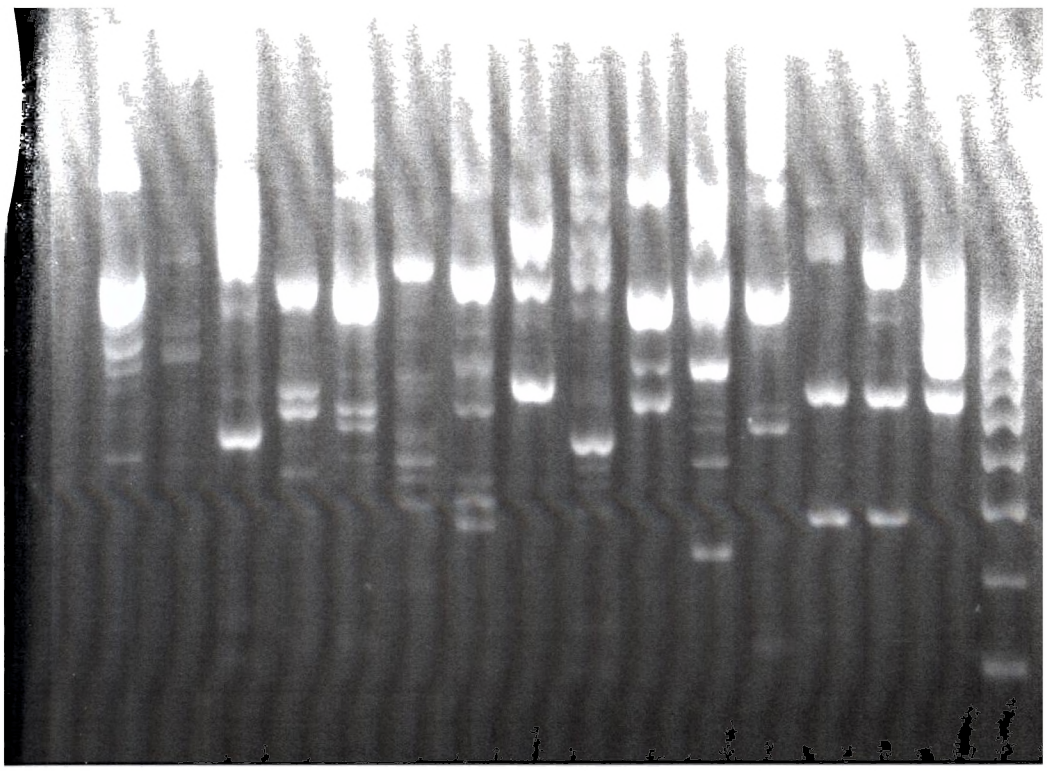


Plate No. 18.1: RAPD Pattern of 15 Isolates of *Mycobacterium* Spp. generated OPA 02

Lane 1 - 15

M1) *M. malmoense*
M2) *M. peregrinum*
M3) *M. marinum*
M4) *M. avium* complex
M5) *M. avium* complex
M6) *M.abscessus*
M7) *M.abscessus*
M8) *M.komossense*
M9) *M.komossense*
M10) *M.kansasii*
M11) *M.kansasii*
M12) *M .asiaticum*
M13) Unknown
M14) Unknown
M15) *M. aurum*

M : DNA Marker (100 bp ladder)

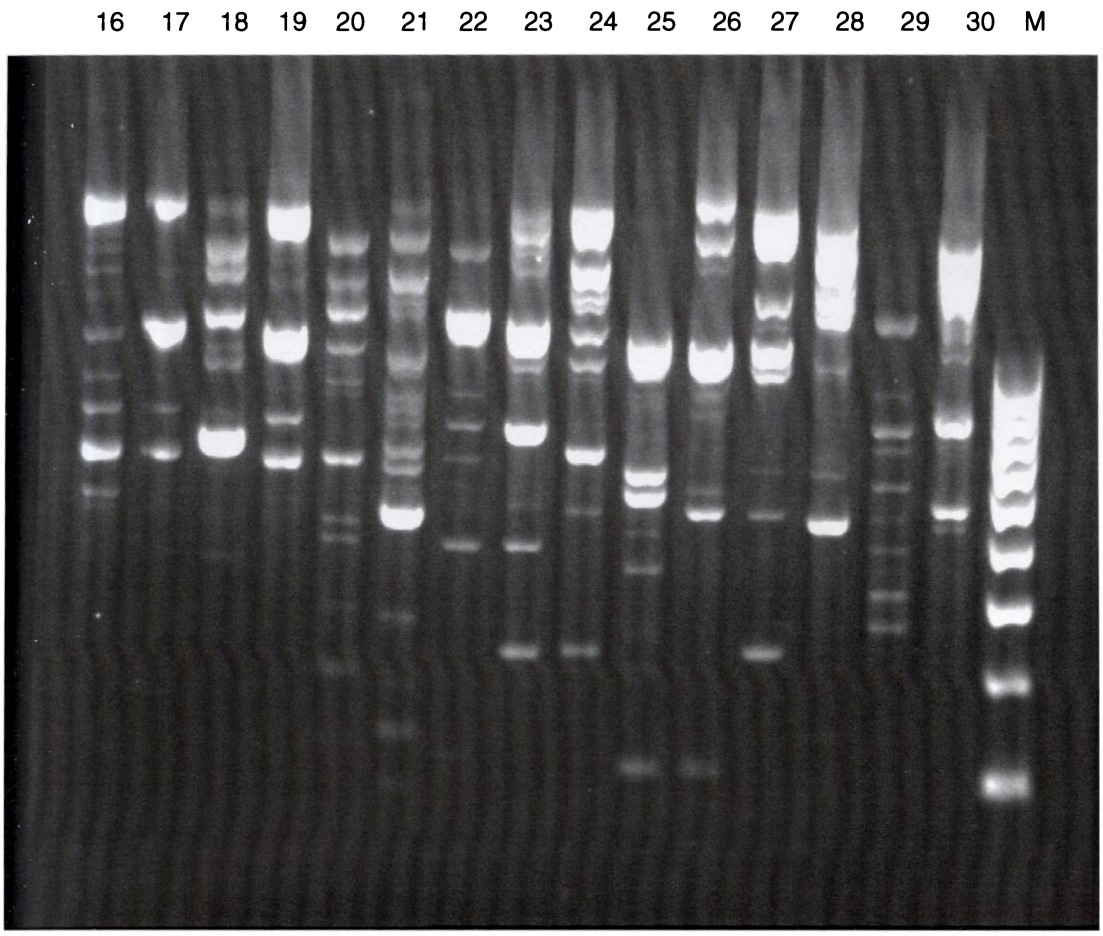


Plate No. 18.2: RAPD Pattern of 15 Isolates of *Mycobacterium* Spp. generated OPA 02

Lane 16 - 30

M16) *M. chelonae*
M17) *M. chelonae*
M18) *M. chelonae*
M19) *M. chelonae*
M20) *M. chelonae*
M21) *M.komossense*
M22) *M.komossense*
M23) *M. marinum*
M24) *M.abscessus*
M25) *M. gastri*
M26) *M. nonchromogenicum*
M27) *M. obuense*
M28) *M. gadium*
M29) *M. chitae*
M30) *M. chitae*

M : DNA Marker (100 bp ladder)

OPA-18

A total of 18 amplicons were generated by OPA-18 ranging in size from 0.35kb to 2.0kb. Though the number of amplification varied in individual isolates, all shared none of the amplicons. The amplicon of 1.6kb was shared by 16 of the isolates. The amplicons of 1.1kb and 1.4kb were found to be common in 12 and 10 isolates respectively. The amplicons generated by OPA-18 from each of the 30 isolates as resolved by agarose gel electrophoresis is presented in Plate 19.1 and 19.2.

Discriminating efficiency of primers used

OPA-18 produced less number of amplicons compared to OPA-02 and was highly discriminating for different species of mycobacteria and polymorphic for strains of the same species. But with both the primers, no polymorphism was observed among all the 30 isolates. Amplicons produced for the isolates by OPA-18 were distinct and reproducible except for 2 strains of *M.komossense*, and single isolates of *M.nonchromogenicum* and *M.obuense* (Plate 19.2) for which no scorable bands were produced. On comparing the banding pattern of different strains of the same species, all the amplicons obtianed with OPA-18 were showing clear-cut intraspecific polymorphism. The unique banding patterns observed for all single isolates of different species, showed considerable species specificity of OPA-18 and OPA-02 primers and can be considered as species specific markers.

Both the isolates of *M.chitae* shared only 3 amplicons and were polymorphic with OPA-18 (Plate 19.2) and hence can be considered as both intra and inter specific markers for the species. This observation was recorded for all the strains of the same species in this study (Plates 19.1 and 19.2).

Even though, the number of amplicons was more for each isolate, observed intraspecies specificity of the isolates was high with OPA-02 (Plates 18.1 and 18.2). These included both polymorphic and monomorphic loci. 5 strains of *M.chelonei* showed 3 polymorphic genic loci along with other nonsharing loci, indicating strain differences within the species. Both the unidentified strains gave only two amplicons with OPA-18 and 3 with OPA-02, and all were polymorphic and may be belonging

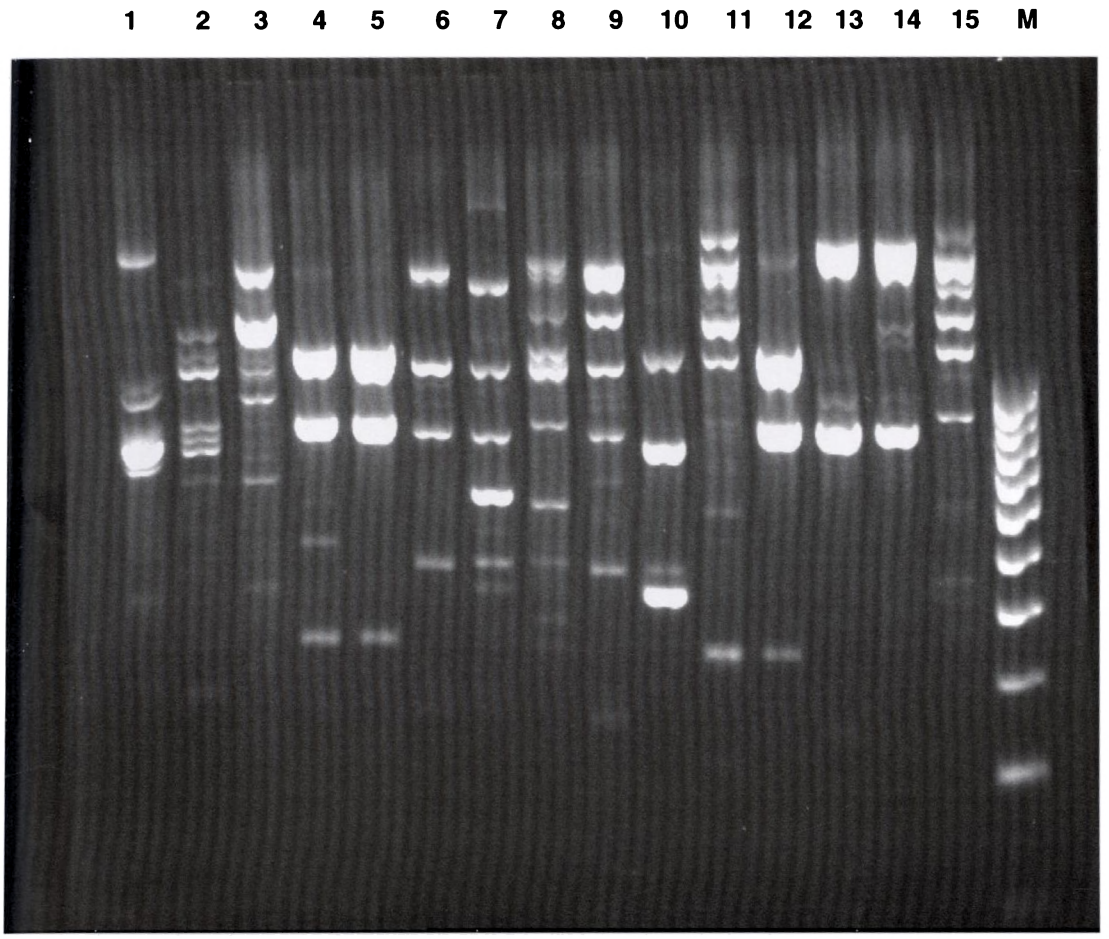


Plate No. 19.1: RAPD Pattern of 15 Isolates of *Mycobacterium* Spp. generated OPA 18

Lane 1 - 15

M1) *M. malmoense*
M2) *M. peregrinum*
M3) *M. marinum*
M4) *M. avium* complex
M5) *M. avium* complex
M6) *M.abscessus*
M7) *M.abscessus*
M8) *M.komossense*
M9) *M.komossense*
M10) *M.kansasii*
M11) *M.kansasii*
M12) *M .asiaticum*
M13) Unknown
M14) Unknown
M15) *M. aurum*

M : DNA Marker (100 bp ladder)

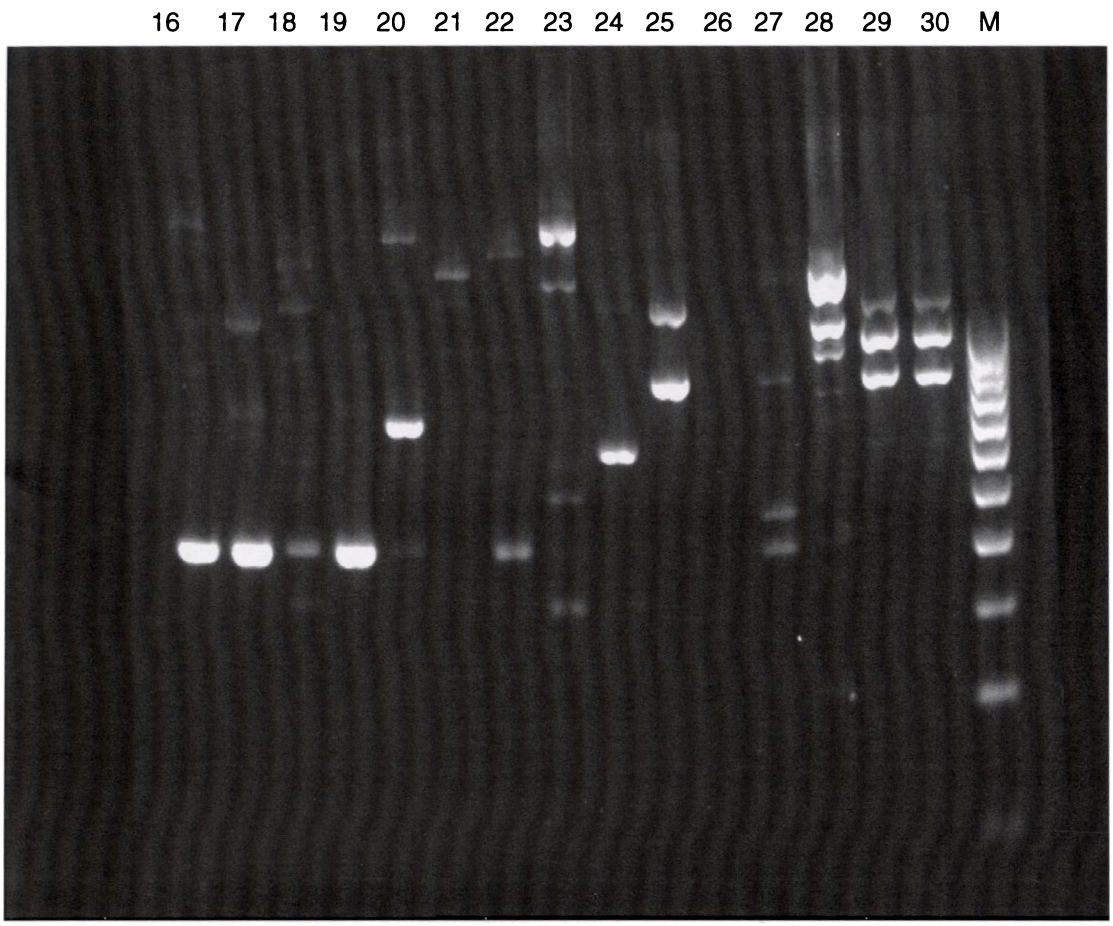


Plate No. 19.2: RAPD Pattern of 15 Isolates of *Mycobacterium* Spp. generated OPA 18

Lane 16 - 30

M16) *M. chelonae*
M17) *M. chelonae*
M18) *M. chelonae*
M19) *M. chelonae*
M20) *M. chelonae*
M21) *M.komossense*
M22) *M.komossense*
M23) *M. marinum*
M24) *M.abscessus*
M25) *M. gastri*
M26) *M. nonchromogenicum*
M27) *M. obuense*
M28) *M. gadium*
M29) *M. chitae*
M30) *M. chitae*

M : DNA Marker (100 bp ladder)

to new species as per RAPD fingerprint. For *M.peregrinum* isolate, no banding pattern was obtained with OPA-02.

The results indicated that OPA-02 and OPA-18 is ideal for getting species-specific amplicons for different *Mycobacterium* Spp., whereas, OPA-02 can also be used for studying the heterogeneity among different strains of the same species of Mycobacterium.

Polymorphism, Similarity index, Genetic distance and Phylogenetic relationship.

The data pertaining to randomly amplified DNA fragments produced by all the primers across the 30 isolates were analyzed by the POPGENE 1.32 software and the resulting estimates are presented below. Comparison of the fingerprints at each of the 39 loci indicated strain- specific RAPD pattern which was quite apparent among the different isolates. The Popgene analysis indicated that overall polymorphism was 100% with these two primers showing not a single band was common among all the 30 isolates.

The average similarity index between species, considering all the amplicons resulting from two primers estimated as Nei's original measures of genetic identity and genetic distance between the 30 isolates are shown in Tables17.1, 17.2 and 17.3. Perusal of the tables shows that the coefficients of genetic identities ranged from 0.4 to 0.95. The mean H values(average gene diversity) ranged from 0.0644 to 0.5000 with an average of 0.3313. Of the 435 pair wise genetic identity estimates, more than 99% were higher than 0.5. Genetic similarity less than 0.5 only in 0.9% cases, only 4 out of the total 435 pair wise comparisons.

Dendrograms in the phylogram form, depicting the phylogenetic relatedness between various isolates and Nei's genetic similarity coefficients, generated by the unweighted pair group method using arithmetic averages. (UPGMA, modified from NEIGHBOR procedure of Phylip Version 3.50) following 1000 bootstrap replications is presented in Fig.7. Examination of the dendrogram showed that the isolates were distinctly grouped into two major clusters, one large cluster with 17 isolates (M1, M3,

Table 17 Nei's original measures of Genetic identity and genetic distance between the *Mycobacterium* spp. isolates

pop ID	1	2	3	4	5	6	7	8	9	10
1	****	0.6750	0.7500	0.6500	0.8000	0.7250	0.6000	0.7000	0.7500	0.7250
2	0.3930	****	0.7250	0.7250	0.6750	0.7000	0.6250	0.6250	0.6750	0.7000
3	0.2877	0.3216	****	0.6500	0.6500	0.6750	0.6000	0.8000	0.8500	0.5750
4	0.4308	0.3216	0.4308	****	0.8000	0.6750	0.7000	0.7000	0.6000	0.6750
5	0.2231	0.3930	0.4308	0.2231	****	0.6750	0.7000	0.7000	0.6500	0.7750
6	0.3216	0.3567	0.3930	0.3930	0.3930	****	0.7750	0.6750	0.7750	0.7500
7	0.5108	0.4700	0.5108	0.3567	0.3567	0.2549	****	0.7000	0.7500	0.7250
8	0.3567	0.4700	0.2231	0.3567	0.3567	0.3930	0.3567	****	0.8000	0.7250
9	0.2877	0.3930	0.1625	0.5108	0.4308	0.2549	0.2877	0.2231	****	0.7250
10	0.3216	0.3567	0.5534	0.3930	0.2549	0.2877	0.3216	0.3216	0.3216	****
11	0.5108	0.7444	0.3567	0.5978	0.4308	0.4700	0.4308	0.3567	0.2877	0.5534
12	0.2877	0.3216	0.3567	0.1625	0.0513	0.3930	0.2877	0.2877	0.3567	0.3216
13	0.3930	0.3567	0.3930	0.3216	0.4700	0.3567	0.4700	0.3216	0.3930	0.4308
14	0.3216	0.5108	0.3216	0.3930	0.3930	0.4308	0.4700	0.2549	0.3216	0.3567
15	0.3216	0.5108	0.2549	0.4700	0.3930	0.4308	0.5534	0.3216	0.3930	0.3567
16	0.4308	0.5534	0.5108	0.6931	0.4308	0.3930	0.6931	0.5108	0.5108	0.3930
17	0.5108	0.3216	0.3567	0.4308	0.3567	0.3216	0.4308	0.3567	0.3567	0.3216
18	0.5108	0.4700	0.5108	0.5978	0.4308	0.3930	0.5108	0.4308	0.4308	0.3216
19	0.4700	0.4308	0.4700	0.5534	0.3930	0.4308	0.4700	0.3930	0.3930	0.3567
20	0.4308	0.3930	0.5108	0.6931	0.5978	0.4700	0.5978	0.6931	0.5108	0.5534
21	0.4700	0.5978	0.3930	0.7444	0.6444	0.5978	0.6444	0.4700	0.3930	0.5978
22	0.5108	0.4700	0.5108	0.5978	0.4308	0.3930	0.5978	0.5108	0.5108	0.3930
23	0.6931	0.6444	0.5978	0.5108	0.5108	0.5534	0.5978	0.5978	0.5108	0.6444
24	0.5534	0.5978	0.6444	0.6444	0.4700	0.5108	0.4700	0.4700	0.5534	0.5108
25	0.3216	0.3567	0.4700	0.2549	0.2549	0.4308	0.3930	0.3216	0.3930	0.2231
26	0.4308	0.3930	0.5978	0.5108	0.4308	0.4700	0.5108	0.5108	0.5108	0.3930
27	0.5108	0.5534	0.6931	0.4308	0.5108	0.5534	0.5978	0.5978	0.6931	0.4700
28	0.4308	0.3216	0.3567	0.4308	0.4308	0.5534	0.5108	0.3567	0.4308	0.4700
29	0.3216	0.4308	0.3216	0.3216	0.2549	0.5108	0.6444	0.3930	0.5534	0.4308
30	0.3567	0.4700	0.5108	0.5978	0.3567	0.5534	0.6931	0.5108	0.6931	0.5534

11	12	13	14	15	16	17	18	19	20	21
6000	0.7500	0.6750	0.7250	0.7250	0.6500	0.6000	0.6000	0.6250	0.6500	0.6250
0.4750	0.7250	0.7000	0.6000	0.6000	0.5750	0.7250	0.6250	0.6500	0.6750	0.5500
0.7000	0.7000	0.6750	0.7250	0.7750	0.6000	0.7000	0.6000	0.6250	0.6000	0.6750
0.5500	0.8500	0.7250	0.6750	0.6250	0.5000	0.6500	0.5500	0.5750	0.5000	0.4750
0.7750	0.6500	0.9500	0.6250	0.6750	0.6750	0.6500	0.7000	0.6500	0.6750	0.5500
0.6250	0.6750	0.7000	0.6500	0.6500	0.6750	0.7250	0.6750	0.6500	0.6250	0.5500
0.6500	0.7500	0.6250	0.6250	0.5750	0.5000	0.6500	0.6000	0.6250	0.5500	0.5250
0.7000	0.7500	0.7250	0.7750	0.7250	0.6000	0.7000	0.6500	0.6750	0.5000	0.6250
0.7500	0.7000	0.6750	0.7250	0.6750	0.6000	0.7000	0.6500	0.6750	0.6000	0.6750
0.5750	0.7250	0.6500	0.7000	0.7000	0.6750	0.7250	0.7250	0.7000	0.5750	0.5500
****	0.6500	0.5750	0.6250	0.6750	0.5500	0.6000	0.6000	0.5750	0.5000	0.5750
0.4308	****	0.6750	0.6750	0.6250	0.6000	0.7500	0.6500	0.7250	0.5500	0.5750
0.5534	0.3930	****	0.9000	0.7000	0.6750	0.7250	0.6250	0.7000	0.5750	0.5500
0.4700	0.3930	0.1054	****	0.8000	0.6750	0.6750	0.5750	0.6500	0.5250	0.6000
0.3930	0.4700	0.3567	0.2231	****	0.6250	0.6250	0.5750	0.5500	0.6250	0.6000
0.5978	0.5108	0.3930	0.3930	0.4700	****	0.8500	0.8500	0.8750	0.7000	0.6750
0.5108	0.2877	0.3216	0.3930	0.4700	0.1625	****	0.8500	0.9250	0.6500	0.6750
0.5108	0.4308	0.4700	0.5534	0.5534	0.1625	0.1625	****	0.8750	0.7500	0.7750
0.5534	0.3216	0.3567	0.4308	0.5978	0.1335	0.0780	0.1335	****	0.6750	0.7500
0.6931	0.5978	0.5534	0.6444	0.4700	0.3567	0.4308	0.2877	0.3930	****	0.6750
0.5534	0.5534	0.5978	0.5108	0.5108	0.3930	0.3930	0.2549	0.2877	0.3930	****
0.5108	0.4308	0.3930	0.4700	0.5534	0.1625	0.1625	0.1625	0.1924	0.2877	0.3930
0.5108	0.5108	0.5534	0.6444	0.6444	0.3567	0.4308	0.3567	0.3216	0.3567	0.4700
0.6444	0.4700	0.5108	0.5108	0.6931	0.1924	0.2549	0.1924	0.1625	0.3930	0.2877
0.6444	0.2549	0.2877	0.2877	0.3567	0.5534	0.3930	0.4700	0.4308	0.5534	0.5978
0.6931	0.4308	0.3930	0.4700	0.5534	0.2877	0.2877	0.2877	0.2549	0.3567	0.3930
0.9163	0.5108	0.4700	0.5534	0.6444	0.3567	0.3567	0.2877	0.3216	0.4308	0.4700
0.5978	0.3567	0.5534	0.5534	0.3930	0.5108	0.4308	0.2877	0.3930	0.3567	0.1924
0.5534	0.3216	0.3567	0.3567	0.3567	0.3930	0.3930	0.5534	0.4308	0.6444	0.5108
0.7985	0.3567	0.5534	0.6444	0.4700	0.3567	0.4308	0.2877	0.3216	0.2231	0.3216

Nei's genetic identity(above diagonal) and genetic distance(below diagonal)

Table 17 continued

22	23	24	25	26	27	28	29	30
0.6000	0.5000	0.5750	0.7250	0.6500	0.6000	0.6500	0.7250	0.7000
0.6250	0.5250	0.5500	0.7000	0.6750	0.5750	0.7250	0.6500	0.6250
0.6000	0.5500	0.5250	0.6250	0.5500	0.5000	0.7000	0.7250	0.6000
0.5500	0.6000	0.5250	0.7750	0.6000	0.6500	0.6500	0.7250	0.5500
0.6500	0.6000	0.6250	0.7750	0.6500	0.6000	0.6500	0.7750	0.7000
0.6750	0.5750	0.6000	0.6500	0.6250	0.5750	0.5750	0.6000	0.5750
0.5500	0.5500	0.6250	0.6750	0.6000	0.5500	0.6000	0.5250	0.5000
0.6000	0.5500	0.6250	0.7250	0.6000	0.5500	0.7000	0.6750	0.6000
0.6000	0.6000	0.5750	0.6750	0.6000	0.5000	0.6500	0.5750	0.5000
0.6750	0.5250	0.6000	0.8000	0.6750	0.6250	0.6250	0.6500	0.5750
0.6000	0.6000	0.5250	0.5250	0.5000	0.4000	0.5500	0.5750	0.4500
0.6500	0.6000	0.6250	0.7750	0.6500	0.6000	0.7000	0.7250	0.7000
0.6750	0.5750	0.6000	0.7500	0.6750	0.6250	0.5750	0.7000	0.5750
0.6250	0.5250	0.6000	0.7500	0.6250	0.5750	0.5750	0.7000	0.5250
0.5750	0.5250	0.5000	0.7000	0.5750	0.5250	0.6750	0.7000	0.6250
0.8500	0.7000	0.8250	0.5750	0.7500	0.7000	0.6000	0.6750	0.7000
0.8500	0.6500	0.7750	0.6750	0.7500	0.7000	0.6500	0.6750	0.6500
0.8500	0.7000	0.8250	0.6250	0.7500	0.7500	0.7500	0.5750	0.7500
0.8250	0.7250	0.8500	0.6500	0.7750	0.7250	0.6750	0.6500	0.7250
0.7500	0.7000	0.6750	0.5750	0.7000	0.6500	0.7000	0.5250	0.8000
0.6750	0.6250	0.7500	0.5500	0.6750	0.6250	0.8250	0.6000	0.7250
****	0.7000	0.7750	0.5750	0.7000	0.7000	0.6500	0.6750	0.7500
0.3567	****	0.7250	0.5750	0.7000	0.7000	0.6500	0.5750	0.7000
0.2549	0.3216	****	0.6000	0.7750	0.7750	0.6750	0.6000	0.7250
0.5534	0.5534	0.5108	****	0.8250	0.7250	0.6750	0.7000	0.6250
0.3567	0.3567	0.2549	0.1924	****	0.8500	0.7000	0.6250	0.7000
0.3567	0.3567	0.2549	0.3216	0.1625	****	0.6500	0.5750	0.7000
0.4308	0.4308	0.3930	0.3930	0.3567	0.4308	****	0.6250	0.8000
0.3930	0.5534	0.5108	0.3567	0.4700	0.5534	0.4700	****	0.6750
0.2877	0.3567	0.3216	0.4700	0.3567	0.3567	0.2231	0.3930	****

Nei's genetic identity(above diagonal) and genetic distance(below diagonal)

Table 18. Summary of Genic Variation Statistics for All Loci (Nei,1987)

	Sample Size	na*	ne	h*	I*
Mean genetic diversity	30	2.0000	1.5536	0.3313	0.5016
Standard Deviation	0.0000	0.2980	0.1360	0.1659	

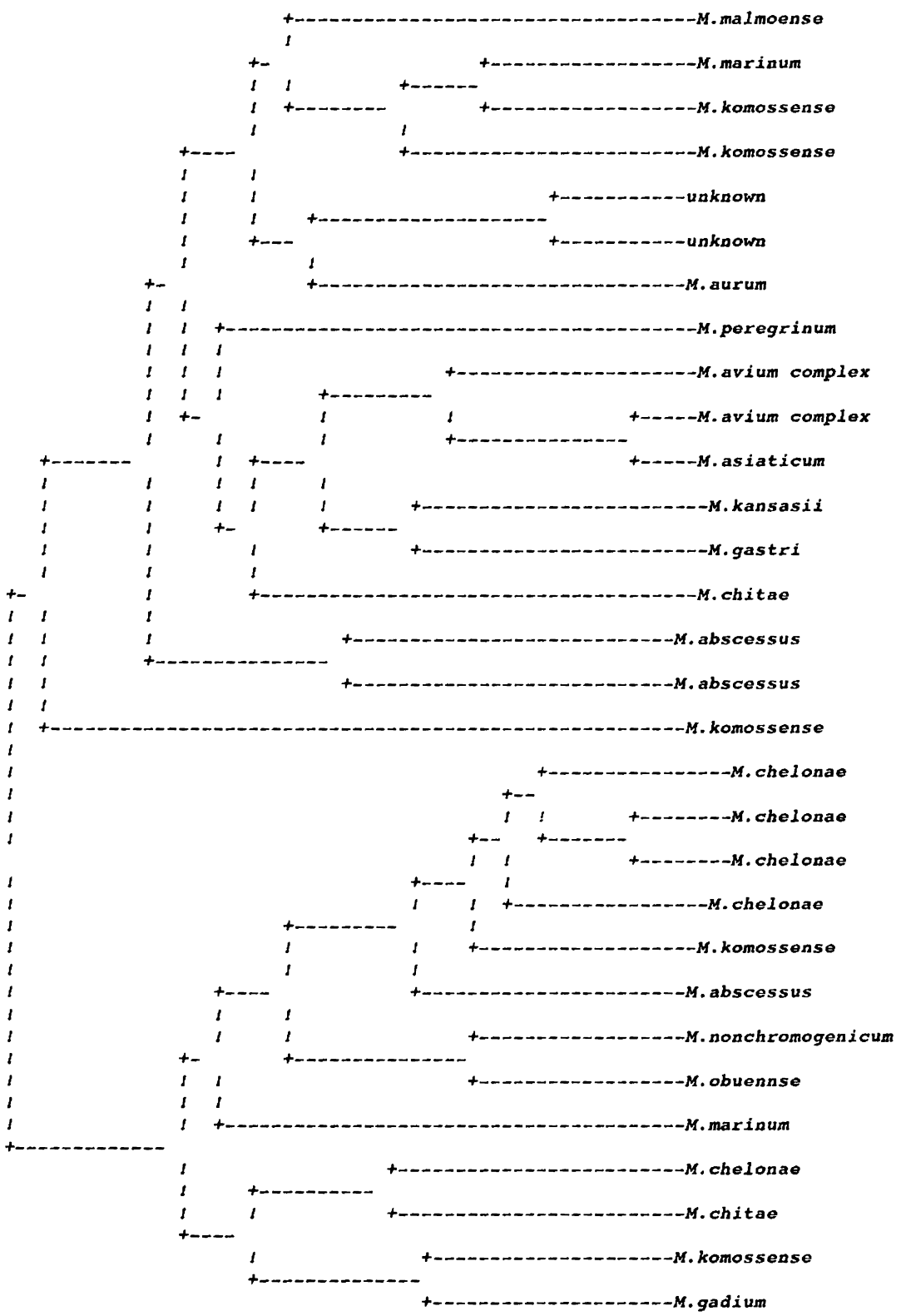
* na = Observed number of alleles

* ne = Effective number of alleles [Kimura and Crow (1964)]

* h = Nei's (1973) gene diversity

* I = Shannon's Information index [Lewontin (1972)]

Fig:7 Dendrogram based on the RAPD data of Mycobacterium spp. Isolates showing genetic relatedness among them



M9, M8, M13, M14, M15, M2, M4, M5, M12, M10, M25,.M29, M6, M7and M11) and minor one with13 isolates (M16, M17, M19, M18, M22, M24, M26, M27, M23, M20, M30, M21 and M28).

Discussion

V DISCUSSION

Bacteria are intimately associated with all life stages of marine organisms. In aquaculture, high densities of microflora often make conditions ideal for opportunistic pathogens, with high mortalities being the result. Knowledge of the different ecological relations between bacteria and different cultivated species is essential if you are t o ensure increased survival. There is a growing awareness in the study of Mycobacteria associated with fish and shell fish. The varied and diverse group of mycobacterial infections arises from the combination of the low innate pathogenicity of the organism and the opportune exposure of the host. The virulence of the particular organism , individual host susceptibility and the timing and the degree of exposure all play crucial roles in the acquisition, progression and the duration of the specific disease produced, although , mycobacteria are classified together in the same genus of bacteria. The various atypical mycobacteria are widely having varied cultural diversity characteristics, histology and responses to water treatement. These very diversities help to define specific microorganisms involved and the spectrum of disease produced in the inmmuno-compromised state of the fish and shellfish. The incidence of these diseases should be aware as all immuno-compromised organisms continuous to increase due to multiple stress factors in the aquaculture programme. Environmental mycobacteria which are called as atypical mycobacteria are acid-fast that are related to tubercle bacilli. Infections caused by these organisms are emvironmentally derived. Ecology of mycobacteria are poorly understood and the reason for the increased number of pathogenic mycobacteria in samples and the appearance of new pathogenic species is unknown.

As the studies on mycobacteria is an unexplored area, in the perennial and pokkali aquaculture systems of Cochin backwaters, a study on **"Ecophysiology of nontuberculous mycobacteria in marine aquaculture ponds"** was initiated and regular collections of cultured tilapia, (*Oreochromis mossambicus*) , sediment and water was made from two fixed stations for a period of one year from March 1999 to Feb 2000. The results of the study show that,

1. Nontuberculous mycobacteria(NTM) was 19.5% of the total heterotrophs observed.
2. Totally, 689 NTM strains were isolated during the period of study and 83.3% (n=574) were identified specifically following standard biochemical identification procedure and molecular studies.
3. Of 33 species of NTM identified, 17 were found pathogenic.
4. Statistically significant correlations observed between mycobacteria and different physico-chemical parameters, revealed profound influence of ecological parameters on occurrence, distribution and activity of different NTM flora in Cochin backwater system.
5. Mycobacterial flora is highly heterogeneous.

Throughout the period of study, station I and II showed almost identical physico-chemical parameters even though slight variations were existed between the two, which can be attributed to the reflection of changes in the Cochin backwater system as the culture ponds are extensions of this estuary. The temperature of the study areas were showing considerable seasonal fluctuations and recorded maximum during premonsoon season, exhibiting up to 34°C, whereas the month of May showed minimum water temperature in both the stations. The change could have been caused due to early monsoon rains during the year of study.

Sharp variations in temperature were observed in the present study during onset and withdrawal of the southwest monsoon, the result reaching parallel to those reported by Lakshmanan *et al* .,(1982). They also reported that salinity gradients in the northern side of the estuary are stronger than the northern side. Present study revealed that there is gradual salinity from monsoon to pre monsoon seasons till the maximum of 24.27% and observed to coming down till 3.14% in May, the previous month of the onset of monsoon. This difference may be caused by the early monsoon and flushing of fresh water into the estuarine system.

The high salinity value in the pre-monson can be attributed to the intrusion of bottom saline waters during this period (Lakshmanan *et al.,* 1987). The range of salinity during monsoon (1.57%-8.58%), in the present study was slightly higher than those reported by Lakshmanan *et al.,* (1982), may be caused by heavy monsoon falls

during the period of study, according to Sreedharan and Mohammed Salih(1974), wide variation observed insalinity may be due to combined action of water movement induced by fresh water discharge, tidal variation and mixing.

The algal bloom that may happened in high concentration of nutrients through monsoon influx and increases proliferation of bacteria followed, could be considered as the reason for low dissolved oxygen levels during the months of March and June 1999.

The range of dissolved oxygen(7-8.4) observed by Venkitesan *et al.,* (2001) were found as parallel to the results of the present investigation. According to the data of the present study, the mean value for organic carbon was showing wide difference in station I with 0.2498mg/gm and in station II with 1.116mg/gm and this difference is revealing the fact that the sediment texture is different in both stations. Organic carbon values indicate that station II is more fertile with clayey silt type of soil capable of holding more organic debris. Low organic carbon content in station I(0.149- 0.385 mg/gm) could be due to high percentage of sand content in the sediment. Low value for organic carbon in station II is only occasional on comparing with station I (Venkatesan *et al.,* 2001; Nair *et al.,* 1993). Maximum organic carbon content was observed during pre monsoon and minimum during monsoon season in thee present study as it was observed by Nair *et al.,* (1993). The maximum organic carbon value observed in station II during February was recorded along with high salinity (22.15%) and this is in accordance with the suggestion by Nair *et al.,* (1993) that increasing salinity promote settling of organic carbon into clay particles.

It appears from the data that annual nitrate nitrogen values were observed to be fluctuating between 0-4.93ugat/l and this is consistent with the earlier reports by Venkatesan *et al.,* (2001) and Lakshmanan *et al.,* (1987). During dry season, the data showed fluctuation between 0.1 and 0.683 but the range exhibited by Sreedharan and Mohammed Salih(1974) was 0.4-1.1ugat/l. Absence of freshwater influx as well as absence of organic decomposition should be considered as the reason for narrow nitrite nitrogen range during the present study.

Eventhough the general range of nitrate nitrogen was observed to be fluctuating from 0.059- 1.564ugat/l in monsoon season of station II, occasional range of 0-2.14ugat/l was recorded. It was in accordance with the study by Lakahmanban *et al.*, (1987). Nil value for nitrite and nitrate nitrogen in the month of June can be attributed to the heavy planktonic growth for full utilization of nutrients and followed bacterial degradation and rain water influx which will cause in abnormal rise in nutrient contents.

Satpathy and Nair (1996) reported a range from 0.19-3.27 reaching almost parallel to the present study and high monsoonal values are not noticeable. The fluctuation of nitrate in pre monsoon and post monsoon in the present study are 0.297-1.564 and 0.059-0.758ugat/l respectively and both these ranges are not same as observed by Sreedharan and Mohammed Salih(1974).

Data of the present study revealed that phosphate content in water in the study areas fluctuated between 0.77(postmonsoon) and 19.81(monsoon) and this range is in consistent with the report of Venkitesan etal (2001). Lowest range observed was only occasional in the post monsoon from 1.03 to 2.01. but in station I, high phosphate content(14.19ugat/l) was observed in pre monsoon than in monsoon(11.86) and parallel results were recorded by Lakshmanan *et al.*, (1987) wit high phosphate content in pre monsoon, possibly caused by local sewage input r industrial waster disposal. Increased bacterial degradation of organic matter could be considered as the reason for increased phosphate considered as the reason for increased phosphgate content in station II. Phosphate distribution s not seasonally correlated with salinity (Lakshmanan *et al.*, 1987).

Studies have been carried out to enumerate heterotrophic bacteria in different ecosystems and according to Chandrika and Nair(1992) and Shyni and Chandrika(1998), variations exist in the bacterial load of different ponds depend on the wide range of biotic, abiotic as well as anthropogenic factors. In the present study, no uniform distribution pattern of heterotrophic bacterial population was observed. The range of total heterotrophic count in stations I and II during pre monsoon was 20.4 $x10^3$ -106.1 $x10^3$. The ranges for monsoon and post monsoon seasons in Station I were almost equal, 12.68 $x10^3$ -64.7 $x10^3$ and 29.68 $x10^3$- 61.4 $x10^3$ respectively and the ranges were 22.48 $x10^3$ -89 and 37.57-82 for station II. Highest TPC in station II and I

were in March and June and the lowest during August and Februay. Chandrika and Nair(1992) found highest TPC in June and lowest in August.

According to Janakiram *et al.,* (2000), the low bacterial load in monsoon is due to low salinity conditions. The minimum TPC observed in monsoon during the present investigation was found to be in accordance with the observation of Chandrika(1983), but according to this, maximum mean TPC was during post monsoon season. the present study revealed result giving high count in water. The reason for this could be the concentration of heterotrophs during membrane filtration before plating. In the two stations, the high nutrient availabitity and organic matter can be attributed as the reason for this high count in the study area

The range of TPC in sediment during the present study was found parallel to that of Janakiram *et al.,* (2000) in ME ponds. During the present study, the count obtained from the surface mucus and skin of Tilapia was found high The high count in skin and mucus can be attributed to the fact that skin is providinbg a better ecological niche for proliferation of heterotrophs.

According to Chandrika and Nair(1992), little baseline data is available on bacterial load from penaeid brackishwater culture ponds. The maximum TPC of the present study during premonsoon was found as parallel to the study by Chandrika and Nair(1992) and they observed this during December to March.

This is the first study designed to enumerate slow growers along with fast growers of NTM from both environment and cultured fish. The results of the study showed that all the samples appear to be rich sources of environmental mycobacteria. The seasonal variations in the occurrence of NTM in two selected aquaculture ponds in the northern part of the Cochin backwater system have been studied and the intensity and the percentage distribution of mycobacteria were estimated by enumerating total mycobacterial count along with total plate count of heterotrophs. The microbial interrelationship in the aquaculture system was determined statistically. The physico-chemical parameters viz. water temperature, salinity, dissolved oxygen, pH, organic carbon, nitrite nitrogen, nitrate nitrogen, ammonia and phosphate were measured

monthly and the possible relationship with mycobacteria and heterotrophs was analyzed statistically and given in correlation matrices. All the physico-chemical parameters except Water temperature and pH showed wide seasonal variations and all parameters were showing distinct positive and negative correlations with occurrence of both heterotrophs and NTM.

The results showed that in both the seasons, nine ecological factors monitored were expressing strong statistically significant positive and negative correlations at 1% and 5% levels during the three seasons separately. Temperature in station I was onbserved to be related with dissloved oxygen, organic carbon($P<0.05$) and pH($P<0.01$, whereas in station II, influence was by factors such as salinity, temperature, dissolved oxygen and phosphate. During postmonsoon in both the stations, temperature was governed positively by pH and negatively by salinity, whereas in station I, nitrate, ammonia and phosphate were factors meant for trempertature maintenance and in station II, they were organic carbon and nitrite. ($P<0.01$). During the same season, the factors which were to be interrelated were different in station I and II is indicating the possibility of other factors that may interfere within the availability of these factors in the sampled area. It is also observed from the data that ammonia can be influence the salinity negatively at 5% level in station I and positively at 5%level in station II, during the same season. During premonsoon, organic carbon and nitrite($P<0.01$) were found to be influencing salinity in station I, but in station II, dissolved oxygen($P<0.01$) and ammonia($P<0.05$) were determining factors. In stations I and II variation in dissolved oxygen content was found to be nfluenced by nitrite, nitrate, ammonia and phosphate and in station I, pH also showed positive correlation with dissolved oxygen at 5% level. Ammonia influenced water pH positively($P<0.01$) in station II and negatively in station I during premonsoon season. As per the data observed temperature in monsoon season of Narakkal was influenced by pH, organic carbon, and nitrate($P<0.01$) but all the factros except phosphate were found to be controlling temperature and salinity in Valappu, both positively and negatively. Organic carbon content in station I duroing monsoon, was positively related with nitrate($P<0.01$) and ammonia($P<0.05$). but in station II, ammonia and phosphate were related with organic carbon negatively($P<0.01$). During postmonsoon, apart from pH and nitrate factors like organic carbon, phosphate and ammonia showed influence with salinity in station I.

The study shows that influence among different ecological factors is natural which can be attributed to unknown activities occurring among various physical, chemical and biological acivities in the system. This distinct difference is also showing high dynamic nature of aquaculture ponds in cochin backwater system. Statistical studies revealed some similarities among both the stations. During premonsoon, dissolved oxygen content in stations was controlled by nitrite, nitrate, ammonia and phosphate. During monsoon, dissolved oxygen content was related with organic carbon ammonia and phosphate($P<0.05$). common factors like pH, phosphate, nitrate($P<0.01$), organic carbon and ammonia($P<0.05$)were observed with positive relationship with salinity during postmonsoon and dissolved oxygen was influenced negatively by ammonia($P<0.01$)in both the stations. These similarities observed in the interrelationship and limiting factors in the stations are showing the continuity of the pond system with Cochin backwaters and ultimate uniqueness of the system with distinct biotic and abiotic factors.

Species of mycobacteria like *M.marinum, M.chlonei, M. abscessus, M.neoaurum, M.scrofulaceum, M.simiae* cause mycobacteriosis in fish, a sub acute to chronic wasting disease known to affect some 167 freshwater and salt water species (Chinabut, 1999). Internal signs of the disease vary according to fish species and typically include granulomas of the spleen, kidney and liver. Just because of this reason, usually for enumeration of NTM from infected and healthy fishes organs like spleen, kidney as well as liver were selected for both microbiological and histopathological examinations (Hedrick *et al.*, 1987; McCormick *et al.*, 1995; Lansdell *et al.*, 1993; Wayne and Kubica 1986) as these are the sites of high mycobacterial replication as well as colonisation. In the present study apart from environmental samples, five fish samples namely skin, gills, stomach, intestine and liver were selected in order to enable the overall assessment in the comparative occurrence of NTM.

According to Portaels *et al.*, (1988), the isolation of mycobacteria from samples such as soil, heavily contaminated with other microbes requires strong decontamination procedures to overcome the multiplication of other bacteria and fungi and the samples were decontaminated using 4 % NaOH, eventhough it is significant to note that only a small percentage of environmental bacteria will survive on pre-treatment with higher concentration of NaOH (Falkinham *et al.*, 1980; Kamala *et al.*, 1994). But, Monique *et*

al., (1979) stated that mycobacterial isolation statistically favours the use of NaOH and they grounded the tissues and added distilled water, centrifuged before inoculation and the method was found efficient when it was followed in the present study also. Membrane filtration and decontamination using 4 % NaOH and centrifugation procedures were followed by Paul, (1969) for retrieving NTM from water sources. Lawhavinit *et al.*,(1993) used 4 % NaOH for 10 minutes to isolate mycobacteria from pejerrey (*Odonthestes bonariensis)* . Kamala *et al.*, (1994) selected Falkinham's method using 4 % NaOH as the best decontamination procedure with which maximum number of strains were retrieved with more species and fewer contamination rate from soil samples in a BCG trial area. The isolation procedure followed by Kent and Kubica (1985) and Lansdell *et al.*, (1993) was similar to that of the present study by homogenizing the tissue with 10ml sterile water, added equal volume of 2-3% NaOH, mixed for 45 seconds and allowed to remain at RT for 15mts, centrifuged at 3000rpm for 15mts and inoculated on selective media. There is scarcity of NTM in water samples and in order to increase the count and number of NTM strains during the present study, 500 ml water samples were membrane filtered and inoculated on selected media for NTM retrieval. In a swimming pool environment, Leoni *et al.*, (1999) filtered different amounts of water and retrieved maximum number of NTM.

Most species of mycobacteria adapt with simple substrates, laboratory adapted strains often grow well on synthetic media containing asparagine, glycerol and mineral salts. Media selection and culture reading schedule are usually based on personal preference or laboratory tradition. Traditionally one agar and one egg media are used for isolation and usage of two media of two different basal compositions will make maximum mycobacterial retrieval possible. According to Portaels *et al.*, (1988) and Portaels, (1995), media used for primary isolation of mycobacteria is important and there is no selective media available for isolation (Steadham, 1980). Nutrient Agar with 5% glycerol, PeizerTB and Loewenstein Jensen were the selective media used in the present study. USA bacteriologists seem to favour Peizer TB medium. Isolation rates of both slow and fast growing NTM in station I and II recorded, highest on NA and lowest on LJ media. The number of strains isolated and the number of species were minimum from LJ than the other two media used. Steadham (1980) observed all the LJ slants as liquefied by proteolytic bacteria and some were completely overgrown by a fungus, and suggested LJ slants as unsatisfactory; frequently was too confluent to allow selection of

a single colony to subculture. Portaels *et al.*, (1988) observed high negativity rate on LJ due to high pH and in the present investigation also, the high contamination rte and the minimum retrieval capacity of LJ has been observed.

High contamination rate (46 %) and high pH optimum (7.0) of LJ well above than that of environmental mycobacteria (pH – 5.4 – 6.5) can be attributed as reason for lowest retrieval capacity of LJ medium. NA with simple and readily available inexpensive reagents (Jenkins *et al.*, 1982) is fulfilling the conditions for ideal culture medium with higher positivity rates, may be due to faster growth of NTM on agar media when compared to egg-based ones. It is observed that the time taken for appearing maximum number of colonies was also less in agar-based media such as NA and PTB (Corner, 1994 and Cousins *et al.*, 1989). In the present study, NA medium allowed highest and effective NTM retrieval with less contamination from all samples tested throughout the entire period. Chapman, (1971) noted the non-fastidious nature of most of the NTM strains that can multiply easily on simple media. Less number of NTM strains obtained from PTB and LJ media in the present study, may be due to inhibitory effect of malachite green and this was found in agreement with the finding of Portaels *et al.*, (1986).

The optimum temperature for mycobacterial isolation was found to be ranging from 35-37 °C. as some important fish pathogenic species like *M.marinum* and *M.chelonei* will grow only at the range of 25-33 °C , the incubation in the present investigation was done at both RT and 37 °C to render the maximum NTM isolation possible.

Even though the toxicity of NaOH towards mycobacteria and the requirement of strict time adherence for the treatment were proved, the reason for high strain number on NA in the present study may be the dilution of the decontaminated and centrifuged samples on 99ml aged sterilized seawater. This result is strongly proving the viable nature of mycobacteria, even after severe decontamination procedure and the capacity to rejuvenate on NA, and the inoculation after proper dilution of the decontaminated samples will nullify the deleterious effect of NaOH and to yield maximum number of environmental strains on agar medium.

In the present study, both TPC and TMC were ranging from 1 x 10^3 to too numerous to count in pond waters and this result was in accordance with that of Covert *et al.*, (1999) for 20% of their samples tested, whereas for 80 % of the samples, the observed TMC was lower than that of the present study. Le Dantec *et al.*, (2002) observed mycobacterial counts like 1 and 50 cfu/l for 78 %, 5 and 500 cfu/l for 21 % of water samples tested and more than 500 cfu/l was recorded only in one sample, but did not observe a count of more than 1000 cfu/l. In the present study all the NTM counts recorded were above this, which may be due to the high organic content of samples examined, and that might have favored NTM colonization. The observed NTM count of water samples was found ranging from 1 cfu/500 ml to too numerous to count and this finding was also parallel to that of Covert *et al.*, (1999).

According to Kubica *et al.*, (1975), the frequency of isolation of environmental mycobacteria in the laboratory depends upon factors like geographic location, season of the year, choice of digesting- contaminating agents used etc. Although ubiquitous in distribution, mycobacteria showed some seasonal and geographical pattern of occurrence and this could be observed in the present investigation among samples and seasons. The number of NTM will be varying according to the presence of organic matter or animal faeces and very little is known about the biodiversity and community structure of indigenous mycobacteria. From the present study, it is evident that maximum mean TMC from Narakkal ($25.3x10^3$) and high mean TMC from Valappu ($30.4x10^3$) were observed during monsoon which was also supported by the findings of Kirschner *et al.*, (1992) and Iivanainen *et al.*, (1993) who observed the rainy periods increased the counts of mycobacteria in the brook waters.

The present study showed both positive and negative influence of various nutrients as well as environmental variables on mycobacterial occurrence and activity. Disease outbreaks are found to be closely related with sudden changes in nutrient profile of the system and hence there is close association between ecology and epidemiology of potentially pathogenic mycobacteria.

In both the sites of study, the occurrence of NTM was negatively correlated with water pH only, during monsoon and this observation was in accordance with the study by Kazda,(1973), Kirschner *et al.*,(1992) and Iivanainen *et al.*,(1993) in natural waters.

In the present investigation, during postmonsoon, positive relationship observed between NTM and alkaline pH shows the diverse effect of pH on NTM occurrence that may be contributed by synergestic effect of other environmental factors in the aquaculture system. As observed by Iivanainen *et al.*, (1993) there was a positive correlation between mycobacteria and heterotrophic bacteria in the present study. According to George and Falkinham (1985), optimal pH for mycobacteria is acidic but present observation recorded the high occurrence of NTM in alkaline waters, which may be enhanced by rightly followed, and favourable procedures for NTM isolation.

Iivanainen *et al.*, (1993) mentioned the requirement of more than20°C for better mycobacterial multiplication but during the present study, in Narakkal culture pond, a strong negative correlation was found between NTM and temperature during monsoon and postmonsoon and in Narakkal only during premonsoon. But the heterotrophs for monsoon and postmonsoon were not affected by water temperature indicating the basic difference between mycobacteria and other heterotrophic bacteria. The strong influence of both organic carbon and nitrate nitrogen content of water was found enhancing the mycobacterial incidence during the seasons.

In station I, nitrate nitrogen was found as the limiting factor for mycobacterial occurrence among the three seasons. During monsoon season of station I, organic carbon showed positive correlation with NTM, whereas dissolved oxygen showed influence during post-monsoon of both the stations. The results indicated the overall influence of nitrite nitrogen, nitrate nitrogen, ammonia and phosphate, both negatively and positively on the occurrence and distribution of NTM in pond waters and it is observed that the occurrence of heterotrophs are also controlled by these nutritional factors.

High alkaline culture systems enabled the high frequency isolation (33 species) of NTM, as environmental mycobacteria are highly tolerant to pH variations (Chapman and Bernard, 1962; Portael and Pattyn, 1982) eventhough Donoghue *et al.*, (1997) regarded an alkaline environment is not primarily optimal for mycobacterial growth.

Culture systems studied were alkaline and harboured NTM in high frequencies with 33 species and can be attributed to the wide pH tolerance to mycobacteria in nature

Brook *et al.*, (1984b) found high numbers of MAI from acidic environment, so this can be attributed as the reason for low MAI prevalence in the present study. *M.avium* can grow in a wide pH range (Kirschner *et al.*, 1999) but growth will reduce at alkaline pH (Portaels and Pattyn, 1982; George and Falkinham, 1985). Acidic pH was found optimum for slow growers (Donoghue *et al.*, 1997) and was found as the reason for low prevalence of slow growers during the present study.

Mainly biochemical characterization is discussed in the present study because it is easiest to learn, the most readily set up and performed and the least subject to bias in interpretation. The reference laboratories of the world, find it convenient to subdivide the mycobacteria on the basis of pigment production and growth rate, thus enabling a more rational selection of the key tests needed to precisely identify an unknown mycobacterium species.

In taxonomic studies of members of mycobacteriaceae, mycobacteriologists (Wayne *et al.*, 1974; Wayne *et al.*, 1976) relied on biochemical and cultural characteristics. Wayne and Kubica (1986) confirmed specific identity of the isolates through different sets of biochemical tests, which are necessary to characterize rapidly growing and slowly growing species separately. Biochemical scheme by Pattyn and Portaels (1972) was selected in the present study for species characterization of isolates as it gives separate keys for rapid and slow growers. Bergey's Manual of Determinative Bacteriology (1974) was also referred for confirmation.

The change in the degree of acid fastness observed accordingly with source of mycobacteria is emphasizing the relationship between the degree of acid fastness and the nutrient availability of NTM. Wayne and Doubek, (1968) reported occasional pink or coral pigment production of mycobacteria upon exposure to light and 18% of the strains produced this pigmentation in present observations.

Tsukamura(1981) studied relationship between photochromogenicity and test temperatures and observed ,100% of *M.marinum* tested were photochromogenic and *M.szulgai* were scotochromogenic at 37°C. They also observed that there is well-flourished growth of *M.marinum* at 37°C. This can be compared with the results of the present study. Tests for photochromogenicity was carried out at 37°C as per the

recommendation of Tsukamura(1981) and they observed 29% strains of *M.kansasii* and 26% of *M.asiaticum* were showing photochromogenicity, which may be due to active metabolism of the strains at this temperature.

According to Jenkins *et al.*, (1982) only some of the tests are useful for distinguishing between two or three species and it would therefore be uneconomic to use all the tests for every strains. Variations in one or two reactions will not prevent the identification of the species (Joan and Mihm, 1959; Gordon and Mihm, 1959). The method of detection of putrescine diamine oxidase (Bonicke and Nolte, 1967) for differentiating rapid and slow growers of NTM was skipped and conducted iron uptake test in peptone agar containing ferric ammonium citrate, as it was easy and reproducible. A further confirmation of slow and fast growers were done using 5%NaCl and was according to Bergey's Manual of Determinative Bacteriology (1974). Among the biochemical tests for slow growers, tests like β-Galactosidase and nicotinamidase were skipped as these amidase tests (Bonicke and Lisboa, 1959) are included in additional tests, which are not compulsory for identification. Slight variations in technique can cause marked inconsistency in characterization (Pattyn and Portaels, 1972).

McFadden *et al.*, (1987) reported that the organisms in *M.avium* complex are not clearly differentiated by biochemical tests. According to Grange (1996), International Working Group on Mycobactrial Taxonomy and mycobacterial systematics, *M.avium* complex includes serovars of *M.avium* and *M.intracellulare* and both these species were together treated as *M.avium* complex in the present investigation.

Stanford and Paul (1973) tried to study mycobacterial occurrence in Ugandan environment and isolated 266 strains from 185 samples and Iivanainen *et al.*,(1993) isolated mycobacteria from all 53 samples, isolated rapid and slow growers from natural water distribution systems. In the present study, 689 isolates were recovered form total 168 samples examined of fish, sediment and water. Portaels(1973) isolated 153 mycobacteria from 332 samples of water, mud, fish and leeches in diverse regions of Bas Zaire shows the highly ubiquitous nature of environmental mycobacteria in all the systems and samples. Viallier and Viallier(1973) identified 564 strains out of 852 isolates, whereas in the present study, 574 strains were identified from total 689 strains.

Total number of NTM species and their respective number of strains were very high in the present study, revealed mycobacterial biodiversity in the area, may be due to higher levels of organic matter and faeces in surface waters (Covert *et al.*, 1999). Cochin backwater system is well connected with sewage and drainage canals from the main land will be contributing to this high nutrient availability in the perennial and pokkali aquaculture systems studied, through under water currents and regular tidal influx and outflow. *M.gastri* was the most frequent species in the study was consistent with the result of these workers. In the present study, the frequency of occurrence of *M.fortuitum, M.peregrinum, M.scrofulaceum, M.avium* in the study were less and were either from water or fish samples. The results of Covert *et al.*, (1999), who recorded these species from surface waters revealed the aquatic nature of environmental mycobacteria.

In the present study, slow growing NTM species such as *M.shimoidei, M.szulgai, M.marinum, M.terrae, M.chelonei, M.flavescens, M.fortuitum, M.absecessus* and *M.aurum* were isolated either sporadically or frequently. Falkinham *et al.*, (2001) observed all these species from surface and raw waters along with *M.gordonae,* which showed continuous and uniform distribution of slow growers I natural as well as aquacultural waters. Viallier and Viallier(1975) reported that the species of mycobacteria observed in sea water environment, were similar to those in fresh water and environment. Puttinaowarat *et al.*, (2000) isolated *M. marinum* from both pond and sea waters, whereas and in the present examination, 11.1% of fishes were positive for the species; one water sample was positive for both *M.fortuitum* and *M.marinum.* There was periodical difference in the sporadic occurrence of *M. avium* complex in the present study as it was observed in Flakinham *et al.*,(2001). According to Carson *et al.*, (1978) and George *et al.*, (1980) these are common residents only in the hot water systems and this may be the reason for the sporadic incidence of *M.avium* complex in backwaters of Cochin, eventhough the species can survive well in aquatic environment.

Sporadic recoveries of *M. flavescens* (0.7 %), *M.phlei* (0.7 %) and *M.terrae* (3.5 %) were made from culture ponds of this study and Leoni *et al.*, (1999) also recorded these species in sporadic frequencies from swimming pool waters. Present study revealed the presence of frequent NTM species such as *M.chelonae* (14.8%), *M.gastri* (8.7%) *M.gadium* (6.8%), and *M.marinum* (7.4%), which was different from swimming

pool environment with, frequent species as *M.gordonae* (73.5%), *M.chelonei* (38.2 %) and *M.fortuitum* (35.3 %). This indicated uniqueness of NTM flora in different geographical are as, eventhough there is difference in the frequency. *M.chelonei* and *M.fortuitum* were isolated from skin and other tissues of tilapia except liver and this was supported by the observation of Humphrey *et al.*, (1987) in which the organisms were isolated from peripheral surface of *Salmo salar* and it is assumed that mycobacteria can be harboured in liver tissues only during the infectious state of the fish.

Species like *M.scrofulaceum*, *M.fortuitum*, *M.terrae*, *M.nonchromogenicum*, *M.parafortuitum*, *M.marinum* etc. were isolated from the present study was parallel with the results from Zaire environment (Portaels, 1995) indicates the similarity among the geographical areas for mycoabcterial flora. But the observation of species like *M. kansasii*, *M. xenopi* and *M. chelonei* in the study reveals the uniqueness and dissimilarity of the pond system from Zaire environment. But the incidence of mycobacteria were different in different geographical locations as it was recorded by Diamant *et al.*, (2000). Torkko et al (2000) isolated *M. xenopi* for the first time from natural waters. Tacquet *et al.*, (1973) and McSwiggin and Collins (1974) isolated *M. kansasii* and *M. xenopi* only from sewage and water supply and the sporadic occurrence of the species from the present study strongly supports their resource as sewage and man made-habitats as the sites of study are continuation of Cochin estuary.

Tsukamura *et al.*, (1974) observed the occurrence of *M. chelonei* in rare occasions is not consistent with the present study in which both the stations show fairly high intensities of the species, may be due to strong interference with environmental variables. NTM species such as *M. terrae*, *M. gastri*, *M. nonchromogenicum*, *M. vaccae*, *M. triviale*, *M. flavescens*, *M. smegmatis* and *M. phlei* were isolated from environmental and fish samples in present study and Collins *et al.*, (1984) observed the same species in waters, prove that their possible source as water. Kazda, 1983, Joynson(1979) and Chapman(1971) reported that mycobacteria replicate on wet or flooded soil but the principal aquatic breeding ground is probably stagnant slow moving water containing rotting vegetation. Rapid growers are in association with aquatic plants and algae in the mud of stagnant pools, can be drained off from wet soil into sea waters and rivers by rains and floods. The seasonal difference observed for various species of mycobacteria strongly supports this possibility. There were numerous

reports of isolation of mycobacteria from water, soil, dust, sawdust and sewage in tropical and subtropical areas (Jones and Jenkins, 1965; Keelberg and Nel, 1973; Reznikov and Leggo, 1974; Corner and Pearson, 1979). In the present investigation surface waters were shown as highly rich for mycobacteria like *M. marinum, M. gordonae, M. scrofulaceum, M. terrae, M. fortuitum, M. aurum, M. phlei* and *M. smegmatis* and the result was found consistent with that of Kasatiya *et al.*, (1974).

In water samples the concentration of *M. avium* complex was >1000cfu/500ml and the result was in parallel with that of Falkinham *et al.*, (2001), who recorded 0.8 to 100,000 cfu/l from drinking water distributions systems. Even though many workers confirmed water as reservoir for mycobacterial proliferation (Carson *et al.*,1978; George *et al.*,1980), high concentration of >10cfu/500ml or >10cfu/gm in Narakkal and Valappu, maybe due to high nutritional availability in a aquaculture ponds. The minimum concentration of mycobacterium observed was 100cfu/500ml and the range was from $0.1x10^3$ to too numerable to count (TNTC). Mean count observed ($4.5x10^3$) observed by Teska *et al.*, (1997) from Japanese medaka, *Oryzias latipes* was considerably higher than that observed during the present study.

The identification of pathogenic NTM apart from saprophytic ones, suggests a proper measure has to be taken to keep essential health status of cultured fishes. Frequent observation of *M. chelonei*, an important fish pathogen in the study was not in consistent with the study by Tsukamra *et al.*,(1974). It is a common species of aquatic environments (Schulze-Robbecke and Buchholtz, 1992) and was isolated by Paramasivan *et al.*, (1981). All the species like *M. xenopi*(Collins *et al.*,1984, Schulze-Roebbecke and Buchholtz,1992), *M. peregrinum*(Covert *et al.*,1999; Le Dantec *et al.*,2002), *M. flavescens*(Collins *et al.*,1984), *M. vaccae*(Kamala *et al.*,1994), *M. .marinum, M. scrofulceum*(Schulze-Robbecke and Buchholtz,1992), *M. smegmatis*(Collins *et al.*,1984, Talaat *et al.*,1999), *M.gastri*(Collins *et al.*,1984) *M.phlei*(Collins *et al.*,1984) *M. parafortuitum, M. shimoidei*(Falkinham *et al.*,2001), *M. diernhoferi*(Kamala *et al.*,1994), *M. triviale, M. szulgai M. aurum* and *M. kansasii*, isolated from different aquatic environments and water distribution systems were observed to be occurred frequently and sporadically , which emphasizes the well oriented distribution of the NTM species. There is gradual emergence of some

mycobacteria in abundance with global changes or microbial biodiversity in the culture environment.

Statistical similarities observed nutritional and bacterial parameters between both the ponds could be attributed to the highest similarity found among the species of NTM observed. Glover *et al.*, (1994); Covert *et al.*, (1999) and Leoni *et al.*, (1999) reported no correlation between heterotrophs and occurrence of NTM. Of the 33 species, only minor difference was recorded as sporadic occurrence of *M. vaccae*, *M. phlei*, *M. gilvum*, *M.aurum* and *M.xenopi* of the systems and the difference may be due the uniqueness of every system for mycobacterial flora (Falkinham *et al.*,2001). *M .terrae* and *M. nonchromogenicum* were not the frequent species in the study but were the frequently observed ones by Portaels(1995) in the urban waters. Le Dantec *et al.*,(2002) isolated saprophytic species like *M. nonchromogenicum*(11.0%), *M.aurum* (1.0%). *M. gadium*(1.0%) and pathogenic species like *M. fortuitum*(3.0%), *M. peregrinum*(10%) and *M. chelonae*(10.0%) from ground or treated waters and the occurrence and distribution of all the above mentioned species in the study was in accordance with above indicating the common mycobacterial spectrum prevailed.

85.1% of all samples were positive for NTM, among which 55% were of environmental samples. The much higher incidence of mycobacteria in the study can be attributed to high organic nature of samples in an aquaculture environment (Covert *et al.*, 1999, Collins *et al.*, 1984; Falkinham III, 1996). From both the stations 16.6% of water samples and 53.3% of fish samples were positive for *M. chelonae*, whereas (102) observed 38.3% of water samples with *M. chelonae* and the difference in the colonization of the species more in fish samples than in pond water may be the reason for this inconsistency. Hatai *et al.*, (1988) isolated the species from *Odonthestes bonariensis* , while sporadic occurrence of the species (4%) was observed from samples of Tilapia and sediment. 40% of *M. shimoidei* were from water samples and this was considered as principal reservoir for the species(Falkinham *et al.*, 2001). Retrieving 8.2% in Narakkal, *M. asiaticum* forms an abundant species, whereas in Valappu, it was nearly sporadic in occurrence (5.6%) and this result is not in accordance with that of Portaels, (1995). High percentage of *M. marinum* and *M. asiaticum* can be attributed as these are the part of natural mycobacterial flora and the possibility of fish mycobacteriosis cannot be denied in the cochin backwater system.

M. marinum, important fish pathogen was the predominant species, accounted for 26.3% and 66.6% of cultured tilapia sampled and the species was isolated frequently from pond water (Puttinaowarat *et al.*,2000) and fishes(Hedrick *et al.*,1987: Humphrey *et al.*,1987). *M. fortuitum*, is the most frequently isolated species from fish (Ashburner, 1977; Hedrick *et al.*,1987) accounted only 2.7 % of all NTM from 4,8 % of all samples, proves low prevalence of the species in aquaculture ponds, whereas, Puttinaowarat *et al.*,(2000) detected 29,6% of samples as positive for *M. fortuitum*. 3% of *M. gadium* strains were recovered from surface water, while in the present study, overall frequency of occurrence of species was high (6.79), may be due to high availability of nutrients in pond water.

Variations in the biotic and abiotic factors in the systems may be influencing the intensity of mycobacterial flora. Teska *et al.*, (1997) isolated *M. abscessus* from healthy Japanese medaka (*Oryzias latipes*) with mean number of cfu/gm of fish was $4{,}5x10^8$, in which IJ gave unsatisfactory result, whereas in the present study 7.8% from Narakkal, and 8.2% from Valappu were frequencies of abundance of the species, recorded from healthy tilapias sampled. The positivity rate from IJ was 28.6%(12 strains) might be due to some cultural conditions prevailed or high intensity of occurrence in the environment (Collins *et al.*, 1984; Neumann *et al.*, 1997). Gruft *et al.*, (1979) and Neumann *et al.*, (1997) isolated *M. terrae* from water samples and are observed as frequent species in natural environments. Frequency was 4.4% and 2.5%(Leoni *et al.*, 1999) in perennial and pokkali ponds, even though the isolation was considerably low from sediment and water and the results were not in agreement with that of Viallier and Viallier(1973), while 12.5% of *M.terrae* compex recorded by Paramasivan *et al.*,(1981) reinforces the presence of the species towards terrestrial and soil environment. 11.8% of *M. peregrinum*, isolated from water sample, disagrees with the lowest isolation rate of the species by Covert *et al.*, (1999).

Single strain of *M. vaccae* identified from skin sample of Narakkal pond only agrees with the results of Portaels, (1995) with sporadic recovery (0.7%) of all samples, whereas Leoni *et al.*, (1999) reported species recovery from 5.9% of the samples.

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Paramasivan *et al.*, (1981) recorded 10.5% of all NTM was *M. scrofulaceum*; whereas the present result accounted 3.5%(10 strains each) only, even though the species is frequent in natural environment (Portaels, 1995)and the decrease in the percentage of the species may be governed by the physico-chemical balance of the system. *M. phlei* was recovered sporadically (2 strains) from fish and water samples and percentage of recovery by Leoni *et al.*,(1999) was higher (1.2%) than that for the present study.

In the present study, molecular genetic characterization of field isolates of NTM using RAPD-PCR technique was carried out. Diverse morphologies and difference in one or two biochemical activities shown by strains of the same species from various sources, forced to conduct RAPD-PCR analysis in order to understand genetic relatedness between species and heterogeneity among strains, if any.

RAPD-PCR technique was found to have many advantages over the conventional methods as well as other molecular techniques. RAPD-PCR method doesn't require knowledge of the genetic structure of the target, since there always will exist low –stringency priming sites for a single primer on both strands of the DNA at positions close enough to permit PCR amplification. It is simple, easy and rapid, compared to the phylogenetic characterization and identification methods, which relies on time-consuming techniques that have limited discriminating power. However, it has wide genomic coverage unlike amplification using specific primers and therefore has higher discrimination. In the present study also RAPD profile was found to be highly discriminatory between species and isolates. DNA hybridization and ribotyping are not sufficient to estimate the extent of genetic heterogeneity between the species or strains. The very high discriminating power of the RAPD-PCR for comparison at species level and within species has been stressed by Goarant *et al.*, (1999).

The PCR based RAPD technique required the least quantity of DNA among the various techniques. It was able to generate reproducible RAPD profiles with as little as 50ng of DNA per PCR reaction, in this study. The advantage of RAPD with respect to sample quantity, result quality and sensitivity has been stressed by Miyata *et al.*, (1995). The modified DNA isolation procedure of Murray and Thompson (1980) yielded quality DNA from mycobacterial cultures. RAPD technique is so simple that it

even eliminates the need for pure DNA, a requirement for other fingerprinting methods (Mazurier *et al.*, 1992).

The results obtained through primer screening indicated that the discriminating level of RAPD varied with primer used, therefore the choice of primer is important. In the present study, all the primers used were commonly being used for mycobacterial RAPD-PCR studies specifically. OPA-18 and OPA-02, the primers selected in the study were found consistent and were used to discriminate among isolates of *M.abscessus* by Zhang *et al.*, (1997) and these primers specifically produced the largest number of strain specific major bands and exhibited than other primers used. In the present study, observed bands ranged from 0.2kb and 2.0kb in length, and this result is also in accordance with these workers.

DNA fingerprint based on RAPD profiles revealed more polymorphism between different species and considerable polymorphism between the strains of same species. Each species produced a unique RAPD pattern and showed distinct inter specific genomic heterogeneity among mycobacteria. Highly reproducible and discriminating banding patterns obtained with both the primers for all the isolates is showing species-specific as well as strain-specific differentiating power of these primers, and as per the result of the study, these can be used for effective intra- and inter-specific identification of mycobacterial isolates. The results obtained by Matsiota-Bernard *et al.*, (1997) in *M.avium* strains was also suggest that primers used for the specific detection of mycobacteria of classical PCR can be used for RAPD analysis. They also observed that both mycobacteria and any other bacterial species, having high GC content could be amplified specifically with same primer.

Distinct genetic identity and banding pattern dissimilarity observed by the two unidentified strains shows that they are of distinct species of mycobacteria , different from all other species identified biochemically. Both these strains gave identical amplicons with OPA-18 and with OPA-02, indicating their strain relatedness.

Estimates of the coefficients of Nei's genetic identities were relatively high and but none of the coefficients reached unity indicating the genetic variance existing among mycobacteria. The estimates of the coefficients of genetic distance also confirm this fact. The phylogenetic tree after bootstrapping confirmed the genetic diversity among different strains as indicated in other species. Occurrences of

interspecific and intraspecific genetic diversity, which remain hidden with other methods, reflect the potential and sensitivity of this approach for systematic studies of mycobactria.

The present study indicate that RAPD is an attractive choice for genetic characterization and evaluation of inter and intra species diversity as well as heterogeneity of mycobacteria. In conclusion, RAPD analysis using OPA-02 and OPA-18 could become reliable and sensitive technique for identification of Mycobacterium *spp*. Assessment of genetic diversity is important in epidemiological studies of nontuberculous mycobacteria (NTM), as data from these studies could be used to monitor trends in the occurrence of new strains, identify possible sources of infection, and differentiate individual strains (Tenover et al,1997).

The unique and uncommon RAPD fingerprint observed for both the unidentified strains is strengthening the need for further screening for new emerging fish pathogenic mycobacteria in the aquaculture systems that may also pause risk for the aquaculturists. The strain-specific RAPD profiles would lead to the development of isolate-specific molecular markers by cloning and hybridisation which would be helpful for accurate diagnosis of new strains of pathogenic mycobacteria.

Both perennial and pokkali fields situated in Cochin back water system were observed as richest geographical area with high species diversity of mycobacteria and the record of fish pathogenic species strengthens the importance of the proper care which has to be taken to avoid possible incidence of fish mycobacteriosis or fish TB, that may be attributed with sudden change in nutrient profile of the aquaculture systems. Possible control measures also have to be studied to control epidemiological occurrence of the disease.

Summary

VI SUMMARY

1. Studies on the "Ecophysiology of Nontuberculous Mycobacteria in marine aquaculture ponds" was carried out based on the samples collected from aquaculture ponds and cultured tilapia(*Oreochromis mossambicus*) at Narakkal(Station I) and Valappu(Station II) during March 1999 to February2000. An account of NTM are given with Intensity charts and Tables.
2. Nontuberulous mycobacteria are relatively common aquatic environmental bacteria. This means that they can live in the aquatic environment and do not require the presence of fish or fish tissue to survive. They have been frequently isolated from coastal waters, sediments and aquaculture facilities.
3. The physico-chemical parameters except temperature was found to be fluctuating widely in both perennial (Station I) and pokkali(Station II) aquaculture ponds studied. The nutrient profile exhibited by station II showed that it is more fertile than Station I.
4. Mean TPC values from fish and environmental samples of pre monsoon, monsoon and post monsoon seasons of both the sites were varying with less count of heterotrophs in gill and liver tissues of the fish.
5. Post monsoon showed less TMC values compared to other two seasons.
6. Membrane filtration technique was found as effective in retrieving maximum TPC and TMC from samples of water.
7. Decontamination technique with 4% NaOH and further treatment with physiological saline recovered NTM from all the samples studied.
8. Both heterotrophic as well as mycobacterial occurrences were observed high in Narakkal.
9. Statistically significant positive and negative correlations observed in the study between heterotrophs, mycobacteria and physico-chemical parameters and within these parameters strongly indicating the overall biotic and abiotic interrelationships, which stabilizes microbial environment in aquaculture systems.

10. Percentage of total NTM observed in Narakkal and Valappu were 21% and 18% respectively. Stomach, intestine and sediment samples showed high intensity of mycobacterial occurrence.
11. NTM occurrence differed in samples from cultured tilapia and environmental samples like water and sediment. Liver recorded low NTM counts. High enzymatic activity of the liver tissue may not be rendering NTM occurrence and so low NTM count from the tissue.
12. Strong seasonal variation in mycobacterial incidence with maximum number of NTM strains (n==135) during post monsoon from Narakkal and during monsoon (n=120) from Valappu.
13. Number of slow growing and fast growing, both pigmented as well as non pigmented NTM strains were observed but generally non pigmented fast growers were isolated more during the study.
14. Among 689 total mycobacterial isolates obtained, 344 from Narakkal and 345 from Valappu of which 293 and 281 were identified specifically from respective stations. From stations I and II, totally 33 species of NTM were identified out of which 19 were fast growers and 14 were slow growers. Common occurrence of most of the species in both the stations indicated the indigenous nature of mycobacterial flora in the Cochin back water system.
15. *M.gastri, M.asiaticum, M.chelonei* and *M.marinum* were the predominant species observed, among which *M.marinum* and *M.chelonae* were important fish pathogens. Other fish pahogenic species identified were only sporadic in occurrence.
16. All the samples harboured significant quantities of NTM. Among the three media selected for NTM isolation, Nutrient agar with 5% glycerol retrieved more than 50% of NTM with maximum species diversity, 23 species of NTM from Narakkal and 27 from Valappu maximum number of strains. This medium is highly recommended for isolation of marine NTM from brackish water.
17. Lowenstein Jensen medium was found to be with minimum NTM retrieval capacity. PTB retrieved 22 and 17 strains and LJ gave 15 and 16 species from Narakkal and Valappu respectively. The number of strains of the same species differed among samples.

18. Strong seasonal influence was observed on the occurrence and distribution of different species apart from the retrieving ability of the media and kind of sample analyzed.
19. *M.vaccae* and *M.marinum* were observed as fully photochromogen, but the percentage of scotochromogen was more among pigmented strains.
20. It is observed that all physico-chemical parameters thoroughly act as limiting factors for incidence of species of mycobacteria.
21. Occurrence of *M.marinum* was influenced positively by dissolved oxygen content, nitrate nitrogen and ammonia and *M. chelonae* by none of the factors.
22. The occurrence of *M.malmoense, M.komossense, and M.abscessus* were found to be affected by ecological factors like ammonia, salinity and water pH respectively, whereas the distribution of *M.fortuitum* was influenced by water temperature, dissolved oxygen content and organic carbon.
23. 30 isolates of different species of Mycobacteria were analyzed by RAPD-PCR using two primers namely OPA-02 and OPA-18 selected from a group of 25 primers after primary screening.
24. Both the primers totally produced 39 amplicons with evident polymorphism in the RAPD profile. Each isolate had a unique pattern.
25. Phylogenetic tree of the 30 isolates depicting the genetic relationship among them was constructed, 17 forming a major cluster and 13 forming a minor one.
26. RAPD was found to be an attractive technique to evaluate inter- and intra-species genomic heterogeneity.
27. The strain-specific RAPD profiles would lead to the development of isolate-specific molecular markers by cloning and hybridization which would be helpful for accurate diagnosis of new strains of pathogenic mycobacteria.
28. RAPD profile shown by unknown strains was distinct and similar and dendrogram depicts their genetic relatedness with *M.aurum*.

Narakkal and Valappu aquaculture ponds in the Cochin backwater system were found to be reservoir of environmental mycobacteria with high microbial biodiversity. Present study strongly reveals the extreme

influence of various ecological factors along with some unknown factors that may also limit the occurrence and distribution. Along with other pathogenic species, record of fish pathogenic NTM like *M.marinum*, *M.chelonei*, *M.kansasii* etc either frequently or sporadically is not excluding the chance of mycobacteriosis in this environment. And the study is revealing the fact that abrupt changes in the biotic and abiotic nature of the Cochin backwaters and the stress followed for cultured fishes may lead to this chronic disease. Fish tank granuloma in hatchery and aquacultural entrepreneurs also cannot be avoided, as the pathogenic species are also prevalent in these waters.

The species of NTM remained unidentified is still pointing into present need of further mycobacteriological study in this area, which may find new fish pathogenic NTM strains and hence to give proper attention in future with respect to piscine TB. Fish mycobacteriosis has been studied for more than a century bur the successful vaccination of fish against mycobacteria is not yet to be achieved, so emphasis should be placed on bio-film research preventing re-growth, improving water quality in aquaculture systems and into the development of mycobacteriosis vaccine. The development of rapid diagnosis technique such as ELISA for detecting mycoacteriosis will be very useful for fish quarantine systems in the near future. It also remains for scientists interested in this disease to apply these tools to completely understand Fish mycobacteriosis at the molecular level. The strain-specific RAPD profiles would lead to the development of isolate-specific molecular markers by cloning and hybridisation, which would be helpful for accurate diagnosis of new strains of pathogenic mycobacteria.

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Appendices

VIII APPENDICES

Appx. 1. ANOVA analysis showing the influence of physico-chemical parameters on the distribution of different NTM species in Station I

Appx. 1.1 *M.agri* and Ammonia

Analysis of Variance

	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.179	1	0.179	737.537	0.001
Residual	0.000	2	0.000		

Appx. 1.2. *M.marinum* and Dissolved oxygen

Analysis of Variance

	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.102	1	0.102	10.716	0.047
Residual	0.029	3	0.010		

Appx. 1. 3. *M.parafortuitum* and Temperature

Analysis of Variance

	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.002	1	0.002	1332872.120	0.001
Residual	0.000	1	0.000		

Appx. 1.4. *M. triviale* and Temperature

	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.002	1	0.002	1332872.120	0.001
Residual	0.000	1	0.000		

Appx. 1.5. *M. shimoidei* and Nitrate notrogen

Analysis of Variance

	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.220	1	0.220	3825.583	0.010
Residual	0.000	1	0.000		

Appx. 2. ANOVA analysis showing the influence of physico-chemical parameters on the distribution of different NTM species in Station II

Appx. 2.1 *M.malmoense* and Ammonia

Analysis of Variance

	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.070	1	0.070	732.181	0.024
Residual	0.000	1	0.000		

Appx. 2.2. *M. obuense* and Nitrite nitrogen

Analysis of Variance

	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.629	1	0.629	3227.902	0.011
Residual	0.000	1	0.000		

Appx. 2.3 *M.gastri* and Ammonia

Analysis of Variance

	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	1.825	1	1.825	73.876	0.003
Residual	0.074	3	0.025		

Appx. 2.4. *M. marinum* and Nitrate nitrogen

Analysis of Variance

	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	1.598	1	1.598	65.934	0.004
Residual	0.073	3	0.024		

Appx. 2.5. *M. marinum* and Ammonia

Analysis of Variance

	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.315	1	0.315	17.023	0.026
Residual	0.056	3	0.019		

Appx. 2.6. *M.asiaticum* and Nitrite nitrogen

Analysis of Variance

	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.732	1	0.732	10.816	0.046
Residual	0.203	3	0.068		

Appx. 2.7. *M. asiaticum* and Phosphate

Analysis of Variance

	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.533	1	0.533	26.044	0.015
Residual	0.061	3	0.020		

Appx. 2.8. *M. scrofulaceum* and water pH

Analysis of Variance

	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.001	1	0.001	183.496	0.047
Residual	0.000	1	0.000		

Appx. 2.9 *M. komossense* and Salinity

Analysis of Variance

	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.098	1	0.098	1749.078	0.015
Residual	0.000	1	0.000		

Appx. 2.10 *M. sphagni* and Ammonia

Analysis of Variance

	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.168	1	0.168	288.881	.037
Residual	0.001	1	0.001		

Appx. 2.11 *M. gilvum* and Ammonia

Analysis of Variance

	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.125	1	0.125	177.065	.048
Residual	0.001	1	0.001		

Appx. 2.12 *M. aurum* and water pH

Analysis of Variance

	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.003	1	0.003	5148.128	0.009
Residual	0.000	1	0.000		

Appx. 2.13 *M. poriferae* and Dissolved oxygen

Analysis of Variance

	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.313	1	0.313	169.485	0.049
Residual	0.002	1	0.002		

Appx. 2.14 M. triviale and Temperature

Analysis of Variance

	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.001	1	0.001	1100.281	0.019
Residual	0.000	1	0.000		

Appx. 2.15 *M. triviale* and Dissolved oxygen

Analysis of Variance

	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.029	1	0.029	488807.445	0.001
Residual	0.000	1	0.000		

Appx. 2.16 *M. fortuitum* and Organic carbon

Analysis of Variance

Sum-of-Squares	df	Mean-Square	F-ratio	P

Appx. 2.17 *M. direrhoferi* and Phosphate

Analysis of Variance

	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.250	1	0.250	73105.847	0.002
Residual	0.000	1	0.000		

Sample preparation methods for isolation of *Mycobacterium* spp. from cultured fish and environmental samples

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ABSTRACT

Fish mycobacteriosis is a problem to which more than 150 species of fish are susceptible. In order to isolate non-pathogenic and fish pathogenic mycobacterial species from environmental samples, three different methods were evaluated. Shaking and membrane filtration methods were adopted to retrieve maximum acid-fast strains from fish and environmental samples. Decontamination with 4% sodium hydroxide facilitated the isolation of acid-fast bacteria in the selective media, killing all the contaminants like heterotrophs and saprophytes. Centrifugation procedure at 4000 r.p.m for 20 minutes eliminated the contaminants from the sample and allowed only acid-fast bacilli to grow in the selective media. Centrifugation was carried out twice with distilled water. Peizer TB and LJ slopes worked well for selective isolation of fish pathogenic Mycobacteria *from the centrifugated samples. It is believed that all the three sample preparation methods will be useful tool to study the fastidious fish pathogenic* Mycobacteria *from environmental samples.*

Introduction

Studies on opportunistic pathogenic bacteria were carried out extensively in Cochin backwaters and aquaculture ponds. But studies on acid-fast *Mycobacteria* from environmental samples are very few. *Mycobacteria* are ubiquitous in nature and are isolated with relative ease from fishes (Kubota *et. al.*, 1970; Land and Abernathy, 1978; Kusuda *et. al.*, 1987) from soil, stream beds, and cattle drinking troughs (Donoghue *et. al.*, 1997) and from water (Ivanainen *et. al.*, 1997; Neumann *et. al.*, 1997; Dailloux *et. al.*, 1992) has not been reported from water and sediments of aquaculture ponds.

The objective of the present study was to find out a standardised and short-cut procedure for the maximum retrieval of environmental *Mycobacteria* species from water, sediment and fish samples collected from perennial aquaculture ponds.

The normal cell morphology and bio-chemical potential of *Mycobacteria* spp. was also examined to distinguish non-pathogenic and pathogenic species especially based on lipid hydrolysis as all lipolytic forms are considered virulent. Virulence in turn is influenced by season and geographic 'niche' in which these pathogens are found. Therefore, a seasonal examination is needed on the three criteria to find out non-pathogenic and pathogenic strains of *Mycobacteria* studied, (1) pleomorphism exhibited by the stains (2) the presence of mycolic acid (3) the hydrolysis of Tween '80 ie. lipolytic strains, The above morphological and bio-chemical potential factors vary in pathogenic and non-pathogenic stains of environmental *Mycobacteria* which is suggested.

Materials and methods

In the present study, the fish, water and sediment samples were collected from perennial aquaculture ponds which were above 6 kms apart and along 9°55' - 10°10'N and 76°20'E. The perennial pond was located at Krishi Vigyan Kendra, Narakkal and other is a polyculture pond of Valappu.

Sample preparation methods for isolation of Mycobacterium

Monthly sampling was carried out from the two stations for the period from October to December 1999. The sample was brought to the CMFRI bacteriology laboratory in asceptic condition within 2 hrs for the bacteriological investigation. Fish (Tilapia - *Oreochromis niloticus*) sediment and water samples were brought to the laboratory in an ice-box (+4°C). Sediment, water as well as skin, gill, stomach, intestinal samples of fish were taken for isolation of *Mycobacteria* to know the occurrence, distribution and percentage composition of *Mycobacteria* in these samples.

In this study, the retrieval of *Mycobacterium spp.* from various samples has been standardised by incorporating methods like membrance filtration, mechanical shaking, decontamination and centrifugation procedures. The above procedures were carried out as the *Mycobacteria* are highly fastidious and won't occur easily in synthetic media. The two synthetic media recommended for isolation of *Mycobacteria* are Loewenstein - Jensen's and Peizer TB media. In the present study total heterotrophs (TPC) was also monitored using Nutrient agar to know the percentage of *Mycobacteria* in these sample. Flowchart -I shows the general procedure followed for isolation.

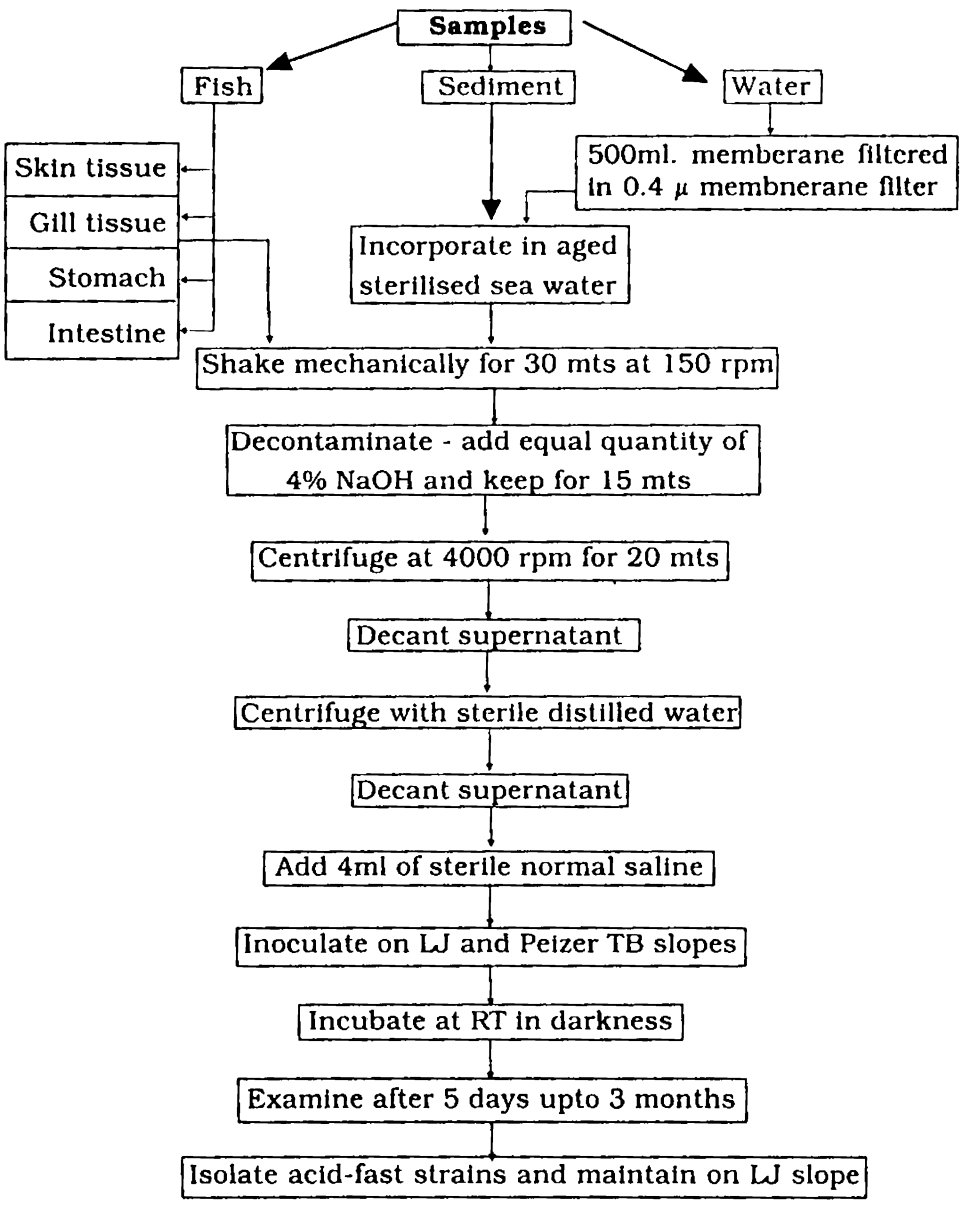
I. *Membrane filtration:* About 500 ml of water sample was membrane filtered (Whatman) using 0.4μm filters in sterile condition and TPC of heterotrophs was taken before and after the membrane filtration.

The membrane filtration technique was followed by mechanical shaking in water samples. Mechanical shaking will dismantle the adhered bacteria from the organic particles into the suspension thereby increasing the incidence of *Mycobacteria*. The decontamination procedure may vary according to the microbiologist and one of the simplest method is adopted in the present study due to its ease in its application.

II. *Mechanical shaking:* Fish and sediment samples were smashed well (1 gm each) placed in 10ml and 99 ml aged, presterilised sea water and was kept for shaking in a mechanical shaker at 150 rpm for 30 mts. The membrane filter used for the filtration of water sample was also subjected

Flow chart - I

Procedure Followed to Isolate Mycobacteria



to shaking. The data on TPC was also collected to compare the difference in total plate count before and after mechanical shaking.

III. *Decontamination procedure:* The decontamination method adopted by Marks and Thomas (1958) was followed and according to the method, 4% NaOH was poured in equal quantity of the shaken sample, mixed well and kept for 15 mts. Only the suspension was used for further procedure. The total plate count of the acid-fast bacteria are also taken before and after the decontamination procedure to understand the effect of the decontamination procedure.

IV. *Centrifugation*: The speed of cetrifugation was 3000 rpm for 20 mts. The supernatant was discarded and the centrifugation was repeated in the same speed with distilled water.

V. *Inoculation:* The supernatant of the centrifugate samples are decanted and added with 4ml of normal saline into each tube and shaken well. Duplicates of LJ and Piszer TB slopes were inoculated with 0.1 ml (for fish and sediment samples) and 0.2 ml (for water sample) of the samples.

VI. *Incubation:* Incubation was done at RT (28±2°C) and in complete darkness because some species of *Mycobacteria* were showing photoactivation capacity which was helpful for the classification by considering, the colour of the pigment produced after one hours' exposure to light. The slopes were kept in slanting portion for the first 24 hours so that the bacterial propagules may get absorbed on the slope and then kept straight, for the rest of the incubation period. In 3-5 days, colonies started to appear on slopes. The general TPC with or without NaOH procedure is also done in the same way with nutrient agar. The mycobacterial colonies on the slopes and plates are stained by Ziehl - Nelson's staining technique. Acid-fast ones were isolated for further bio-chemical and physiological studies.

Results

Membrance filtration

Mycobacteria spp. are sparsely distributed in surface water, hence sample preparation methods are suggested to retrieve maximum of them

on selective media. The membrane filtration method was adopted as an additional sample preparation method for surface pond water. The total plate count before and after membrane filtration, the TPC of aerobic hetrotrophic bacteria in two stations from October to December 99 is given in Table 1 and 2. In KVK (Table 1) the highest count obtained was during November 99 the count being 200 x 10^{-3}, and the lowest TPC encountered during October and December 1999 the count being 10 x 10^{-5}. Compared to this the TPC obtained without membrane filtration was much lower, the count ranging from 2 x 10^{-5} at the month of October to 45 x 10^{-3} at the month of November.

Total Heterotrophs (TPC) encountered in Valappu is given in Table 2. In this station, after membrane-filtration the highest and lowest values obtained were during November, the count being 36 x 10^{-3} and 10 x 10^{-4} respectively. The lowest count encountered without membrane filtration was 2 x 10^{-5}in the post-monsoon month of October 1999.

There is every possibility of obtaining more *Mycobacterium* by sample preparation methods and the incidence of *Mycobacteria* will be more in fish and environmental sample after membrance - filtration. This study will highlight the importance of these sample preparation methods in obtaining the highly fastidious *Mycobacteria* in synthetic organic media.

Table 1. Effect of membrane filtration on general TPC from water samples of KVK aquaculture pond

Months / Procedure	With filtration	Without filtration
October	10 x 10^{-5}	15 x 10^{-3}
	83 x 10^{-3}	2 x 10^{-5}
November	200 x 10^{-3}	45 x 10^{-3}
	34 x 10^{-5}	3 x 10^{-5}
December	28 x 10^{-3}	8 x 10^{-3}
	10 x 10^{-5}	4 x 10^{-5}

Table 2. Effect of membrance filtration on general TPC from water samples of Valappu aquaculture pond.

Months / Procedure	With filtration	Without filtration
October	23×10^{-3}	9×10^{-3}
	18×10^{-5}	2×10^{-5}
November	36×10^{-3}	11×10^{-3}
	10×10^{-4}	3×10^{-4}
December	21×10^{-3}	13×10^{-3}
	28×10^{-5}	6×10^{-5}

Mechanical shaking

Table 3 and Table 4 shows the count obtained before and after the mechanical shaking procedure at 150 rpm for 30 mts. The procedure was found to be effective in increasing the TPC of heterotrophic bacteria. During the sample preparation method, all the samples are subjected to this procedure and considerable difference in bacterial count was obtained in water samples.

The total heterotrophs (TPC) encountered was very high in Valappu and recorded upto 176×10^{-3} during the month of November. The TPC in the same month was only 11×10^{-3}/ml without the shaking procedure. In all the three months, the TPC was recorded high in which the lowest count recorded as 24×10^{-4}/ml in the month of November. The lowest TPC count was recorded 1×10^{-3}/ml without mechanical shaking procedure during the month of December.

Table 3. Effect of mechanical shaking on general TPC from water sample of Valappu aquaculture pond.

Months / Procedure	After shaking water sample	Before shaking water sample
October	96×10^{-3}	16×10^{-3}
	27×10^{-5}	2×10^{-5}
November	176×10^{-3}	111×10^{-3}
	24×10^{-4}	7×10^{-4}
December	39×10^{-3}	1×10^{-3}
	43×10^{-5}	12×10^{-2}

Table 4. Effect of mechanical shaking on general TPC from water samples of KVK aquaculture pond.

Months / Procedure	After shaking water sample	Before shaking water sample
October	122×10^{-3}	31×10^{-3}
	22×10^{-5}	2×10^{-5}
November	210×10^{-3}	42×10^{-3}
	40×10^{-5}	8×10^{-5}
Dcember	27×10^{-3}	3×10^{-3}
	6×10^{-5}	11×10^{-5}

Table 4, illustrates TPC of KVK, the highest count encountered was during November (210×10^{-3}/ml) and lowest during December (6×10^{-5}). In October, highest count obtained was 122×10^{-3}/ml, but the highest count before shaking was 42×10^{-3}/ml and the lowest being 2×10^{-5}/ml in October.

From this account, it is clear that there is defenite enhanced occurrence of growth in TPC and *Mycobacteria* after the shaking procedure. So it is concluded that the sample preparation methods are essential for the maximum number of *Mycobacterial* spp. retrieval from environmental samples.

Decontamination procedure

This is considered to be the most important sample preparation method as it is making the isolation of *Mycobacteria* more easy by selectively enhancing only the occurrence of *Mycobacteria* as 4% NaOH will permit only the bacteria having mycollic acid in their cell wall to grow.

Table 5 and 6 shows the TPC of *Mycobacteria* at the station of KVK before and after the decontamination procedure. All the six samples like skin, gill, stomach, intestine of tilapia and sediment and water samples were subjected to decontamination procedure.

Table 5. Retrieval of *Mycobacteria* from samples of KVK aquaculture pond before decontamination.

Months/ Samples	Skin	Gill	Stomach	Intestine	Sediment	Water
October	-	-	5×10^{-4}	-	12.14×10^{-2} 3.7×10^{-4}	-
November	37×10^{-6} 17.2×10^{-4}	-	5×10^{-4} 2.88×10^{-6}	7.69×10^{-4} -	- -	-
December	0.85×10^{-6}	1.85×10^{-2} 1.85×10^{-4}	12.5×10^{-4}	2.6×10^{-4}	-	-

Table 6. Retrieval of *Mycobacteria* from samples of KVK aquaculture pond after decontamination.

Month/ Samples	Skin	Gill	Stomach	Intestine	Sediment	Water
October	0.76×10^{-3}	3×10^{-4}	1.66×10^{-3} 106×10^{-5}	2×10^{-3} 1.55×10^{-5}	4×10^{-4}	-
November	-	0.86×10^{-2} 2.58×10^{-4}	14.6×10^{-3} 1.12×10^{-5}	0.90×10^{-5} 1.8×10^{-3}	4×10^{-4} 18×10^{-2}	
December	41.4×10^{-3} 57.57×10^{-4}	0.94×10^{-4}	5.35×10^{-3}	21.6×10^{-5}	8.57×10^{-2}	-

Table 3 shows the count of TPC and *Mycobacteria* obtained in gill, stomach, intestine, sediment and water samples mycobacterial TPC in all the three months. Skin sample was showing the absence of *Mycobacteria* during November '99. The lowest *Mycobacterial* count was encountered in skin sample the count being 0.76×10^{-3}/gm during October. The skin sample harboured the highest count 37×10^{-6} and 17.2×10^{-4} during November '99. Water samples in all the three months before and after decontamination procedure is devoid of *Mycobacteria*. During December, the lowest *Mycobacterial* count observed was 0.85×10^{-6} from the skin sample.

Table 7 and 8 shows *Mycobacteria* TPC encounted at Valappu before and after decontamination procedure respectively. After decontamination, all the six samples showed mycobacterial occurrence except stomach during December. In the month of October, skin sample harboured highest TPC before decontamination procedure the count being 140.4 x

10^{-4}/gm and 130.28 x 10^{-6}. But the corresponding value after decontamination was 3.3 x 10^{-3}/gm and 9.16 x 10^{-5}/gm. In Table 7 the lowest TPC was recorded from stomach the count being 0.775 x 10^{-4}/gm. In water samples, *Mycobacteria* occurred only in the month of December '99, the count encountered as 2 x 10^{-2}/ml. The highest TPC encountered was 53.68 x 10^{-5} in skin during November and lowest was recorded during December, the count being 0.95 x 10^{-2}. In all the three months, water samples were showing mycobacterial representation, the highest count recorded during October and lowest count during December. The decontamination procedure was effective for the retrieval of *Mycobacteria* from sediments as the count obtained after decontamination was more when compared to the count obtained before decontamination, except during October'99.

Table 7. Retrieval of *Mycobacteria* from samples of Valappu aquaculture pond before decontamination.

Months/ Samples	Skin	Gill	Stomach	Intestine	Sediment	Water
October	140.4 x 10^{-4} - 130.28 x 10^{-6}	-	.775 x 10^{-4}	2.4 x 10^{-4}	213 x 10^{-2} - 0.9 x 10^{-4}	
November	1.08 x 10^{-6}	4.16 x 10^{-4}	-	2 x 10^{-4}		
December	0.95 x 10^{-6}	6.9 x 10^{-3}	-	-	-	2 x 10^{-2}

Table 8 - Retrieval of *Mycobacteria* from samples of Valappu aquaculture pond after decontamination

Months/ Samples	Skin	Gill	Stomach	Intestine	Sediment	Water
October	3.3 x 10^{-3} 9.16 x 10^{-5} -	10.8 x 10^{-3} 11.6 x 10-5 1 x 10^{-4}	5.5 x 10^{-3} 2 x 10^{-5} 1.7 x 10^{-5}	6.9 x 10^{-3} 3 x 10^{-5} 12.38 x 10^{-5}	2.94 x 10^{-3}	12 x 10^{-3} 16 x 10^{-4}
November	53.68 x 10^{-5}	4 x 10^{-2}	1.7 x 10^{-3}	10.6 x 10^{-3}	5 x 10^{-4}	1 x 10^{-4} 4 x 10^{-3}
December	2.06 x 10^{-3} 2.06 x 10^{-4}	0.95 x 10^{-3}	-	1.8 x 10^{-3}	32.14 x 10^{-3} 1.78 x 10^{-4}	1 x 10^{-3}

Discussion

Mycobacteriosis or fish tuberculosis is a serious infectious disease and a threat to aquaculturists. The causal organism *Mycobacterium* (the same genus of bacteria that causes tuberculosis in humans) has been isolated from different sources. Bacterial tuberculosis had been studied in yellow tails by Kubota *et. al.* (1970) and in mountain white fish by Land and Abernathy (1978). An epizootic of mycobacteriosis had been reported in yellow tails in 1987 by Kusuda *et. al.* Some mycobacteria are highly pathogenic but when isolated from environmental samples most of them are non-pathogenic. They are simply there as normal flora. Certain generalisation can be made regarding disease-causing *Mycobacteria.*

1) Most of them are aerobic.

2) All are acid-fast rod shaped or highly pleomorphic cocco-bacilli in adverse environmental conditions.

According to Dailloux *et. al.* (1992), water is the natural habitat of *Mycobacteria*, both fresh and salt water. Neumann *et. al.* (1997) has isolated *Mycobacteria* from water and they had incubated the samples on LJ slopes after enriching by filtration. It the present study, membrane-filtration procedure was adopted for making the incidence of *Mycobacterium* maximum possible. Throughout the study period, water samples were devoid of *Mycobacteria.* This may be due to the less number of mycobacterial cells which is insufficient to grow into the medium. Surface water showed high TPC in three months the count being 16 x 10^{-4}/ ml in October. Mechanial shaking was not used by any of the workers during the isolation procedure, and it is suggested that as it gives enhanced mycobacterial occurrence it may also be included in the isolation procedure.

For the decontamination procedure of the various samples, each laboratory was having their own standardised method of choice. Dalsgaard *et. al.* (1992) used the decontamination procedure by Beerwerth *et. al.* (1967) given in Procedure I. With this method, it was possible to isolate the fastidious *Mycobacteria.* After adding the decontaminant, a mixture

Peizer medium slopes.

Twelve methods for the isolation of mycobacterium were compared by Neumann *et.al.* (1997) from surface and treated waters and in each method a particular combination of decontaminants, growth medium and incubation temperature was used. The efficiency of each method was determined by calculating the positivity rate, negativity rate, contamination rate, mean number of mycobacterial colonies formed etc. It was found that 0.005% CPC was found best for treated waters. The general method adopted in the present study was found to be good for the maximum retrieval of *Mycobacteria* from water.

Isolation of *Mycobacteria* was very high using 4% NaOH as decontaminant, in all the six samples of Valappu and KVK except water sample of KVK during the study period. The absence of *Mycobacterium* in water may be due to the pH variation attained during decontamination procedure. *Mycobacteria* was absent in skin sample during November but recorded high during December. Intestine and sediment samples of KVK also showed higher values than gill and stomach.

Water samples recorded highest TPC in Valappu during the three months of study, the lowest value being $1x10^{-2}$/ml. Skin sample showed lowest count during October. Before decontamination the count encountered is $140.4x10^{-4}$ and $130.28x10^{-6}$. The corresponding count after decontamination is being $3.3x10^{-3}$ and $9.16x10^{-5}$. Sediment in October also showed high count before decontamination the count being $0.9x10^{-4}$/ gm. After decontamination, the corresponding value is only $2.94x10^{-2}$/ gm. The occurrence of highest mycobacterial count in the samples before decontamination will be quiet accidental. In the present study, both the stations were showing high mycobacterial count, after decontamination procedure.

Some of the *Mycobacteria* from environmental samples require large incubation period as they are slow-growing forms in synthetic media despite of their predominance in the environment. Diagnosis is both difficult and time consuming using conventional methods. All the sample preparation methods suggested in the present study are efficient in retrieving mycobateria from water, sediment, faeces, fishes and fish blood samples and will be useful tool in the study of both pathogenic and non-pathogenic mycobacteria from environmental samples.

of 5% oxalic acid and 0.1% malachite green are added to neutralise the sample. Ivanainen *et. al.* (1997) tried two decontamination methods to isolate *Mycobacteria* from brook waters. The decontaminants used were : 0.7 mol/litre NaOH followed by 50g/litre oxalic acid and 0.9 mol/litre H Sub (2) SO Sub (4) combined with 0.5g/litre cycloheximide. The NaOH-oxalic acid method generally resulted in lower contamination and higher isolation of mycobacteria. Dalsgaard *et. al.* (1992) and Ivanainen *et. al.* (1997) used oxalic acid to neutralise the mixture. But in the present study, the neutralisation procedure was not done, the decontaminant used was 4% NaOH for 15 mts. which gave maximum retrieval of *Mycobacteria spp.*

Procedure - I

Beerwerth (1967)

Isolation procedure for *Mycobacteria*

1) Tissue treated with equal volume of 4% NaOH for 15 mts.
2) Centrifuged at 3000 rpm for 15 mts.
3) Decant supernatant.
4) Neutralise with 5% oxalic acid to which is added 0.1% malachite green for 15 mts.
5) Decant supernatant.
6) Suspend sediment in 4 ml physiological saline.
7) Inoculate 0.1 ml of this suspension on to four slants of LJ medium.

Lansdell *et. al.* (1993) isolated several *Mycobacterium* species from marine fish caught in the wild and fresh water ornamental fishes. After excising the infected tissues, the tissues were homogenized in 10 ml sterile water and an equal volume of 2-3% NaOH was added as in the present study. Each sample is mixed in a vortex mixture and allowed to remain for 15 mts at RT. Samples were centrifuged at 3000 rpm to effect 95% sedimentation rate of all bacilli present. The centrifugate was neutralised with 2NHcl. In the present observation all these methods were followed except the neutralisation procedure. Eventhough neutralisation procedure was not adopted, high counts of several *Mycobacterium* species has been recorded on Nutrient agar, LJ medium and

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