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 to ensure increased survival. There is a growing awareness in the study of Mycobacteria associated with fish and shellfish. 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 treatment. These very diversities help to define specific microorganisms involved and the spectrum of disease produced in the immuno-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 environmentally 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-monsoon 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 0.2498 mg/gm and in station II with 1.116 mg/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 the 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 0-4.93 ugat/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 0.4-1.1 ugat/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 x10³ -106.1 x10³. The ranges for monsoon and post monsoon seasons in Station I were almost equal, 12.68 x10³ -64.7 x10³ and 29.68 x10³ - 61.4 x10³ respectively and the ranges were 22.48 x10³ -89 and 37.57-82 for station II. Highest ITPC in station II and I
were in March and June and the lowest during August and February. 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 availability 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 providing 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 observed to be related with dissolved 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 temperature 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 influenced 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 factors except phosphate were found to be controlling temperature and salinity in Valappu, both positively and negatively. Organic carbon content in station I during 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 activities 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. chelonii*, *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, even though it is significant to note that only a small percentage of environmental bacteria will survive on pre-treatment with higher concentration of NaOH (Falkingham 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 (Odontesthes 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 rate 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 IJ 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 $1 \times 10^3$ 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 $(25.3 \times 10^3)$ and high mean TMC from Valappu $(30.4 \times 10^3)$ were observed during monsoon which was also supported by the findings of Kirschner et al., (1992) and Ivannainen 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 Ivannainen 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 synergistic 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 than 20°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 1, nitrate nitrogen was found as the limiting factor for mycobacterial occurrence among the three seasons. During monsoon season of station 1, 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) even though 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 \textit{M. kansasii} and 26% of \textit{M. asiaticum} were showing photochromogenicity, which may be due to active metabolism of the strains at this temperature.

According to Jenkins \textit{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 \textit{et al.}, (1987) reported that the organisms in \textit{M. avium} complex are not clearly differentiated by biochemical tests. According to Grange (1996), International Working Group on Mycobacterial Taxonomy and mycobacterial systematics, \textit{M. avium} complex includes serovars of \textit{M. avium} and \textit{M. intracellulare} and both these species were together treated as \textit{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 livanainen \textit{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 from 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. shimoides, M. szulgai, M. marinum, M. terrae, M. chelonei, M. flavescens, M. fortuitum, M. abscessus* 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 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 Falkinham 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. cheloneae* (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 areas, even though 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 mycobacterial 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. flavescentis, 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, Joyson (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 \textit{M. marinum}, \textit{M. gordonae}, \textit{M. scrofulaceum}, \textit{M. terrae}, \textit{M. fortuitum}, \textit{M. aurum}, \textit{M. phlei} and \textit{M. smegmatis} and the result was found consistent with that of Kasatiya \textit{et al.}, (1974).

In water samples the concentration of \textit{M. avium} complex was $\geq 1000 \text{cfu/500ml}$ and the result was in parallel with that of Falkingham \textit{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 \textit{et al.}, 1978; George \textit{et al.}, 1980), high concentration of $\geq 10 \text{cfu/500ml}$ or $\geq 10 \text{cfu/gm}$ 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 $0.1 \times 10^3$ to too numerable to count (TNPC). Mean count observed ($4.5 \times 10^3$) observed by Teska \textit{et al.}, (1997) from Japanese medaka, \textit{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 \textit{M. chelonei}, an important fish pathogen in the study was not in consistent with the study by Tsukamra \textit{et al.}, (1974). It is a common species of aquatic environments (Schulze-Robbecke and Buchholtz, 1992) and was isolated by Paramasivan \textit{et al.}, (1981). All the species like \textit{M. xenopi} (Collins \textit{et al.}, 1984, Schulze-Roebbecke and Buchholtz, 1992), \textit{M. peregrinum} (Covert \textit{et al.}, 1999; Le Dantec \textit{et al.}, 2002), \textit{M. flavescens} (Collins \textit{et al.}, 1984), \textit{M. vaccae} (Kamala \textit{et al.}, 1994), \textit{M. marinum}, \textit{M. scrofulaceum} (Schulze-Robbecke and Buchholtz, 1992), \textit{M. smegmatis} (Collins \textit{et al.}, 1984, Talaat \textit{et al.}, 1999), \textit{M. gastrii} (Collins \textit{et al.}, 1984) \textit{M. phlei} (Collins \textit{et al.}, 1984) \textit{M. parafortuitum}, \textit{M. shimoidei} (Falkingham \textit{et al.}, 2001), \textit{M. diernhoferi} (Kamala \textit{et al.}, 1994), \textit{M. triviale}, \textit{M. szulgai} \textit{M. aurum} and \textit{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 (Falkingham et al.,2001). *M. terrae* and *M. nonchormogenicum* 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(Falkingham 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 (6.7%), 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 $4.5 \times 10^8$, 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 mycobacteria.

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.