ECOPHYSIOLOGY OF PATHOGENIC VIBRIONACEAE FROM CULTURED *OREOCHROMIS MOSSAMBICUS*

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Dedication

To my father dearest

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सारांश

विब्रियोनेसिए के जातिवार वितरण और शक्यता और जलकृषि में इन पर पड जाने वाले पारिस्थितिक प्रभाव का निर्धारण करने केलिए दो स्टेशनों में वर्ष 1997 दिसंबर से 1999 मई तक तालाब के पानी, तलछट और पालन की गई तिलापिया मछली में 18 महीनों के "दौरान पालन किए गए *ओरियोक्रोमिस मोसाम्बिकस* में रोगजनक विब्रियोनेसिए का पारिस्थितिक शरीर क्रिया विज्ञान" विषय पर अध्ययन किया गया।

पालन के दोनों तालाबों में से 10 विब्रियो जातियों जैसे *वी. ऐंग्विल्लेरम, वी पाराहीमोलिटिकस, वी. फिशेरी, वी. फ्लूवियालिस, वी. फरनीसी, वि. हार्वेई, वी. कामबेल्ली, वी. मेश्निकोवी, वी सिनसिनेटियान्सिस* और *वी. वल्निफिकस* का विलगन किया गया। अध्ययन की पूरी अवधि में विलगन किए गए वनस्पतिजातों में *वी. ऐंग्विल्लेरम* प्रमुख था। मानसून पूर्व मौसम में सबसे अधिक और मानसून के दौरान कम विब्रयोनेसिए विलगनों को पाया गया। स्टेशन - 1 में पाए गए विब्रियों में मौसमों की विभिन्नता ($P<0.01$) दिखाई पडी, बल्कि स्टेशन - 11 में पाए गए विब्रियों में मौसमों की विभिन्नता नहीं दिखाई पडी फिर भी 5% स्तर पर विब्रियो जातियों के बीच उल्लेखनीय विभिन्नता दिखाई पडी।

अध्ययन की पूरी अवधि में तीन गतिशील *ऐरोमोनस* जातियों यानी *ए. हाइड्रोफिला, ए. काविए* और *ए. सोब्रिया* का विलगन किया गया और *ए. हाइड्रोफिला* प्रमुख देखा गया। *ऐरोमोनस* जातियों में मौसमिक तथा जातिवार विभिन्नता नहीं दिखाई पडी।

वी. कामबेल्ली तथा 9 पारिस्थितिक प्रचलों के बीच समाश्रयण विश्लेषण करने पर विश्लेषण सार्थक ($P<0.05$) देखा गया और 85.5% परिवर्तनशीलता दिखाई पडी और *ए. हाइड्रोफिला* के साथ यही विश्लेषण करने पर भी सार्थक ($P<0.05$) निकला और 82.9% परिवर्तनशीलता दिखाई पडी। इनके बीच के इस सहसंबंध से विब्रियोनेसिए के जातिवार वितरण पर पारिस्थितिक प्राचलों का प्रभाव व्यक्त हो जाता है।

विब्रियोनोसिए ने पेनसिलिन और आंपिसिलिन के प्रति प्रतिरोध दिखाया और बहुविध औषधों के प्रति इसका प्रतिरोध भी साबित हुआ है।

रोगजनकता पर परीक्षण किए गए 4 विभेदों (strains) (*वी. ऐंग्विल्लेरम, वी. फ्लूवियालिस, ए. हाइड्रोफिला* तथा *ए. काविए*) में *वी. ऐंग्विल्लेरम* और *ए. हाइड्रोफिला* ने उच्च रोगजनका की शक्यता दिखाई।

ABSTRACT

The present study, "Ecophysiology of Pathogenic Vibrionaceae from cultured *Oreochromis mossambicus*" was conducted in pond water, sediment and cultured tilapia for a period of 18 months from December 1997 to May 1999 at two stations to assess the species wise distribution and pathogenic potential of Vibrionaceae and the effect of environmental parameters on them in aquaculture.

10 *Vibrio* sp. were isolated from both the culture ponds, *V. anguillarum, V. parahaemolyticus, V. fischeri, V. fluvialis, V. furnissii, V. harveyi, V. campbellii, V. metschnikovii, V.cincinnatiensis and V. vulnificus. V. anguillarum* was the predominant flora isolated throughout the study period. Maximum isolates of Vibrionaceae were obtained during the pre monsoon season, while the monsoon recorded less numbers. In the case of vibrios, at station I, significant difference ($P<0.01$) between seasons was observed, whereas at Station II, no significant difference was noted between seasons but there was significant difference between the *Vibrio* spp. at 5% level.

The three motile *Aeromonas* sp., *A. hydrophila, A. caviae* and *A. sobria* were isolated throughout the period of study with *A. hydrophila* being the most predominant form. No significant difference was obtained in the *Aeromonas* spp. between seasons and between species.

The fitted regression analysis between *V. campbellii* and the 9 environmental parameters was significant ($P<0.05$) explaining 85.5% of the variability and *A. hydrophila* was also significant ($P<0.05$) explaining 82.9% of the variability. Matrices of correlation showed the influence of environmental parameters on the species wise distribution of Vibrionaceae.

Vibrionaceae exhibited maximum resistance towards penicillin and ampicillin and multiple drug resistance was very evident.

Of the 4 strains tested (*V.anguillarum, V.fluvialis, A. hydrophila* and *A. caviae)* for their pathogenicity, *V. anguillarum* and *A. hydrophila* exhibited high pathogenic potential.

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1. INTRODUCTION

The UN FAO (1997) estimates that half of the world's sea food demand will be met by aquaculture in 2020, as wild capture fisheries are over exploited and are in decline. Though shrimp culture is widespread throughout the tropical world, the industry is beset by disease mostly due to bacteria and viruses. The high density of animals in hatchery tanks and ponds is conducive to the spread of pathogens, and the aquatic environment, with regular applications of protein rich feed, is ideal for culturing bacteria (Moriarty, 1999).

The pride of India's aquaculture is the shrimp industry, however, the appearance of diseases affecting marine shrimp have exposed the weakness of relying on a single product. The private sector investing in such projects has suffered significant losses with no solution in sight. The main finfish aquaculture activity in India is the culture production of the Indian Major Carps. Great strides have been made in this sector. The production has crossed 2 million tons and is growing at a rate of 10% annually. However, carp has no value in foreign markets and except in West Bengal, Assam and a few other states, it is difficult to market major carps in other states (Cohen, 2001).

Thus both of India's major aquaculture crops are inflicted by problems. While the marine shrimp industry is environmentally unsustainable the carp industry is economically less viable. India must find an aquaculture concept, which is environmentally, economically and socially sustainable one, with a frame work which will allow equitable participation of all sectors of the population. To be truly sustainable, such a concept must have an export component and take into consideration global trends which affect the agribusiness environment, including market integration, product transformation, changing consumer preferences, concentration and integration of production means, ecology, biotechnology and changing government roles.

It is in this context, that the importance of adopting other culturable species such as tilapia, *Oreochromis mossambicus*, Trewavas (1983) (formerly *Oreochromis mossambica*, Peters (1852)), which are easily adaptable, palatable and resistant to diseases becomes important. Worldwide production of tilapia exceeds 1 million metric tons per year (FAO, 1997), making them one of the most important aquatic species in culture today. In 1999, the total US consumption of tilapia was about 135 million pounds (USDA, 2000).

There are two principal reasons, why diseases of tilapias have been less well studied than those of many other groups of cultured fishes. (1) Such fishes are generally found in countries where diagnostic facilities are less than adequately developed so that losses cannot be investigated properly (2) Culture of tilapias has only been intensified recently. At low stocking densities, environmental water quality is usually high and opportunities for infections to build up are thus limited. Equally, the observation of disease conditions is much more difficult at low densities in large water bodies than under the controlled conditions of the high density tank or cage systems (Roberts and Sommerville, 1982).

As there was paucity of information regarding the occurrence and seasonal distribution of species of *Vibrio* in water, sediment and tilapia sp., the present study "Ecophysiology of pathogenic Vibrionaceae from cultured *Oreochromis mossambicus*" was conducted for a period of 18 months from December 1997 to May 1999 from two aquaculture ponds. The study concentrated on the estimation, occurrence and distribution of vibrios in water, sediment and different parts of cultured *Oreochromis mossambicus* during different seasons as recently vibriosis has been widely reported Actis *et.al.* (1999) throughout the world in cultured fishes.

The family Vibrionaceae consists of 4 genera. They are *Vibrio*, *Aeromonas*, *Photobacterium* and *Plesiomonas* (Baumann and Schubert, 1984). Species from each of these genera have been isolated from the surface and intestinal contents of marine and fresh water fish (Austin, 1988). These micro organisms are world wide in distribution, with species of *Vibrio* and *Aeromonas* being considered as normal constituents of the microflora of aquatic animals (Austin, 1988).

Pathogenic species are normally present in low numbers when compared with the more abundant saprophytes but their presence in a certain environment always means a risk of transmission to higher organisms including man, especially if they become concentrated by filter feeding organisms living in the same habitat (Arias *et.al.*, 1999). In recent years, vibriosis has become one of the most important bacterial disease in maricultured organisms, affecting a large number of species of fish and shell fish (Sano and Fukuda, 1987., Lightner *et.al.*, 1988., Karunasagar *et.al.*, 1994; Huq and Colwell, 1994; Actis *et.al.* 1999).

Based upon their motility, the genus *Aeromonas* have been classified into non motile and motile aeromonads. The motile group consists of *A. hydrophila, A. caviae* and *A. sobria* (Popoff, 1984). Of the three mesophilic motile aeromondas, *A. hydrophila* is found to be significantly related with fish and fish diseases (Schaperclaus *et.al.*, 1992; Sugita *et.al.*, 1994). *A. caviae* the most prevalent species in water with a high degree of pollution has been studied by Fiorentini *et.al.*(1998, Araujo *et.al.*, 1990,1991). The non-motile aeromonad, *A. salmonicida* is one of the most studied fish pathogens, because of its wide spread distribution, diverse host range and economically devastating impact on cultured fishes, particularly the salmonids (Austin and Austin, 1993) but its significance in our waters is restricted because of its very infrequent occurrence in tropical waters (Reddy *et.al.*, 1994).

Plesiomonas shigelloides currently resides in the family Vibrionaceae but whose true taxonomic position is still the subject of controversy (Brenden *et. al.*, 1988). Strains of *P.shigelloides* are ubiquitous, normally found in the environment, in fresh water, in fish and birds and therefore the potential for transmission to humans is high (Arai *et.al.*, 1980). *Plesiomonas shigelloides* isolated from fin fishes has been studied (Esteve and Garay, 1991; Sugita *et.al.*, 1993; Twiddy and Reilly, 1995; Nedoluha and Westhoff, 1995).

The genus *Photobacterium* was created to accommodate luminescent bacteria that have been isolated from sea water and marine fauna but which could not be identified as vibrios. This genus includes *P.phosphoreum*, *P.leiognathi* and *P.angustum*. *P.phosphoreum* and *P.leiognathi* are wide spread in the marine environment and have been isolated from sea water, the surfaces and intestinal

contents of marine animals and from the specialized luminous organs of marine fish (Reichelt and Baumann, 1973; Ruby and Morin, 1979). *Photobacterium* sp. colonizing the surface of healthy turbots were examined by Austin (1982).

1.1 Objectives Of The Present Study

Quantitative and qualitative analysis of Vibrionaceae in water, sediment and fish.

Estimation of hydrobiological parameters.

Isolation and identification of Vibrionaceae from pond water, sediment and various parts of *Oreochromis mossambicus* like skin, stomach and intestine by means of phenotypic tests and diagnostic tables.

Antibiotic resistance of Vibrionaceae for resistance frequency analysis.

Selection of pathogenic Vibrionaceae with virulence from diseased *Oreochromis mossambicus.*

To study the virulence of the isolated strains.

Statistical analysis.

2. REVIEW OF LITERATURE

Members of the family Vibrionaceae can be isolated from fresh water, estuarine and marine environment as well as from the alimentary canal of man and warm blooded animals. Some species are pathogenic to marine animals while some species comprise the ectocommensal flora of finfish and shellfish and some others participate in recycling of organic matter (West and Colwell, 1984). Although species like *Vibrio cholerae* itself is the cause of a serious disease, most species in the genus are saprophytes or apparently harmless parasites (Pelczar, 1977). The primary fish pathogens of the family Vibrionaceae are *Vibrio anguillarum* and *Aeromonas salmonicida* while others are opportunistic pathogens, which can cause epizootics or at least high rates of endemic disease under certain circumstances (Watkins *et.al.*, 1981).

2.1 Vibriosis

Egidius *et.al.* (1983), Egidius and Andersen (1984), Sindermann, (1984) Devesa *et.al.* (1985), Austin (1988) and Colwell (1984) has contributed a lot to the study of vibriosis in fin fishes. Vibriosis has become the economically most important disease in cultured fishes as well as wild fish populations due to its epizootic nature and can be defined as an infection caused by a bacterial species of the genus *Vibrio* (Egidius, 1987).

2.1.1 Vibriosis due to *Vibrio anguillarum*

The main causative agent of vibriosis, *V.anguillarum* was first described in 1909 by Bergman, as the etiological agent of the 'red pest' of eels in the Baltic sea. The losses produced by this disease are so disastrous that vibriosis caused by *V.anguillarum* has been recognized as a major obstacle for salmonid marine culture (Schiewe, 1983 and Trust, 1986).

V.anguillarum has been repeatedly demonstrated to be the cause of enzootic diseases among various fish species from marine, brackish and fresh waters (Mattheis, 1964 and McCarthy *et al.*, 1974.). Horne (1982) has made a detailed study on the characteristics of *V.anguillarum* and the occurrence of vibriosis in fish, its route of entry into the host and its pathogenicity.

Epidemic outbreaks of vibriosis in cultured fishes due to *V.anguillarum* infections have been reported by many researchers (Faranda *et.al.*, 1982; Muroga and Tatani, 1982; Dumitrescu and Voicu, 1984; Dalsgaard, 1986). High percentage of *V.anguillarum* in a modified extensive shrimp culture system has been reported by Anand *et.al.* (1996). They noticed that the nutrient enriched aquaculture system supported higher microbial growth than the open estuarine system.

Although *V. anguillarum* is part of the normal microflora of water (Larsen, 1982) and fish (Mattheis, 1964) the precise mode of infection is unclear, but probably involves attachment to and then colonization of the intestine of host followed by penetration of the tissues. It has been postulated that infection may begin with colonization of the posterior gastro-intestinal tract and rectum (Ransom, 1978) and with adequate susceptibility, the pathogens reach into the organism not only by way of the intestinal tract but also via the skin. It has been reported that *V.anguillarum* is a minor component of the alimentary microflora of healthy fish both cultured and wild, but the incubation period varies with temperature, strain virulence and the degree of stress under which the fishes are living (Roberts, 1978). Virulent strains of *V.anguillarum* can be isolated from the intestine of healthy fish (Schaperclaus, 1991).

2.1.2 Vibriosis in marine fin fishes

Vibriosis in marine fin fishes have been reported by Muroga and Tatani, (1982), Egidius *et.al.* (1983), Tareen, (1984), Lewis, (1985), and Farkas and Malik, (1986). Review works on vibriosis in fishes have been published by Lio-po (1984), Egidius, (1987) Sano and Fukuda (1987), Stoskopf, (1993) and Huq and Colwell, (1994).

Pomadasys hasta exhibiting fin and tail erosion were studied by Santha *et.al.* (1985). The fishes infected with vibrios exhibited extensive necrosis of fin

edges, separation of rays and gaps in fins, haemorrhage and skin erosion. Georgekutty (1989) isolated *V.fischeri*, *V.vulnificus*, *V.costicola*, *V.cholerae*, *V.anguillarum*, *V.alginolyticus*, and *V.parahaemolyticus* from diseased fishes caught from the different water bodies of Trivandrum implying the synergestic role of vibrios in causing infections.

2.1.3 Vibriosis in cultured fish

Vibriosis has acquired a new diamension and significance with the occurrence of epizootics in culture operations (Egusa, 1983; Grimes *et.al.*, 1984; Pradeep and Lakshmanaperumalsamy, 1986; Sano and Fukuda, 1987 and Ni *et.al.*, 1995). Colorni *et.al.* (1981) studied the *Vibrio* sp. isolated from diseased gilt head sea bream reared in the laboratory and *V.alginolyticus* was found to be the etiological agent but pathogenicity studies gave negative results. Hatai *et.al.* (1981) studied the mortalities that occurred in cultured horse mackerel infected with a *Vibrio* sp. closely resembling *V. parahaemolyticus.*

Toranzo *et. al.* (1984) made investigations on the *Vibrio* epizootics (68%) which occurred in cultured striped bass in estuarine water. *Vibrio* and *Aeromonas* infections causing mortalities in some cultured tilapia and occasional serious epizootics in others have been reported (Lightner *et.al.*, 1988). Serovar E of *V. vulnificus* is highly virulent to eels and has been recovered from diseased cultured Japanese and European eels (Tison *et.al.*, 1982; Biosca *et.al.*, 1991, 1997). *V. anguillarum*, *V.alginolyticus*, *V.harveyi* and *V.splendidus* were suggested to be pathogenic to sea breams (Balebone *et.al.*, 1998). Li *et.al.* (1999) isolated the potential pathogenic vibrios associated with moribund silver sea breams namely *V. alginolyticus*, *V.vulnificus*, *V. parahaemolyticus*, *V.logei*, *V. pelagicus*, *V. fluvialis* and *V.meditterranei.* Of the 7 strains tested, *V.alginolyticus*, *V.vulnificus* and *V.parahaemolyticus* were confirmed to be virulent by challenge experiments. Diggles *et.al.*(2000) conducted studies to determine the cause of acute mortality of juvenile turbot *Colistium nudipinnis* and *C. guntheri. V. splendidius* I and *V. campbellii* like were isolated from the hatchery reared turbots. The mortalities were due to infection of opportunist bacteria in fish predisposed by a combination of adverse factors including pond water quality.

2.1.4 Vibriosis in Crustaceans

Mass mortalities of cultured penaeid shrimps have been frequently reported by many workers such as Lightner (1983), Nash *et.al.* (1992), Abraham and Manley (1995). Luminiscent Bacteria ("LB") are autochthonous flora of coastal waters and many crustaceans, being inhabitants of that environment harbour these organisms (Lavilla pitago *et. al.*, 1992). Therefore they are opportunistic pathogens and are often responsible for large scale mortalities in hatcheries (Sunaryanto and Mariam, 1986; Karunasagar *et.al.*, 1994). An epizootic which occurred regularly among larvae of *P. indicus* was due to *V.campbellii* in a hatchery at Narakkal Cochin (Hameed *et.al.*, 1996). Sugumar *et.al.* (1998) carried out a comparative study between the luminescent bacterial load in diseased and healthy shrimps revealing the dominance of "LB" in infected prawns. 90.12% of the total bacteria isolated were *Vibrio* sp. of which *V. harveyi* contributed 27.91% and *V. splendidus II* (13.37%). Raj (2000) have found that antibiotic resistant *V. harveyi* colonise larval tanks in biofilms causing mass mortalities. Relationships between disease outbreak in cultured tiger shrimp (*P. monodon*) and the composition of *Vibrio* communities in pond water and shrimp were investigated by Sung *et.al.*(2001). The composition of the *Vibrio* community remained diverse with the dominant species being *V. parahaemolyticus* (20%) and *V. harveyi* (53.3%). Immediately before mass mortality of the shrimp, *V. parahaemolyticus* dominated. The LD_{50} of *P.monodon* experimentally challenged with a virulent strain of *V. parahaemolyticus* was found to be 1 x 10^5cfu/prawn (Sudheesh and Xu, 2001).

2.2 Vibrios in the gastro intestinal tract of fishes

Bacteriological analysis of marine fishes, water and sediment have revealed that maximum bacterial counts occurs in the gut of fishes, followed by the gills, body surface and sediment (Karthiayani and Iyer, 1975). Variations occurring in the intestinal microflora of mullets maintained under different rearing conditions were studied by Hamid *et.al.* (1978).

The intestinal microflora of *Tilapia zilli* reared in sea water and fresh water were investigated by Sakata *et.al.* (1980,1984). *Vibrio* and *Aeromonas* were

the dominant genera isolated, and variations in the intensity of both these bacteria were noticed during the different seasons. Ramesh and Venugopal (1988) while studying the distribution of luminous bacteria in the different body parts of fish found that the highest density of luminous bacteria occurred in the gut. They found that the bacterial load of the environment and type of food influenced the distribution of luminous bacteria. The works done by Olsson *et.al.* (1998) substantiate the fact that turbot intestinal tract and feaces can serve as an enrichment site for *V. anguillarum*.

2.3 Vibrios in the sediment

Sediments have proved to be the most stable ecological niche for the survival of vibrios (Natarajan *et.al.*, 1979). The incidence, ecology and pathogenicity of vibrios isolated from marine sediments were studied by Hood and Ness (1982), West *et.al.* (1983), Oliver *et.al.* (1983) and Chandrika (1983).

2.4 Percentage distribution of Vibrios

The percentage distribution of vibrios in aquatic organisms and their environments have been worked upon by Toranzo *et.al.* (1983). Nair *et.al.* (1980) conducted a seasonal study to elucidate the incidence of *V. parahaemolyticus* in fishes collected from different sources. *V. parahaemolyticus* was more readily recovered from market samples of fish (44%) when compared with freshly caught brackish water fishes (37.5%). Studies on the *Vibrio* sp. isolated from mullets collected from coastal lagoons and captive populations, 40.29% of cultured mullets and 13.43% of captured specimens yielded vibrios (Aguirre *et.al.*, 1982). Austin (1988) has reported that vibrios comprise 32% of the total bacteria isolated from the marine environment. Alavandi (1989) has reported that vibrios share 5% of the total heterotrophic bacteria in the coastal waters of Cochin.

During their studies on the luminescent bacteria in the Arabian sea Ramaiah and Chandramohan (1992) isolated over 65% of *V. harveyi* and over 25% of *V. fischeri* from algae collected from the beaches of Goa and Lakshwadeep. The incidence of *V.parahaemolyticus* both in and around Cochin were studied by Sanjeev and Stephen (1993) and they were able to isolate 51.26% from fin fishes and 78.57% from shell fishes.

Matte *et.al.* (1994) analyzed the percentage distribution of *Vibrio* sp. in the oysters originating from the southern coast of Brazil and the highest incidence was observed for *V.alginolyticus* (81%) followed by *V. parahaemolyticus* (77%) *V.cholerae* (31%), *V.fluvialis* (27%), *V.furnissii* (19%), *V.mimicus*(12%) and *V. vulnificus* (12%).

Balebone *et.al.* (1998) studied the percentage distribution of bacteria in intensively cultured sea breams and they found that vibrios constitute 67.8% of the total isolated bacteria. The percentage distribution of *V.vulnificus* in marine and brackish water fish and shell fish from coastal areas of Cochin was found to be 16.6% (Thampuran and Surendran, 1998). Quantitative and qualitative studies on vibrios at different sites of the Spanish Mediterranean coast was done by Arias *et.al.* (1999). Their study reported that *V.splendidus* accounted for 36% of the total isolates, followed by *V. harveyi* (19%), *V.mediterranei* (11%), *V.pelagicus* (6%) and *V.tubiashi* (5%) with a marked seasonality in the *Vibrio* sp.

The study of the bacterial diseases of fish is hindered by our less than adequate understanding of the ecological processes involving interactions between bacteria and their hosts in the aquatic ecosystem. The invading micro-organisms and their relationship with their environment and host is to be studied to understand bacterial diseases of fish.

2.5 Impact Of Hydrological Parameters On Vibrios

Snieszko (1972) has pointed out that communicable diseases of fish occur only when susceptible host and virulent pathogen meet in proper environmental conditions requisite for disease induction. This is highly applicable in aquaculture because with a few exceptions such as *A. salmonicida* and *V. anguillarum* most of the bacteria are secondary pathogens of limited invasive capacity (Inglis *et.al.*, 1993). These secondary pathogens express themselves only under conditions of stress. Stress can be defined "as a stage produced by environmental or other factors which extends the adaptive responses of the individual beyond the normal range, such that its chances of survival are significantly reduced (Brett, 1958). Chandrasekharan *et. al.* (1991) have reported that under extreme

conditions, each individual environmental factor has an independent influence on the growth of bacteria. Thampuran *et. al.* (1997) while studying the qualitative and quantitative aspects of the occurrence of vibrios in the coastal areas of Cochin observed that the percentage of vibrios in sea water was a function of not only total bacterial population but also of environmental factors.

2.5.1 Salinity

Many of the hydrological parameters are found to influence aquaculture practices but salinity has been found to be the most significant. Studies conducted by Natarajan *et.al.* (1979) have stated that changes in populations of *Vibrio* sp. in samples of water, plankton and sediment could be correlated to salinity. The variations occurring in the gastrointestinal microflora of *Tilapia nilotica* when shifted to sea water from fresh water was studied by Sugita *et.al.* (1982) and they found that the changes occurring during the process was found to be due to the sensitivity of some bacteria to salinity.

V. parahaemolyticus occurred throughout the year in Cochin backwater and the monsoon rains and thereby salinity influenced their distribution (Pradeep and Lakshmanaperumalswamy, 1984). Muroga *et. al.* (1986) detected *V. anguillarum* from sea water especially when water temperature was below 20^{0}C but not from fresh water in spite of the occurrence of vibriosis in some ponds. It was demonstrated by "in vitro" experiments that the organism persisted in sea water but perished within 3-5 hr in fresh water specifying the significance of salinity on the incidence of vibrios. Ramesh *et.al.* (1987) and Sivasankar and Jayabalan (1994) during their studies on the influence of hydrological parameters on the seasonal distribution of *V. harveyi* found that salinity has a greater influence than temperature and oxygen on the bacteria. Chowdhury *et.al.* (1989) while conducting quantitative analysis on aquatic environments of Dhaka and Okayama found the brackish water environment with an average salinity of 4ppt to be the optimal condition for pathogenic bacteria. Venkateswaran *et. al.* (1989) while studying the distribution of *Vibrio* sp. in water and sediment of Seto inland sea concluded that salinity indirectly governs the distribution of total vibrios. Jayasree *et.al.* (2000) have reported that high salinities favour the outbreak of luminous vibriosis.

2.5.2 Temperature

Temperature is found to influence the density of vibrios in different aquatic organisms and their environments. The influence of environmental factors on the density of vibrios were studied by Larsen and Willeberg (1984) and they found that the densities were highly influenced by temperature while salinity was of minor significance. Saxena and Kulshrestha (1985) while studying the effect of temperature on *V. parahaemolyticus* in fish found that temperatures as low as -20^0C and as high as 80^0C adversely affected the growth and count of *V. parahaemolyticus*. Marchand (1986) studied 203 strains of *V. metschnikovii* isolated from the Arcachon Bay and they found that low temperatures always promoted the growth of the bacterium and that they are very tolerant towards salinity. The growth pattern of *V. parahaemolyticus, V. vulnificus, V.alginolyticus, V.mimicus* and V.*harveyi* isolated from fishes of Cochin were studied for their temperature tolerance (Sudha *et.al.*, 1998) and the optimal temperature for all the above mentioned *Vibrio* sp. was found to be 37^0C. Arias *et.al.* (1999) reported that very low numbers of *V. vulnificus* occurred at temperatures below 25^0C. Nishiguchi (2000) while studying the influence of temperature on the symbiotic population of *Vibrio* spp. found that *V. fischeri* and *V. logei* co-exists and their concentration depend on abiotic factors including temperature. *V. fischeri* dominated at 26^0C and *V. logei* at 18^0C.

The influence of hydrological parameters such as temperature and salinity on the incidence, growth and seasonal variations of vibrios have been studied by a number of workers (Itami and Kusuda, 1984; Garry *et. al.*, 1985; Bockemuhl *et.al.*, 1986; Powell and Loutit, 1994; Faublee *et. al.*, 1995). Tamplin *et.al.* (1982) enumerated *V. vulnificus* in sea water and shell fishes from estuaries during the different seasons and significant fluctuations were noticed in their presence and density. High temperature and salinity were found to favour the presence of *V. vulnificus* in both the groups. *V.vulnificus* strains were frequently isolated during summer seasons from environments of relatively low salinity and their results suggests that *V. vulnificus* is commonly found in Gulf coast environments and that the occurrence of the organism is favoured by warm temperature and low salinity.

Dumitrescu and Voicu (1984) studied the influence of environmental parameters on the occurrence of vibriosis in rainbow trouts reared in sea water. *V parahaemolyticus* and *V. anguillarum* were identified as the etiological agents. The studies proved that the propagation of the infection was more pronounced during summer and that it was directly related to high temperature, low dissolved oxygen, high ammonia and organic matter. The association between low dissolved oxygen and the density of vibrios in culture sites were studied by Watkins and Cabelli (1985). Thune *et.al.* (1991) has reported that elevated temperature and low dissolved oxygen accelerated the systemic infections due to *V.mimicus* in red swamp craw fish.

2.5.3. Organic carbon

On studying the density of heterotrophic bacterial population in different environments, Kannan and Vasantha (1986) and Jones and Brason (1998) found that the particulate organic carbon coincided with the higher densities of bacteria and *V. vulnificus* concentrations respectively.

2.5.4. Nutrients

In addition to the above mentioned parameters the nutrient salts are also found to significantly influence *Vibrio* sp. A. series of papers dealing with the dissimilatory reduction of nitrate to ammonia by *Vibrio* sp. are available (Herbert and Brown, 1980; Herbert *et.al., 1980)* and Bonin, 1996).

Shinoda *et.al.* (1985) studied the distribution of *V.parahaemolyticus* and *V. fluvialis* in the estuarine regions and found that *V.parahaemolyticus* preferred brackish water areas than sea water due to the optimum concentration of salt contents such as phosphates and nitrates. Benny and Kurup (1991) while studying the variations in microbial populations of estuarine sediments and molluscs depending on the phosphate, nitrate and organic carbon content found that the microbial population increased with increase of organic carbon content and decreased with increase of phosphate and nitrate. Burford *et.al* (1998) has reported that bacterial numbers are highly correlated with organic carbon and nitrogen in the sediment suggesting that they are limiting factors to bacterial growth.

2.6 Seasonality in the occurrence of vibrios

Vibrios are greatly influenced by seasonal variations, particularly summer due to enhanced temperature. Vibriosis normally occurs in fishes of salt and brackish waters especially in shallow waters during late summer when temperatures are high (Roberts, 1978).

The seasonal occurrence of *V.parahaemolyticus* in fresh water fishes were reported by Sarkar *et.al.*(1985). The *Vibrio* numbers were found to increase significantly during summer and warm water temperature were thought to be responsible for the increase. *V.parahaemolyticus* is a marine bacterium found virtually in almost all coastal areas. This species is isolated more frequently and in great numbers from water, sediment and shell fishes during the summer months (Kaysner *et.al.*, 1997). Qualitative studies of *V.vulnificus* in the gastro intestinal tract of fishes were done by Depaola *et.al.* (1994) and they found the density of *V.vulnificus* to be low during winter and high during April-October in estuarine fish. Maximum density was observed in bottom dwelling fishes. De *et.al.* (1977) after conducting analysis of water and fish samples collected from different sources have established a positive correlation between the high incidence of *V.parahaemolyticus* in May, June and October and the increased occurrence of gastro enteritis in Calcutta. Croci *et.al.* (2001) while studying the seasonal trend of Vibrionaceae in mussels found that the *Vibrio* genus was particularly prevalent in summer and scarce in the winter months.

2.7 Sensitivity Studies

Even though broad spectrum antibiotics are frequently administered in aquaculture practices, certain antibiotics like tetracycline, gentamycin, chloramphenicol, oxytetracycline and sulphonamides are found to be highly sensitive towards *Vibrio*. Hence, these antibiotic sensitivity studies are considered significant in the bio-chemical characterization of vibrios. Antibiotic sensitivity studies on vibrios isolated from different sources have been carried out by Surendran and Iyer (1971), Pradeep and Lakshmanaperumalsamy (1985), Bakhrouf *et.al.*(1995), Abraham *et.al.*(1997), Li *et.al.*(1999),Sanjeev(1999) and Praveena (2000).

Observations were made on the antibiotic sensitivity of *V.parahaemolyticus* isolated from water, sediment and fish of Cochin backwaters to 10 antibiotics (Pradeep and Lakshmanaperumalsamy, 1985). Rosily *et.al.*(1987) have reported that *Vibrio* sp. isolated from the larval stages of *P.indicus* are resistant towards ampicillin and penicillin and sensitive towards chloramphenicol. Antibiotic sensitivity of *V.parahaemolyticus* isolated from fishes marketed in Cochin were studied by Sanjeev and Stephen (1992). Antibiotic sensitivity of *V.campbellii* towards 11 antibiotics were studied by Hameed and Rao (1994). Investigations carried out in integrated fish farms of south East Asian countries showed that significant levels of antibiotic resistant pathogenic bacteria occurred in aquaculture ponds where antibiotics are routinely incorporated into animal feeds (Twiddy and Reilly, 1995).

The antibiotic sensitivity of 3 sp. of *V.fluvialis* II isolated from the foot of abalones affected by the pustule disease were studied by Li *et.al.*(1996). The studies imply that different strains have different sensitivities and that the mechanism of resistance is related to gene mutation. Antibiotic sensitivity of *V. carchariae* isolated from infected groupers were investigated by Yii *et.al.*(1997). The antibiotic sensitivity of *V.harveyi* isolated from the various body parts of *P.indicus* were tested against 9 antibiotics (Pillai and Jayabalan,1996). *V.harveyi* strains isolated from an aquaculture system were screened for their antibiotic sensitivity and the isolates were found to exhibit resistance towards 5-18 antibacterial agents (Abraham *et.al.*, 1997). The antibiotic sensitivity of *V.parahaemolyticus* strains isolated from a brackish water culture pond situated near Cochin was investigated by Sanjeev (1999). The antibiotic sensitivity of *V. harveyi* isolated from *P.indicus* were studied by Raj (2000).

2.8 Pathogenicity Studies In Fin Fishes

Numerous experimental studies have been conducted to confirm the pathogenicity of vibrios isolated from different sources (Sako and Hara, 1981; Nordmo *et.al.*,1997 and Farto *et.al.*, 1999). The pathogenesis of *V.parahaemolyticus* to tilapia was investigated by Hubbert (1989). Experiments conducted by Sakata and Kawazu (1992) have shown that a 300 minute incubation of tilapia in cell suspension of *V.damsela* caused complete haemolysis. Comparative studies were conducted on fishes and mice with *V.mimicus* isolated from both wild and hatchery reared fishes to

determine its pathogenicity (Lupiani *et.al.*, 1993). *V.mimicus* isolates were found to be pathogenic to fishes whereas they were not toxic to mice. Austin and Austin (1994) used rainbow trout and Atlantic salmons as models to prove the potential pathogenicity of vibrios. The intraperitoneal injections at 10^5-10^6cells/ml of *V.anguillarum*, *V.salmonicida*, *P.damsela*, *V.esteuranianus*, *V.carchariae* and *V.costicola* resulted in infection while, rest of the 25 species tested showed no evidence of pathogenicity.

Jaikumari *et.al.* (1995) carried out experimental studies on *Oreochromis mossambicus* by injecting *V.anguillarum* ATCC 19264 at different dilutions. 50% mortality occurred at 10^3 dilution. *V.furnissii* strains isolated from eel culture systems were used for virulence studies by intraperitoneal injections (Esteve *et.al.*, 1995). The LD_{50} was estimated at 6.5 to 7.6 $\log_{10}$. The overall observations have indicated that *V. furnissii* is a potential pathogen of European eels. Numerous trials were done by Nordmo *et.al.* (1997) to determine the best method of inducing cold water vibrosis in fishes and they found intraperitoneal injection to be the most reliable one. Pedersen *et.al.* (1997) conducted experimental studies to determine the LD_{50} of *V.damsela* in cultured fishes and the value was found to occur in the range of $1x10^3$ to $5x10^5$cells/fish.

2.9 Species Specificity Of Vibrios

Egidius (1977) conducted species specificity experiments to study the possible spread of vibriosis from young salmons to coal fishes and vice versa in fish pools. *V.anguillarum* from rainbow trouts injected into the same species or into young salmons gave rise to vibriosis with death in 6-8 days and isolation of the organism from the kidneys but when it was injected into coal fishes only 9% developed the disease and the organism was not found in the kidneys. He also observed that *V. anguillarum* from coal fishes had no effect on rainbow trouts but caused swelling and abscesses in other coal fishes suggesting that pathogenicity of *V. anguillarum* is highly species specific. Austin and Austin (1994) conducted experiments to determine the pathogenicity of fish and shellfish associated vibrios to salmons and trouts. *V. anguillarum*, *V. salmonicida* and *P.damsela* produced

generalized septicaemia when injected intraperitoneally and their studies accentuate the fact that vibrios are highly species specific.

2.10 Plesiomonas shigelloides

2.10.1 Isolation of *Plesiomonas shigelloides* from fin fishes

Plesiomonas shigelloides currently resides in the family Vibrionaceae but its true taxonomic position is a subject of controversy (Brenden *et.al.*, 1988). It was considered a member of the genus *Aeromonas* but due to morphological and biochemical differences, it is now placed in the genus *Plesiomonas* (Schubert, 1974). Strains of *Plesiomonas shigelloides* are ubiquitous and therefore the potential for transmission to humans is high (Arai *et.al.*, 1980).

Sakata and Todaka (1987) used a selective medium for the isolation of *P.shigelloides* from water and tilapia from culture ponds and the recovery rate was 93%. The general occurrence of *P.shigelloides* in farm reared European eels were reported by Esteve and Garay (1991) and Esteve *et.al.* (1993). Nedoluha and Westhoff (1993) conducted quantitative and qualitative studies on the microbiological flora of cultured hybrid striped bass, *P.shigelloides* formed 7% of the total bacterial isolates.

Sugita *et.al.* (1993) detected *P.shigelloides* from 29 out of the 51 fish specimens and 4 out of 10 water samples. Black bass, Japanese eel, ayu and tilapia harboured high densities of *P.shigelloides* ranging from 10^4-10^8 cfu/g, with high frequency of occurrence (73-100%). Nedoluha and Westhoff (1995) made quantitative and qualitative studies on cultured striped bass reared in flow through tanks. Of the total bacteria isolated, *P.shigelloides* formed (13%) of the predominant bacterial group. 9 integrated fish farms in a South East Asian country were studied to detect the level of pathogenic bacteria and 22% of the fish samples showed the presence of *Plesiomonas* (Twiddy and Reilly, 1995).

2.10.2 *Plesiomonas shigelloides* isolated from the intestine of fin fishes

Damme and Vandepitte (1980) studied the intestinal contents of 59 Zairese fresh water fish collected from rivers and lakes and *P.shigelloides* was isolated from 59% of the samples with higher densities in river fish. The intestinal microflora of *Sarotherodon niloticus* was examined by Sugita *et.al.* (1985) and they found that *P.shigelloides* predominated the intestinal flora. Investigations were carried out by Sakata and Koreeda (1986) on the bacterial strains isolated from tilapia intestine. *P.shigelloides* and *A.hydrophila* were the most frequently isolated forms. The bacterial flora in the alimentary tract of farm raised channel cat fish were qualitatively examined and the seasonal trends exhibited by *P.shigelloides* were reported by Mac Millian and Santucci (1990).

2.10.3 *Plesiomonas shigelloides* infections in fishes

P.shigelloides isolated from carps suffering from erythrodermatitis were studied by Schulz and Bulling (1981). Galli and Perez (1986) investigated the outbreak of *haemorrhagic septicaemia* in cage cultured catfish and *A.formicans* and *P.shigelloides* were isolated from the infected fishes. Lesel *et.al.* (1989) reported the occurrence of pathogenic *P.shigelloides* strains in tropical fishes.

2.10.4 Pathogenicity studies

P.shigelloides strains associated with moribund cat fishes cultured in Kedah were investigated by Taufik and Wong (1990). They studied the pathogenicity exhibited by 73 strains towards tilapia and only 5 strains were found to be pathogenic to this fish. Mc Garey *et.al.* (1991) assayed *P.shigelloides* strains isolated from UDS affected fish and healthy fish for virulence associated factors.

2.10.5 Antibiotic sensitivity

Twiddy and Reilly(1995) studied the antibiotic resistance exhibited by *P.shigelloides* isolated from integrated fish farms. They were found to be resistant

towards chloramphenicol, oxytetracycline, tetracycline, neomycin, nalidixic acid and oxolonic acid. Marshall *et.al.* (1996) studied the antibiotic sensitivity of *P.shigelloides* strains isolated from crabs. The isolates were found to be sensitive towards gentamycin and tetracycline and resistant to ampicillin, kanamycin and streptomycin.

2.11 *Photobacterium*

Photobacteria are gram negative, non motile chemoorganotrophs capable of both respiratory and fermentative metabolism and that which has the ability to grow in the absence or presence of oxygen (Baumann and Baumann, 1984).

Photobacterium sp. are difficult to distinguish from luminescent vibrios but they do not grow well on TCBS medium and some strains grow poorly at 37^{0}C. This genus includes *P.phosphoreum*, *P.leiognathi* and *P.angustum*. Won *et.al.*(2000) have formulated a new microbial culture medium by using a by-product of kanary, supplemented with N source and growth factor.

2.11.1 Distribution

Due to their growth temperature profiles, *P.phosphoreum* is found in colder waters and in association with the luminous organs of fish from mid water and bathyal habitats (Ruby and Morin, 1978). During a study on the distribution of luminous species in the open ocean of the North Atlantic Ruby *et.al.* (1980) noted that the density of *P.phosphoreum* increased at depths ranging from 200-1000m with maximal concentrations ranging from 3-8 cfu/100ml of sea water. *P.leiognathi* was found in lesser numbers in the water column.

Photobacterium strains isolated from the China coast has been reported by Yang *et.al.* (1983). Ramaiah and Chandramohan (1992) have reported the occurrence of *P.leiognathi* as the bait organ symbiont in the frogfish, *Antennarius hispidus* collected from the coralline habitat off Bombay at a depth of 50m. They have highlighted the scanty distribution of *P.leiognathi* in the water column of both coastal and offshore environments of the Arabian sea.

Prayitno and Latchford (1995) have isolated *P.phosphoreum* from moribund shrimp larvae from hatcheries. The pathogenicity studies have revealed that *P.phosphoreum* is pathogenic to *P.monodon* larvae at 10^3 cfu/ml. Their studies have revealed that exposure of luminous bacterial pathogens to low salinities such as 10 and 15 ppt for 12 hr prior to immersion challenge experiments with *P.monodon* larvae resulted in significantly higher mortality ($P<0.05$). The results imply that environmental factors play a key role in disease out breaks and thus explains the seasonality in disease outbreaks.

2.12 *Aeromonas salmonicida*

Emmerich and Weibel (1894) made the first authentic report of the isolation of *A. salmonicida* during a disease outbreak at a Bovarian brown trout hatchery, the manifestations of the disease including furuncle-like swelling and at a later stage, ulcerative lesions on infected trout.

Aeromonas salmonicida is one of the most studied fish pathogens, because of its widespread distribution, diverse host range and economically devastating impact on cultivated fish, particularly the salmonids (Austin and Austin, 1993). A number of reviews on *Aeromonas salmonicida* have been published (Mc Carthy and Roberts, 1980; Austin and Austin, 1993 and Wilkund and Dalsgaard, 1998) *Aeromonas salmonicida* was traditionally thought of as a pathogen of salmonids, but recently *Aeromonas salmonicida* have been associated with clinical or covert disease in a variety of salmonid and non-salmonid species in fresh water, brackish water and sea water (Hiney and Olivier, 1999).

2.12.1 *Aeromonas salmonicida* infections in fishes

Investigations on ulcer disease in gold fish was carried out by Elliott *et.al.* (1977). The principal causative agent was found to be *Aeromonas salmonicida* while *A. hydrophila* was found to be the secondary invader. Mass mortalities in minnows due to *Aeromonas salmonicida* infection has been studied by Hastein *et. al.* (1978). The main feature exhibited was the development of extensive haemorrhagic skin lesions from which the bacteria was isolated. Novotony (1978) while studying the mass mortality in net pen culture found that, though furunculosis is

restricted to fresh water, it can be transmitted to sea water and in culture systems it can reach epizootic proportions. Boomker *et.al.* (1984) reported the incidence of *Aeromonas salmonicida* infections in rainbow trouts reared in sea water exhibiting ulcerative skin lesions.

Aeromonas strains isolated from ulcers of carps were studied by Csaba *et.al.* (1984). The infected carps exhibited characteristic ulcers or small sized papulae 5-6 days after scarification. *Aeromonas salmonicida* strains isolated from cultured turbot, sea bass, sea breams and salmons were investigated by Vigneulle (1986). Mass mortality of tilapias and carps reared in reservoirs due to *Aeromonas salmonicida* infection was investigated by Reddy *et.al.* (1994). The infection resulted in septicaemia leading to per acute condition and was reproduced in carps by bath challenge.

2.12.2 Route of entry

Sakai (1979) conducted experimental studies in fishes to determine the portal of entry of *Aeromonas salmonicida*. Intraperitoneal and intra muscular injections of *Aeromonas salmonicida* in *Oncorhynchus* caused 100% mortality, whereas a bath in cell suspension caused 80-60% mortality only in fin cliffed fishes. The results suggests that there are 2 invasive routes, a percutaneous infection through the epidermal injury and an infection through the digestive tract.

2.12.3 Percentage distribution of *Aeromonas salmonicida*

The occurrence of ulcerative disease in gold fishes were studied by Elliott and Shotts (1980). *Aeromonas salmonicida* was isolated from 77% and *A. hydrophila* from 34% of the total lesions cultured and was most prevalent in early lesions.

2.13 Identification Studies

A series of tests for the identification of *Aeromonas salmonicida* was designed by Tranquet and Michel (1985). The biochemical characteristics and pathogenicity of *Aeromonas salmonicida* in marine fishes were investigated by

Izumikawa and Ueki (1997) and Iida *et.al.* (1997). Dalsgaard *et.al.* (1998) standardized the biochemical tests for the identification of *Aeromonas salmonicida*.

2.14 Antibiotic sensitivity studies

Sensitivity studies on moribund fishes from pen culture systems were carried out by Novotony (1978). They were found resistant to oxytetracycline and sulpha drugs. The antibiotic sensitivity of *Aeromonas salmonicida* isolated from diseased fishes were studied by Nusbaum and Shotts (1981) and Barnes *et.al.* (1994). Antibiotic resistance of *A. salmonicida* isolated from Atlantic salmons was investigated by Hawkins *et.al.* (1997). They were found sensitive towards chloramphenicol, kanamycin and neomycin. Some of the strains were found resistant to erythromycin and oxytetracycline.

2.15 Pathogenicity studies

Cipriano (1982) carried out a series of experiments to study the method of infection of *Aeromonas salmonicida* in trouts and he found that a 15 minute exposure to *Aeromonas salmonicida* cells caused 70-100% mortality within 14 days. Morikawa and Tashiro (1982) carried out lethality studies on salmons with *Aeromonas salmonicida*. The LD_{50} of *Aeromonas salmonicida* in salmons were studied by 3 different infection routes, intraperitoneal, intramuscular and bath challenge. Mc Carthy (1983) carried out studies on the experimental bath challenge method for fish furunculosis caused by *Aeromonas salmonicida* and cohabitation challenge tests. Acute furunculosis with high mortality was reported by Nordmo *et.al.*(1998).

2.16 Influence of environmental factors

The viability of *Aeromonas salmonicida* inoculated in fishes under different temperatures was observed by Sako and Hara (1981). The studies showed that viable counts of *Aeromonas salmonicida* in all organs of *Oncorhynchus masou* increased faster and the period required for death became shorter as water temperature increased, suggesting that temperature strongly affects the growth rate both 'in vivo and in vitro'.

2.17 Motile Aeromonads

Shotts *et.al.* (1972) have stated that aeromonads are autochthonous inhabitants of aquatic environments and also belong to the flora of fish and amphibia. These bacteria are not in general, considered to be normal inhabitants of the human gastrointestinal tract. *A. hydrophila* is typically recognized as an opportunistic pathogen or secondary invader (Austin and Austin, 1987). Isolates differ greatly in their pathogenicity with some strains being highly virulent and others non virulent. Based on their motility aeromonads have been classified into nonmotile and motile aeromonads. The motile group consists of *A.hydrophila*, *A. caviae* and *A. sobria* (Popoff, 1981, 1984).

2.17.1 Incidence of *Aeromonas* in fishes

Of the 3 mesophilic motile aeromonads, *A. hydrophila* is found to be significantly related with fish and fish diseases. Motile aeromonads of the *A. hydrophila* complex cause haemorrhagic septicaemia in fish (Schaperclaus *et.al.*, 1992). The incidence of *A. hydrophila* infection in cultured sea bass and *Salmo salar* have been studied by Candan *et.al.* (1995) and Boira (1996). Mass mortality of cultured sea bass caused by *A. hydrophila* was reported by Doukas *et.al.* (1998). The outbreak of septicaemic condition in farmed rainbow trout was studied by Ogara *et.al.* (1998). The farmed trouts infected with *A. hydrophila* and *A.caviae* exhibited various degrees of eye pathology, ulcertion and cutaneous and internal haemorrhage.

An epizootic which occurred in pond cultured ayu due to *A.hydrophila* infection causing exophthalmia and subcutaneous haemorrhage were studied by Jo and Ohnishi (1980). The destructive role of *Aeromonas* spp. in carp erythrodermatitis has been explained by Schulz and Bulling (1981). Mortalities in coloured carps due to haemorrhagic septicaemia caused by *A.hydrophila* were studied by Bang (1983). Xu *et.al.* (1986) studied the physiological and biochemical properties of *A. sobria* affecting the caudal peduncle of grass carp. Huang *et.al.* (1991) conducted detailed studies on *A. sobria* causing *haemorrhagic asciteosis* of crucian carps.

The density of *A. hydrophila* in the different organs of infected cat fishes were reported by Pathak *et.al.* (1993). The total aerobic bacterial density on water

exposed organs was found to be higher than the visceral organs (skin>gills>visceral organs). The infections were found to be maximum during summer (26%) followed by monsoon (12%) and winter (5%). The pathogenic and non pathogenic species of *Aeromonas* distributed in the different internal organs of EUS infected *Channa striatus* was reported by Qureshi *et.al.* (1999). The *A. hydrophila* strains isolated from their internal organs were found to be highly pathogenic, while *A. sobria* was found to be less virulent.

The occurance of A.*hydrophila* in fin fishes were reported by Boonyaratpalin (1983) and Rahim *et.al.* (1985). The bacterium is distributed widely in fresh water and bottom sediments containing organic materials, as well as in the intestinal tract of fish (Egusa, 1978 and Sugita *et.al.*, 1994). Comparative studies on the bacterial load in skin, gills and intestine of wild and pond reared sea bass was conducted by Nedoluha and Westhoff (1995).

Aeromonas sp. isolated from different sources such as fishes, fish eggs, shrimps and water samples were reported by Hansen and Olafsen (1989) and Hannien *et.al* (1997). Garcia *et.al.* (1999) have investigated the incidence of *Aeromonas* sp. (*A.sobria, A.schubertii* and *A. hydrophila*) in some cultured fishes like Mozambique tilapia, red tilapia and silver mullets.

2.17.2 *Aeromonas* Infection In Tilapia

A. hydrophila infection is the most common of haemorrhagic septicaemias in tilapia and usually manifests itself by causing affected fish to darken, loose appetite and cluster around exit screens (Roberts and Sommerville, 1982).

Avault and shell (1967) found that *T. rendalli* and *S.mossambicus* became more susceptible to fin rot at temperatures below 14^0C when they were being over wintered in heated troughs. Almeida *et.al.* (1968) list 11 distinct types of bacteria which were isolated from diseased eyes, fin rot, skin lesions and gill infection of *S.mossambicus*. Wang and Xu (1985) have reported 'skin rot' in Nile tilapia where infection rate has reached upto 80% causing great loss in some districts in China. The circular or elliptic erosions which appear on both sides of the dorsal fin and sometimes on all parts of the body were found to be caused by *A.hydrophila* of the

many forms of bacteria that are present in the intestine of tilapia, *A. hydrophila* was found to be the most destructive (Lightner *et. al.*, 1988). They found that the affected golden hybrid tilapia developed severe epidermal ulcers, often with tail and fin rot and severe mortality. A salt tolerant TCBS +ve strain of *A.hydrophila* was isolated from the kidneys of the affected fish. The bacterial population in sediment was found to be about 200 times that of the water column. This bacterial count of sediment when compared to the fish is much less where the diseased skin carried a higher bacterial load than the healthy normal fish (Okpokwasili and Alapiki, 1990). Bacterial counts in tissues of diseased and healthy fish were in the order of intestine, skin, liver and kidneys (Okpokwasili and Obah, 1991). Yambot and Inglis (1994) studied the phenomenal eye disease affecting Nile tilapia. 70% mortality was reported due to *A. hydrophila* infection where the animals exhibited whitening, exophthalmia and bursting of the eye. Okpokwasili and Ogbulie (2001) studied the brown patch syndrome, which occurred during the dry season in tilapia (*O.niloticus*). Pathogenicity tests revealed that the disease cannot be initiated by bacterial inoculation alone.

2.17.3 Intestinal Microflora of tilapia

The intestinal microbes of *Tilapia nilotica* were studied by Sugita *et.al.* (1980) using 5 different media and *Vibrio* and *Aeromonas* were found to be the predominant groups irrespective of the media used. Sugita *et.al.* (1982) while studying the variations in the bacterial flora of the gastrointestine of *Tilapia nilotica* adopted to sea water from fresh water noticed that as salinity changes, the bacterial flora also changes indicating the sensitivity of some bacteria to high salinity. The composition and characteristics of the predominant bacteria in the intestine of tilapia were reported by Sakata *et.al.* (1980, 1984) and Sakata and Koreeda (1986).

2.17.4 Intestinal microflora of other fin fishes

Ventura and Grizzle (1987) investigated the portal of entry of *A. hydrophila* and they found that the bacterium gained access through the digestive tract or injured skin under stress. Mac Millan and Santucci (1990) studied the seasonality in the distribution and predominance of *A. sobria* and *A hydrophila* in the intestine of cultured channel cat fish. The different *Aeromonas* sp. isolated from the

intestinal tract of cultured fresh water fishes were reported by Sugita *et.al.* (1994). Gilda *et.al.* (1996) carried out experimental studies on walking cat fish to determine the virulence and portals of entry of *A hydrophila* and they found that only intramuscular injection treatment induced dermo muscular pathology in the test cat fish. As many as 48% of the genotypically identified *A. caviae, A hydrophila* and *A. sobria* isolates differed from the type strains of corresponding sp. in 1-3 phenotypic characters. The *A.caviae* strains isolated from the gastro intestinal tracts of 2 marine fishes were studied by Ohmori *et.al.* (1998).

2.18 Distribution Of Motile Aeromonads In Aquatic Environments

2.18.1 Distribution in estuarine waters

Fiorentini *et.al.* (1998) have found *A.caviae* to be the most prevalent species in water with a high degree of pollution while *A.hydrophila* strains were more commonly isolated from cleaner waters. No correlation was established between temperature and the number of aeromonads in either estuary. Identical types of aeromonads were found in both estuaries at different times of the year indicating that certain *Aeromonas* strains can survive in more widely varying physico-chemical conditions.

2.18.2 Aeromonads in culture ponds

Kasornachan and Reungpraeh (1983) studied the correlation between the occurrence of *A. hydrophila* in natural waters and culture ponds of Thailand and mortality rate. No positive correlation between mortality rate and density of the bacterium in water was evident. Sugita *et.al.* (1985) made quantitative and qualitative studies on the bacterial populations in water and sediment of carp culture ponds.

2.18.3 Aeromonads in polluted waters

The distribution of motile aeromonad sp. in riverine and marine environments were surveyed for one year (Nakano *et.al.*, 1990). High counts of

motile aeromonads were found in polluted marine samples. Statistical analysis suggested that the densities of *Aeromonas* were related to the cumulative effect of various physico-chemical parameters rather than to a single factor. Araujo *et.al.* (1990, 1991) studied the distribution of aeromonads in waters with different levels of pollution. *A.caviae* predominated in sewage and waters with a high degree of faecal pollution. In less polluted waters, both fresh and marine, *A.caviae* and *A hydrophila* were almost equally distributed. In waters with no faecal pollution *A. sobria* dominated.

2.19 The percentage distribution of motile aeromonads

Chandrika and Nair (1992) studied the bacterial flora of the coastal waters of Trivandrum and they found that *Aeromonas* spp. contributed 20.9% of the total 43 bacterial strains isolated. 9 integrated fish farms were studied in a south east Asian country (Twiddy and Reilly, 1995) to detect the level of pathogenic bacteria and the contribution of *A hydrophila* was found to be 67%. Noterdaeme *et.al.* (1996) reported the percentage distribution of fish associated motile aeromonads. Of the 208 isolates studied, *A. hydrophila* predominated forming (43.8%) followed by *A. sobria* (26.9%) and *A.caviae* (16.3%). Garcia *et.al.* (1999) studied 109 isolates of *Aeromonas* sp. obtained from different cultured finfishes and *A hydrophila* was found to contribute 50.7% followed by *A.sobria* and *A.schubertii.*

2.20 Antibiotic sensitivity

Van Impe (1977) reported the biochemical characteristics and the sensitivity of 29 antibiotics and 15 antiseptics to 50 strains of *A. hydrophila* isolated from fish and compared them with strains implicated in fish pathology. The results indicated a good agreement between the 2 strains demonstrating the potential hazard to humans. The antibiotic sensitivity studies of *A. hydrophila* isolated from cultured black porgy infected with tail rot disease were done by Chen and Chien (1978). The strains were found to be sensitive towards neomycin and resistant towards penicillin, streptomycin, tetracycline and chloramphenicol.

Among the 9 antibiotics used, the isolate was found to be highly

sensitive to neomycin. Reungprach and Kasornachan (1983) while studying the antibiotic sensitivity of *A. hydrophila* found the strains to be highly sensitive to neomycin, chloramphenicol, erythromycin and tetracycline.

The antibiotic resistance, enterotoxicity and haemolytic activity of *A. hydrophila* isolated from different infected fishes were studied by Zeaur *et.al.* (1984). The antibiotic resistance of bacteria isolated from the internal organs of cultured eels were reported by Chen and Kou (1987). Ishimura *et.al.* (1988) made observations on the antibiotic sensitivity of motile aeromonads isolatd from different aquatic environmental samples. All isolates except one were found to be sensitive towards tetracycline, chloramphenicol and gentamycin and majority of the isolates were resistant to ampicillin.

Dixon *et.al.* (1990) studied the antibacterial resistance exhibited by *Aeromonas* sp. towards 11 antibacterials isolated from tropical fishes. *A.sobria* proved to be the most resistant often showing susceptibility to only 3 of the 12 test drugs while *A. hydrophila* was consistently the least resistant. The antibiotic sensitivity of *A. hydrophila* isolated from diseased carps were studied by Lipton (1991). Among the 10 antibiotics, gentamycin, tetracycline, streptomycin, penicillin and neomycin were found to be sensitive.

Okpokwasili and Okpokwasili (1994) studied the antibiotic sensitivity of *A. hydrophila* strains isolated from tilapia infected with "Brown Patch Disease". The strains were found to be sensitive towards streptomycin. The antibiotic resistance of *A. hydrophila* isolated from *Tilapia mossambica* towards 5 antibiotics were studied by Son *et.al.* (1997). Of the 21 fish isolates examined, all were found sensitive to gentamycin and resistant to ampicillin. Most strains were found to be resistant towards streptomycin (57%), tetracycline (48%) and erythromycin (43%).

Recently a rapid molecular assay for the detection of antibiotic resistance determinants has been introduced (Moore *et.al.*,2001). The PCR amplification system for the detection of antibiotic resistance genes may reduce the use of inappropriate antibiotic agents and may allow more directed antibiotic therapy and help in the earlier identification of resistant organisms.

2.21 Influence of environmental parameters

Flagg and Hinck (1978) while studying the effect of ammonia on the susceptibility of fish found that the ammonia concentration and time of exposure influenced the host susceptibility. They observed that the bacterial density in host livers increased as the concentration of un-ionized ammonia increased. Goberg *et.al.* (1978) carried out experiments to study the influence of temperature on *A. hydrophila* infections in fishes. High densities of *A. hydrophila* were found to be significantly correlated with temperature and pH, whereas no correlation was noticed with organic carbon. The concentration of *A. hydrophila* was found to increase in March, June and October and this was positively correlated with the incidence of infection in Bass. Hazen *et.al.* (1978) while studying the abundance of *A. hydrophila* in different natural aquatic habitats at Puerto Rico, found that saline systems had higher densities of *A. hydrophila* than did fresh water systems. Of the water quality parameters measured, only conductivity was significantly regressed with density of *A. hydrophila*.

Meyer (1978) has stated that summer infection due to *A. hydrophila* are related to oxygen depletion. Hazen (1979) while monitoring the densities of *A. hydrophila* in a cooling reservoir found that the temperature, redox potential and pH were all significantly correlated with densities of *A. hydrophila* in the water column, whereas they were not related to dissolved oxygen and organic carbon.

The influence of temperature and nutrients on the growth of *Aeromonas* have been studied by Vanderkooj *et.al.* (1980), Rippey and Cabelli, (1985) and Araujo *et.al.* (1989). Walters and Plumb (1980) while studying the influence of stress factors on the virulence of *A. hydrophila* in cat fish found that low oxygen (1.5mg/l) and high ammonia (1.2mg/l) significantly influenced the % mortality. Plumb (1981) has reported the occurrence of infectious diseases in cultured channel cat fish caused by *A. hydrophila* under the influence of high levels of carbondioxide, ammonia, nitrite and low oxygen. Though each factor is individually stressful to fish, they reduce disease resistance more significantly in combination.

The influence of environmental parameters such as oxygen, temperature, nitrogen and ammonia on the abundance of *A. hydrophila* were studied

by Hazen (1983). The multiple regression and correlation of the data produced a best-fit regression which explained 38% of the variation observed in *A. hydrophila* density. The relationship between water quality and facultative pathogen such as *Vibrio* and *Aeromonas* were investigated by Sindermann (1984). Okpokwasili and Obah (1991) while studying the correlation between the bacterial load and hydrological parameters during the different seasons found that the bacterial counts of water and sediment samples increased during the dry season when the hydrological parameters such as ammonia, carbondioxide, nitrite, nitrate, sulphate and temperature were high. A significant negative correlation ($P<0.05$) existed between the bacterial counts and dissolved oxygen concentration.

The reports of Huq and Colwell (1994) have shown that concentration and availability of nutrients directly affect bacterial survival and multiplication. The association of aeromonads with high levels of nutrients such as phosphates, nitrates and organic matter were studied by Boira (1996). Ortega *et.al.* (1996) studied the association between prevalence of *Aeromonas* spp. in fish farms with oxygen concentration, ammonia concentration and total dissolved solids. They found that oxygen and ammonia concentrations acted as risk factors when their values were lower than 7.06mg/l or higher than 0.05mg/l. Constantino *et.al.* (1997) while studying the occurrence of *A. hydrophila* infections in poorly managed poly culture systems found that a combination of stress and deficient farm management contributed towards the infection.

2.22 Seasonality in the occurrence of motile aeromonads

The influence of temperature on the occurrence and survival of *Aeromonas* during the different seasons were investigated by Cavari *et.al.* (1981). Pathak *et.al.* (1988) have reported that the highest isolation rate of *A. hydrophila* occurred in water during the late winter followed by a progressive decline in density during the summer and monsoon seasons. The organism was recovered from fish throughout the period from which it was concluded that they form a reservoir which is unrelated to their density in water. Boussaid *et.al.* (1991) studied the abundance of *Aeromonas* during the different seasons and its correlation with water temperature. The highest numbers occurred during the cold months while the lowest appeared

during the warm months. The temporal changes were found to be negatively correlated with water temperature values.

2.23 Pathogenicity Studies

Studies on the toxicity of *A.sobria* to fin fishes were done by many researchers, Shieh (1988), McGarey *et.al.* (1990) and Ma *et.al.* (1996). Figueiredo and Plumb (1977) while studying the virulence of *A. hydrophila* isolated from diseased cat fish fingerlings found the LD_{50} of water organisms to be significantly less virulent than the isolates from diseased fish. The lethality studies of *A. hydrophila* isolated from diseased fishes and water on channel cat fish fingerlings proved that the isolates from water were significantly ($P<-0.05$) less virulent than the isolates from the diseased fishes.

In the course of bacteriological investigations of tropical fishes, Almeida *et.al.* (1968) found a strain of *A. hydrophila* which, with intramuscular and intraperitoneal injections, killed *Sarotherodon mossambicus* with the symptoms of a generalized nonhaemorrhagic septicaemia within 16-18hr. Elliott and Shotts (1980) carried out both injections and scale removal experiments on *Carassius auratus* with *A. hydrophila* isolated from cutaneous ulcers. They observed that *A. hydrophila* and *A. hydrophila* complex could not produce lesions as in the original specimen though *A. salmonicida* could initiate cutaneous lesions. The biochemical characteristics and virulence of *A hydrophila* isolated from different sources were studied by Wakabayashi *et.al.* (1981). They carried out virulence studies of motile aeromonads on rainbow trouts isolated from healthy and moribund fishes. Of the 20 selected strains, 4 *A. hydrophila* and 2 *A.sobria* were found to be highly virulent to fish. The virulence assays of *A. sobria* strains isolated from moribund shads on rainbow trouts revealed that the strains were highly pathogenic with a mean LD_{50} of $2x10^5$.

Virulence studies of motile aeromonads on fishes isolated from different microhabitats were done by Carmen *et.al.* (1990). 72% of *A. hydrophila* and 63% of *A. sobria* isolates were found to be virulent to fish by intramuscular injection, but all *A.caviae* isolates were found to be avirulent. Liu *et.al.* (1990) while studying the pathogenesis of bacterial septicaemia in cultured tilapia experimentally infected with

A. hydrophila observed that mortality occurred in fishes which were orally inoculated or immersed in *A. hydrophila* and that more than 5 days were required for exhibiting significant septicaemia with 10^4 cells/g, proving that *A. hydrophila* is a causative factor for bacterial septicaemia of cultured tilapia.

The role of *A. hydrophila* in causing mass mortalities in cultured cat fish larvae were investigated by Kuge *et.al.* (1992). Experiments on intra-abdominal injection of isolates into cat fish and carp showed that the isolates killed 80-100% of the fishes and the bacterium could be successfully reisolated. Wu *et.al.* (1994) studied the correlation between the density of *A. hydrophila* in water and haemorrhagic septicaemia in fishes. The test results of immersion infection on fish body and fish ponds showed that a positive correlation exsisted between the amount of *A. hydrophila* in water and diseases in fishes. They also observed that high ammonia and low dissolved oxygen concentration accentuated the occurrence of the disease.

Ringo and Vadstein (1998) while carrying out experiments on the mortality of turbot larvae found that 100% mortality occurred on the seventh day of administering *A.caviae*. The larval gut was colonized by *A.caviae* and it is therefore concluded that there is a good correlation between the bacterial load of *A.caviae* and mortality. Shakila *et.al.* (1999) carried out intraperitoneal injections with *A. hydrophila* on *Cirrhinus mrigala*. Disease symptoms were expressed by fishes but all of them survived through experimental inoculation and no mortality was recorded.

2.24 Keys employed in identification

The number of described sp. in the family Vibrionaceae, particularly the genus *Vibrio* has been expanding rapidly. The number of *Vibrio* spp. increased from 5 in Bergey's manual of determinative Bacteriology in 1974 (Shewan and Veron, 1974) to 20 in Bergey's manual of systematic Bacteriology in 1984 (Baumann and Schubert, 1984) and now exceeds 34 (Urdaci *et.al.*,1991). Among more than 30 spp. of the genus *Vibrio* at present defined and characterized, only 12 have been implicated in human infections (Janda *et.al.*,1988 and Kelly,*et.al.*,1991).

The bio-chemical determination and identification of environmental *Vibrio* species presents certain difficulties because of their great diversity (Alsina and Blanch, 1994). The important discrepancies occurring in the results of certain tests for the same species (Baumann and Schubert, 1984; Austin and Lee, 1992; Farmer and Hickman-Brenner, 1992) produces a constant change in the taxonomy of Vibrionaceae which has been reflected in the number of new references (Egidius *et.al.*, 1986; Austin and Lee, 1992; Farmer and Hickman-Brenner, 1992).

A set of biochemical keys specially designed for environmental isolates is developed by Alsina and Blanch (1994), which can be used for strains of gram-negative oxidase positive, facultative anaerobes growing on TCBS medium. Also a new improved version of a set of biochemical keys was developed by Alsina and Blanch (1994) consisting of 29 tests and a maximum of 10 tests is still sufficient for the most complicated identification. The new keys maintain the same criteria and characteristics of the original set of keys. Abraham *et.al.* (1999) have formulated a simple taxonomic key for the identification of marine luminous bacteria based on 10 biochemical tests.

The Bergey's manual of Systematic Bacteriology (1984) placed the genera *Vibrio*, *Aeromonas*, *Plesiomonas* and *Photobacterium* under the family Vibrionaceae but at present, the genus *Aeromonas* constitutes a new family Aeromonadaceae (International Committee on Systematic Bacteriology, subcommittee on the Taxonomy of Vibrionaceae, 1992).

2.25 New Species In Vibrionaceae

Based on the taxonomic studies performed on 93 bacterial strains isolated from bacterial haemorrhagic septicaemia in cultured fishes in fresh water, Xu *et al.*(1993) have proposed a new bio-var, *V. fluvialis III*. Iwamoto *et.al.* (1995) have isolated and studied a new species, *V. trachuri* in Japanese horse mackerel. Ishimaru *et.al.* (1996) investigated the occurrence of a new sp. of *Vibrio*, namely *V. ichthyoenteri* in Japanese flounder larvae with opaque intestines. Ma *et.al.* (1999) isolated a new sp. of fresh water luminescent bacterium *V. quinghaiensis* from the

body surface of *Cynocypris prezewalskii* cultured in China. The species exhibited a wide pH range and can be luminous in fresh water.

Currently the following additional *Aeromonas* sp. have been recognized. *A.media* (Allen *et.al.*, 1983), *A. veronii* (Hickman - Brenner *et.al.*, 1987). *A. schubertii* (Hickman-Brenner *et. al.*, 1988), *A. trota* (Carnahan *et.al.*, 1991) *A.jandaei* (Carnahan *et.al.*, 1991), *A.allosaccharophila* (Martinez Murcia *et.al.*,1992), *A.eurcenophila* (Martinez-Murcia *et.al.*, 1992) *A.eletropelogenes* and *A. ichthiosmia* (Collins *et.al.*, 1993), *A.encheleia* (Esteve *et.al.*, 1995).

3. MATERIALS AND METHODS

3.1 Area of Study

The study was conducted for a period of 18 months from December 1997 to May 1999. Two perennial polyculture ponds located along $10^0 00^1$N $76^0 13.5^1$E and $10^0 03^1$N $76^0 12.5^1$E in the Vypeen islands were selected to study the monthly occurrence, distribution and seasonal variations of Vibrionaceae isolated from water, sediment and tilapia (*Oreochromis mossambicus*) (Plates 1&2). An attempt was also made to prove their pathogenicity in these cultured fishes. Estimation of physico chemical parameters were carried out to study the effect of ecological parameters favouring the growth and distribution of these microbes.

Sampling sites (fig.1)

Station I	Valappu, Vypeen (Co-operative farm)	Iha/1.5m	*Oreochromis mossambicus, Chanos chanos Penaeus indicus*
Station II	Krishi Vigyan Kendra of CMFRI, Narakkal	0.8ha/1m	*Oreochromis mossambicus Etroplus suratensis and Penaeus indicus*

The pond fixed as first station was fertilized once a year and it was very fertile with clayey soil and luxuriant growth of phytoplankton (Plate 3). The second pond was sparsely fertilized with poor phytoplankton growth and sandy porous soil (Plate 4). Both the ponds were of the modified extensive type in which fishes were fed naturally as the water exchange was influenced by tidal influx and out flow.

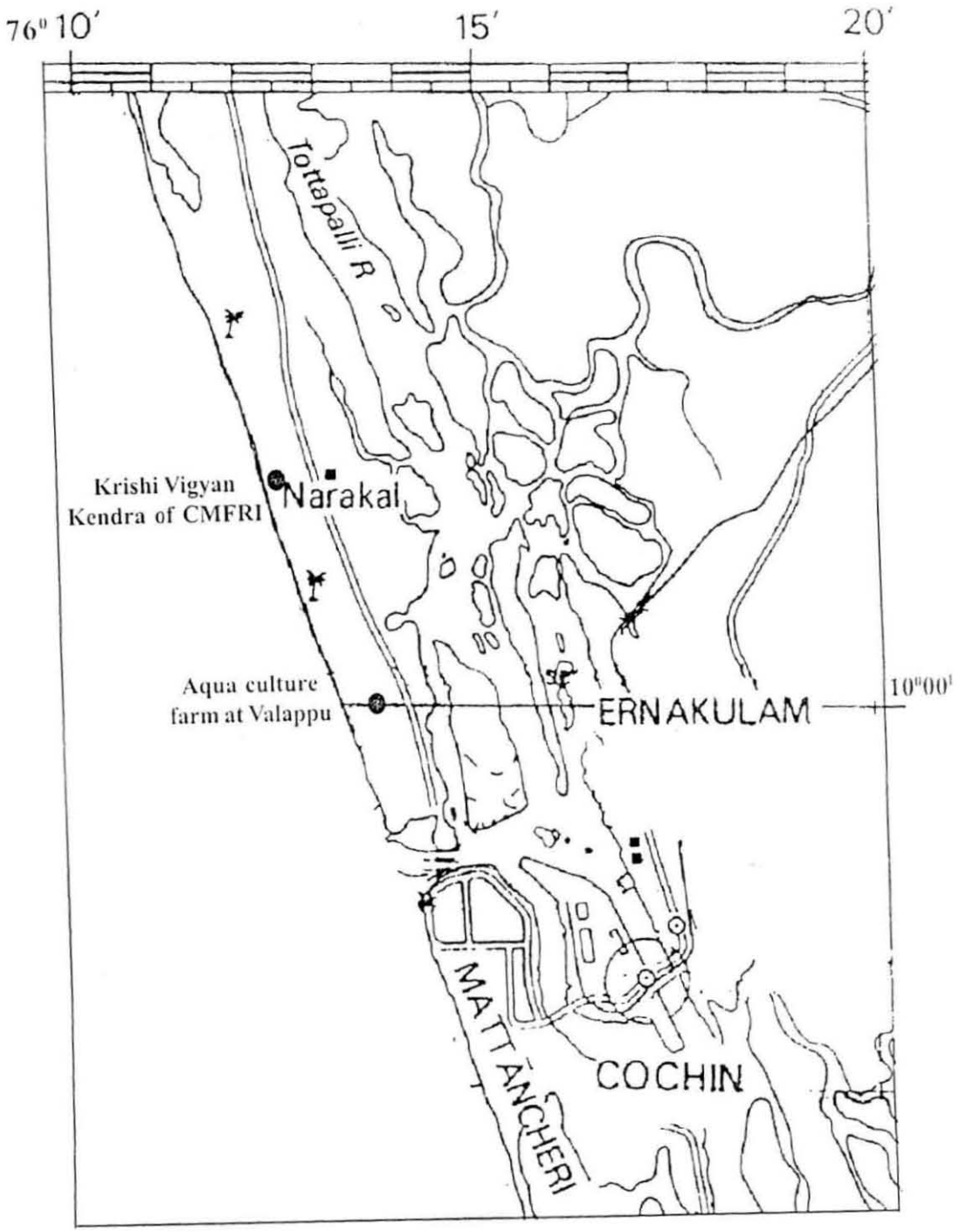

FIG. 1 MAP SHOWING THE STUDY AREA

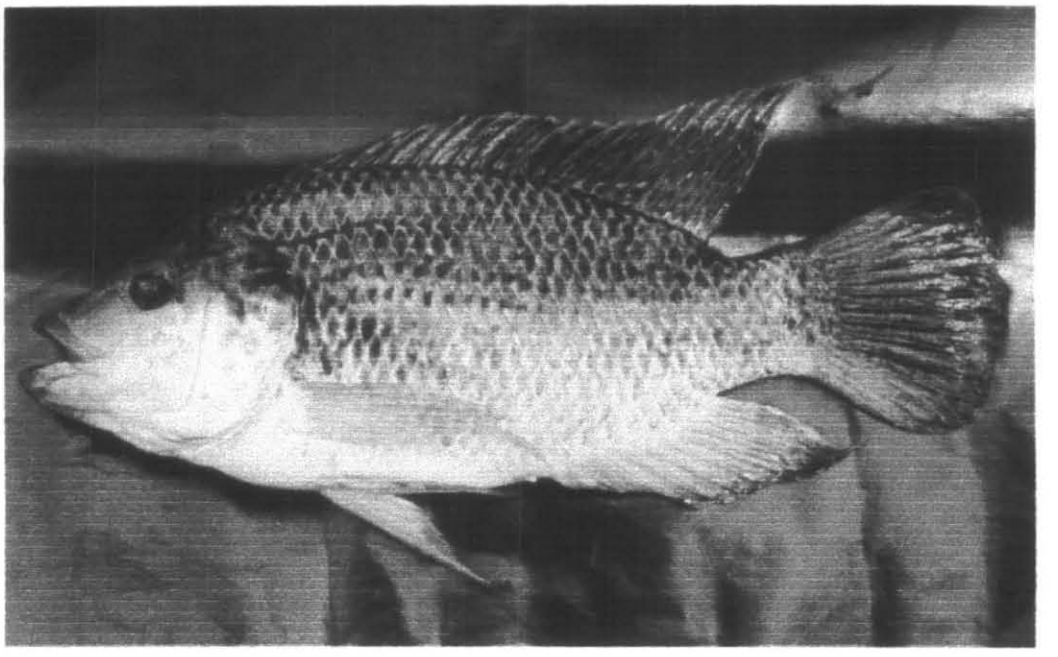

Plate 1 The study animal-*Oreochromis mossambicus* - Male specimen

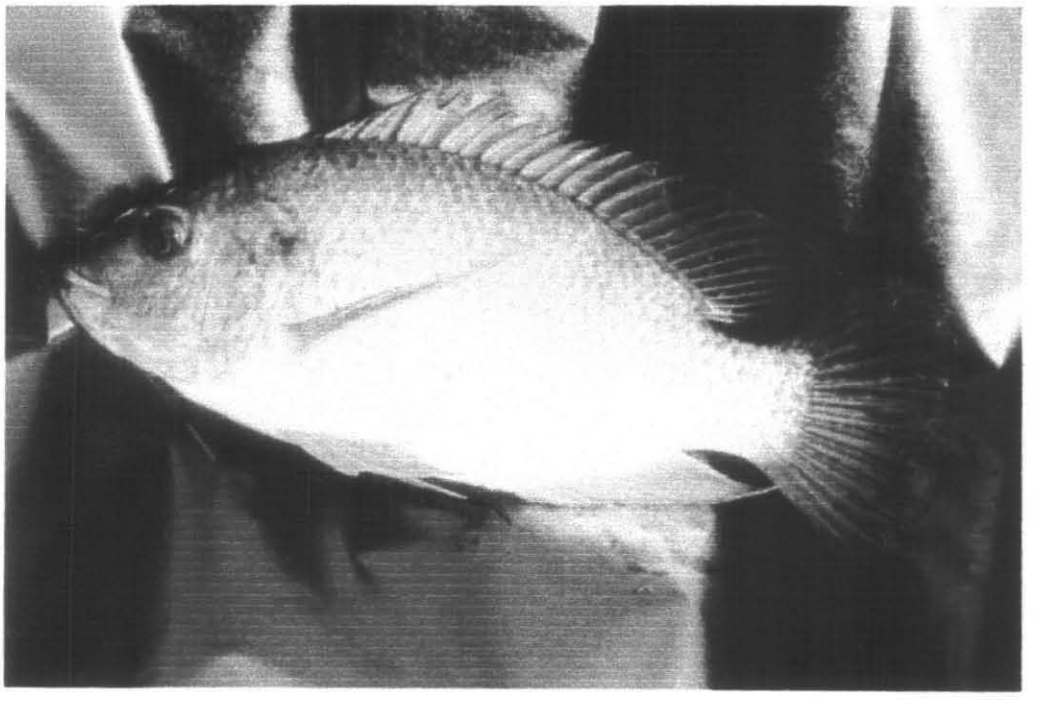

Plate 2 The study animal-*Oreochromis mossambicus* - Female specimen

Plate 3 Station I - Aquaculture pond at Valappu (1ha/1.5m)

Plate 4 Station II - Aquaculture pond at KVK (0.8ha/1m)

3.2 Sample Collection

Regular monthly samples were collected from both the stations for the study. Tilapia collected by cast netting were transported to the laboratory in sterile polythene bags in live condition. Following examination of the external body surface and gills, 1gm of the skin just below the dorsal fin was taken for bacteriological analysis. The body cavity was opened aseptically after swabbing the area with disinfectant. An incision was made with sterile scalpel through the body at the base of pectoral fins. Any adhesions between the body wall and the under lying viscera were carefully separated and the 2 sides of the body wall pinned back to the dissecting surface and the stomach and intestine were separated out adopting all aseptic procedures. Water was collected in sterile plastic bottles and sediment in sterile polythene covers for quantitative and qualitative analysis of Vibrionaceae.

3.3 Bacteriological investigations

Bacteriological investigations were carried out for quantitative and qualitative analysis of total plate count (TPC), total *Vibrio* count (TVC), total aeromonad count (TAC).

3.3.1 Quantitative analysis

The total heterotrophic bacteria were estimated by serial dilution technique and pour plating method (Rodina, 1972). Approximately 1g of the sample (skin, stomach, intestine) were transferred to a mortar and pestle and ground well with 99ml of aged sea water. This was transferred into a sterile conical flask and shaken for 30 mts at 250 rpm in a bacteriological shaker. After thorough shaking, serial dilutions were made according to the standard procedure. 1ml of the inoculum was transferred into sterile 10cm dia glass petriplates and pour plated with Zobell's marine agar (Hi-media). These plates were incubated at room temperature for 24 hr and the total plate count (TPC) was estimated.

3.3.2 Quantification of vibrios

Estimation of total vibrios was done by MPN method using Alkaline peptone water as well as spread plate technique using the selective medium TCBS agar (Hi-Media). Thiosuplhate citrate bile salt sucrose agar (proposed by Nakanishi (1962), modified by Kobayashi et.al (1963) was used for the isolation and selective cultivation of *Vibrio anguillarum* and other enteropathogenic vibrios. Biochemical characterization was done as per the scheme of Alsina and Blanch (1994).

3.3.3 Quantification of *Aeromonas*

For the quantification and isolation of *Aeromonas*, MPN method using Trypticase soy agar with ampicillin was adopted as well as pour plating was done on modified Furunculosis agar as per standard procedures.

Modified Furunculosis

Tryptone	-	10g/l
Yeast extract	-	5g/l
Tyrosine	-	1g/l
NaCl	-	2.5g/l
Starch	-	100g/l
Ampicillin	-	1000mg/l

Suspended 33.5g/l of the media in distilled water. Heated to boiling and dissolved completely. Dispensed and sterilized by autoclaving at 15lbs pressure (121^0C) for 15 minutes.

Biochemical characterization was done as per the scheme of Popoff and veron (1976)

3.3.4 Antibiotic sensitivity

Antibiotic Sensitivity tests were done as per the standardized method of Bauer *et.al.* (1966). The antibiotic discs were acquired from Hi-media.

3.4 Collection and maintenance of infected fishes

Infected fishes which exhibited fin erosion, sloughening of scales, darkening of skin and corneal opacity were collected from brackish water farms and transferred to fibre glass tanks imparting minimum stress. The salinity of the water was maintained at 18-20 ppt with proper aeration. They were fed on formulated feed and the disease symptoms observed. The fishes were maintained for 30 days.

3.5 Sampling of infected specimens

Bacteriological investigations were carried out by 2 methods to isolate the etiological agents.

1) In the first method, samples were taken from infected portions such as fins, skin and eyes and inoculated into 1% peptone water and incubated at room temperature. After overnight incubation, loopful of broths were streaked on to both TCBS and Furunculosis agar plates. The colonies which developed on the respective plates were isolated and subcultured on sea water agar slants until their biochemical studies were done.

2) In the second method, swabs were taken directly from the infected parts and streaked on TCBS agar, Furunculosis agar and Zobell's agar plates. The colonies which developed overnight were isolated and subcultured in sea water agar slants until biochemical characterization were done.

These strains were preserved in semi solid nutrient agar and stored as stock cultures in refrigerated condition for further studies.

3.6 Maintenance of fish for infectivity trials

Tilapia of mean body weight 30-35gms and body length of 13-15cms were held in fibre glass tanks (Plate 5) for at least 1 week for acclimatization of the fish to laboratory conditions. Salinity of the water was maintained at 18-20ppt and the fishes were stocked at the rate of 6 fishes/tank. 18 fishes were used for each set of experiment. The tanks were well aerated (Plate 6) and fishes were fed on formulated feed.

Infectivity trials were performed with tilapia to confirm the pathogenicity of 4 isolates, namely *V.anguillarum*, *V. fluvialis*, *A. hydrophila* and *A.caviae*.

3.7 Bio-chemical tests done for bacterial identification

Gram staining	**Sugar fermentation**
Motility	Lactose Maltose Mannitol Sucrose Glucose
Decarboxylation of amino acids	**Tolerance tests**
Arginine Lysine Ornithine	Tolerance to Salinity 0%NaCl 6%NaCl 8%NaCl
Nitrate reduction test Ammonia production test Oxidase test Catalase test	**Tolerance to temperature** Temperature at +4^{0}C Temperature at +40^{0}C
Indole production Voges-proskauer test Citrate utilization	**Sensitivity studies** Sensitivity to 0/129 (150μg)

Hydrolysis	Antibiotic sensitivity
Starch	Tetracycline
Gelatin	Erythromycin
Caesin	Streptomycin
Urease	Gentamycin
Aesculin	Chloramphenicol
	Penicillin
	Ampicillin
	Novobiocin
Hydrogen sulphide Production test	

3.8 Pathogenicity Assays In Tilapia

3.9 Sample preparation

Pathogenicity of the different pure culture bacterial isolates were determined "in-vivo" following the protocols described by Watkins *et.al.* (1981). A pure culture of each strain to be tested was grown for 36 hr at RT in peptone broth. The culture which was centrifuged at 2500 rpm for 15 mts was washed thrice in phosphate buffered saline (PBS) and the pellet was resuspended in PBS and used for injection trials. The turbidity was adjusted by optical density to obtain 10^3, 10^4, 10^5 and 10^6 cells/ml using a spectrophotometer at 550nm. Viable bacteria in the original suspensions were estimated by the standard plate count method on TSA.

Intraperitoneal injections were carried out with *V.anguillarum* (strain1)*V.fluvialis* (strain2), *A.hydrophila* (strain3) and *A. caviae* (strain 4)for pathogenicity studies.

3.10 Pathogenicity assay – Challenge concentration site and type of injection

Strains	Challenge Concentration	Site &type of injection
Strain 1,2,3&4	10^3cells/ml	Intraperitoneal (posterior to the vent)
"	10^4cells/ml	"
"	10^5cells/ml	"
	10^6cells/ml	"
"	10^6cells/ml	Injected into the eye socket
"	10^6cells/ml	Bath challenge

Plate 5 Experimental set up

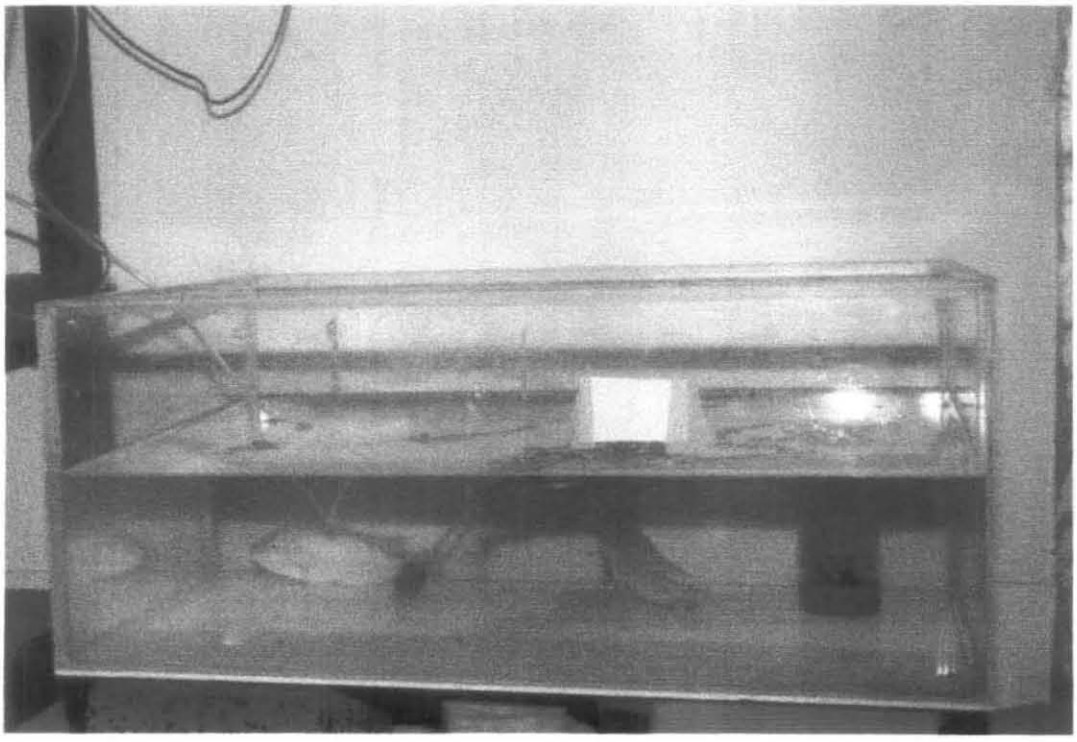

Plate 6 Experimental set up

3.11 Intraperitoneal injection challenge

A total of 4 intraperitoneal challenges were done. The challenge inoculum was drawn into 1ml disposable syringe with a total measure as per the details given above. The fish were netted out individually and the inoculum injected posterior to the anal vent. The needle was held at an angle of 45^0 to the abdomen and the inoculum injected just inside the peritoneum. One-tenth millilitre volumes of appropriate PBS dilutions of the final suspension were injected to the control fish. After injection, the fishes were released into the fibre glass tanks, maintained for 14 days and observations were made. The number of viable cells inoculated was determined from mean count obtained from triplicate TCBS plates and furunculosis agar plates

3.12 Cell suspension

The cell suspensions of the different bacterial isolates were prepared in the same way as that of the intraperitoneal injection studies.

3.13 Bath challenge

The bath challenges were performed in fibre glass tanks with 15 litres of well aerated water of 18-20ppt. The fishes were exposed to10^6 cells/fish in triplicates. The conrol consisted of fishes exposed to PBS.

3.14 Experiments to determine the mode of entry of the pathogen

Caudal cliffed fishes were used for the experiment. The fishes were introduced into 15litres of water maintained just as in the bath challenge studies. These fishes were observed for a period of one week. Controls were also maintained.

Biochemical characterization of the strains re-isolated from the experimentally infected fishes were done to establish Koch's postulates. Median Lethal dose was calculated by the technique of Reed and Muench (1938).

3.15 Sediment and water samples

The following procedure was adopted in the case of sediment and water where the *Vibrio* counts were less.

3.15.1 Sediment Sample

Ten gram of sediment was weighed out into 90ml of sterile sea water and kept in a bacteriological shaker for shaking. This was then filtered through a membrane filter(0.02μ Sartorius), the flask was closed with a stopper and again shaken for 20 minutes. The sediment mixture was allowed to stand for 1-2 minutes to allow large particles to precipitate. Then 50ml of the suspension was placed in the funnel of the filter apparatus fitted with a membrane filter. Once all the liquid was filtered the filter paper was placed in a sterile petridish. The filter paper was washed down and serial dilution was carried out.

3.15.2 Water Sample

50 ml of water sample asceptically collected from the sampling site was filtered through a (0.02μ Sartorius) membrane filter. The filter paper was later transferred into 99ml of sterile seawater. This was thoroughly shaken in a bacteriological shaker and from this, serial dilutions were carried out. *Vibrio* count was determined by the MPN method using Alkaline peptone water as pre-enrichment broth and aeromonad count was determined by the MPN method using Trypticase Soy Broth with ampicillin.

Alkaline peptone water

Peptone - 10g.

NaCl - 5g.

Dissolved ingredients to 1 litre distilled water and adjusted pH to 9.1 ± 0.1. Distributed in 225ml conical flasks and autoclaved at 121^{0}C for 15 minutes.

Trypticase Soy Broth with ampicillin was prepared according to the manufacturer's directions (DIFCO). After autoclaving at 121^{0}C for 15 minutes ampicillin (Sigma) was added to the broth to give a final concentration of 30 mg/l.

To the enrichment broths 1g of sediment and 1ml of water were inoculated and incubated at RT for 24hr.

One loopful enriched inoculum was then streaked on TCBS Agar and modified Furunculosis agar plates and incubated at RT for 18-24 hr. Colonies that occurred were randomly selected for further bio-chemical tests by confirmatory tests for identification.

3.16 Measurement of hydrological parameters

3.16.1 Temperature

Temperature of water was noted in the field by using a mercury high precision thermometer of ±0.1^{0}C at 8A.M on every sampling day.

3.16.2 Hydrogen ion concentration

p^H value of water was determined using a digital pH meter. The instrument was calibrated with the help of the pH buffers (4.2 and 9.1)

3.16.3 Salinity

The water samples were collected in narrow mouthed, polyethylene bottles of 300ml capacity. The bottles were washed with the same water before collecting samples. Salinity was estimated by following the Mohr titration Method (Strickland and Parsons, 1968).

10 ml of water sample was titrated against the silver nitrate solution of normality 0.14 N (24.5g of silver nitrate dissolved in 1000 ml of distilled water) with 10% potassium chromate as an indicator. Care was taken to arrive at the exact end point colouration in all the samples. The volume of silver nitrate consumed for each sample was noted. Silver nitrate solution was standardized during every set of

titration using the standard sea water of known salinity. Salinity of the sample was calculated using the following formula:-

$$Salinity\ ppt = \frac{V_2 x S}{V_1}$$

where V_2 = Volume of silver nitrate for 10ml of sea water sample.

V_1 = Volume of silver nitrate for 10ml of std. sea water.

S = Salinity of standard sea water (34.99 ppt).

3.16.4 Dissolved oxygen

The dissolved oxygen in the water samples was determined using Winkler method (Strickland and Parsons, 1968). The water samples were collected in 125ml capacity Corning reagent bottle with BOD stoppers. The collection bottles were washed twice with ambient water before sampling. Care was taken to ensure filling of water into the bottle with air- bubble free unagitated water. One ml of Winkler A (365 g of manganous sulphate dissolved in 1000ml of distilled water) and one ml of Winkler B (mixture of sodium hydroxide and potassium iodide dissolved in distilled water) solutions were added immediately to the bottle containing the water sample. The stopper was carefully replaced without trapping any air bubble and the precipitate was dispersed uniformly throughout the bottle by shaking.

The precipitate was dissolved using 1 ml of concentrated sulphuric acid. The solution was then titrated against sodium thiosulphate solution (0.01N) using starch as an end point indicator. The dissolved oxygen was estimated using the formula.

$$\text{Oxygen (ml/l)} = \frac{V_1 x N x 8 x 1000 x R x 0.698}{V_2}$$

where

V_1 = Volume of sodium thiosulphate

N = Normality of sodium thiosulphate

V_2 = Volume of water sample taken for titration against the sodium thiosulphate

R = Correction factor

0.698 = To convert parts per million to ml of oxygen/ litre

8 = Equivalent weight of oxygen.

3.16.5 Nitrite

Nitrite was estimated by the Azo-dye method (Bendschneider and Robinson, 1952). 50ml of water sample was taken in a conical flask and 1ml of sulphanilamide solution (prepared by dissolving 5g of sulphanilamide in 50ml of concentrated hydrochloric acid and the same solution was made upto 500ml using distilled water) was added. The sample was mixed thoroughly and kept aside for reaction. After 3 minutes, 1ml of NNED (N-1-Naphthylethylenediamine dihydrochloride) solution (0.5g of NNED dissolved in 500ml of distilled water) was added and the sample was mixed well. Blank was prepared using distilled water and the analysis was carried out in the same manner. Standard nitrite solutions of known concentration were prepared and analysis was carried out in the same manner mentioned for water sample. Between 10 minutes and 2 hours the absorbance of sample was measured against blank in the spectrophotometer at 545 nm. The sample values were calculated from the standard values and expressed in μg – at/L.

3.16.6 Nitrate

Nitrate was estimated following the method of Mullin and Rily (1955). To 50ml of water sample, 2 ml of buffer solution (a mixture of phenol and sodium hydroxide solutions) was added. After rapid mixing, 1ml of reducing agent (a mixture of copper sulphate and hydrazine sulphate solutions) was also added and the flasks containing the samples were kept in a dark place for about 20 hours in order to effect the reduction of nitrate. Later, 2ml of acetone was added to the sample. After 2 minutes, 1ml of sulphanilamide solution was added and after keeping the sample for 2 minutes, the absorbance of the samples were measured against the distilled water blank in the spectrophotometer at 545 nm. Standard graph was prepared for

standard nitrate solutions of known concentrations. The sample values were plotted in the graph and expressed in μg – at/L.

3.16.7 Phosphate

The method described by Murphy and Riley (1962) and followed by Strickland and Parsons (1968) was used for the analysis. 50 ml of water sample was taken in a clear conical flask and 5ml of mixed reagent (prepared by mixing the ammonium molybdate solution, diluted sulphuric acid, ascorbic acid solution and antimonyl tartarate solution) was added. The sample was mixed thoroughly. After 5 minutes, the absorbance of the sample was measured against distilled water blank using spectrophotometer at 885 nm. The sample values were calculated from the standard phosphate values. The amount of phosphate was represented in μg – at/L.

3.16.8 Ammonia

Ammonia was determined following the phenol hypochlorite method (Solarzano,1969). To 50ml of the sample, 2ml of the phenol solution (prepared by dissolving 10g of phenol in 100ml of ethanol) and 2ml of sodium nitro prusside solution (1g of sodium nitro prusside dissolved in 200ml of de-ionized water) were added. After mixing well, 5ml of oxidizing solution (prepared by mixing 100ml of sodium citrate and 5g of sodium hydroxide dissolved in 500ml of de-ionized water and 25ml of 1.5N sodium hypochlorite solution) was added to the sample and mixed thoroughly. After 1 hour the developed colour was read spectrophotometrically against distilled water blank at 640nm absorbance. The sample values were then calculated from the standard ammonia values and the amount of ammonia was represented in μg – at/L.

3.16.9 Total organic carbon

The method of Walkley and Black (1934) was used to determine the organic carbon content of the sediment. The sediment sample was dried in the oven at 100^{0}C for 24 hours and the sample was ground in an agate mortar and passed through a 0.2mm brass sieve. To a 0.5 g of powdered sample, 10ml of 1N potassium dichromate solution (49.04g of potassium chromate in 1 litre of distilled water) and

20ml of concentrated sulphuric acid containing 1.25g of silver nitrate for every 100ml of acid were added and swirled. The sample was heated in water bath for 30 minutes. The material was cooled and 200ml of distilled water was added. To this 100ml of concentrated phosphoric acid and 1ml of diphenylamine (0.5g diphenyl amine in a mixture of 20ml distilled water and 100ml concentrated sulphuric acid) solution were also added. The sample was then back titrated with 0.5N ferrous ammonium sulphate (139.0g of ferrous ammonium sulphate in distilled water, to which 7.5ml of concentrated sulphuric acid was added and diluted to 1 litre by adding distilled water). The change from blue colour to sharp brilliant green colour was noted as the end point. Analysis was carried out in the same manner for blank using distilled water. The values were calculated using the following equation.

$$\text{Organic carbon} = \frac{3.951}{g}\,1-\frac{T}{S} \qquad \text{(mg/g)}$$

where

S = volume of titrant used against blank (ml)

T = Volume of titrant used against sample (ml)

g = Sample weight in gms.

Analysis of variance and correlation studies were carried out according to the methods of Snedecor and Cochran (1968).

3.17 Bacteriological Analysis-Composition, Media and Methods

Gram Staining

1) Ammonium oxalate – crystal violet solution

Crystal violet	2g
Ethanol (95%)	20ml
Ammonium oxalate	0.8g
Distilled water	80ml

Dissolved crystal violet in ethanol

Dissolved ammonium oxalate in distilled water

Mixed the two solutions, and allowed to stand for 24 hr and was filtered.

2) Iodine Solution

Iodine	1g
Potassium iodide	2g
Distilled water	300ml

Dissolved potassium iodide and then iodine in about 5ml distilled water and remaining volume of water was added.

3) Safranin Solution

Safranin	0.25g
Ethanol (95%)	10ml
Distilled water	90ml

Dissolved safranin in ethanol and added with the distilled water

Method

1. Ammonium Oxalate – crystal violet solution was added to heat – fixed smear for 1 minute.
2. Washed with water
3. Iodine solution was applied for 1 minute
4. Iodine solution was tipped off
5. Decolourised with alcohol (95%) until no more violet colour emanated from the smear.
6. Washed thoroughly with water

7. Safranin solution was applied for 2 minutes.

8. Washed with water, drained and blot dried and examined

Gram positive organisms gave blue/purple colour

Gram negative organisms gave pink/red colour

Motility by hanging drop

To 5ml of peptone broth the culture was inoculated and incubated for 24 hr. A clean dry cover slip was taken and a little vaseline was smeared round the edge of the cover slip. A small loopful of culture was transferred into the centre of cover slip. A cavity slide was inverted over the cover slip so that the drop of the culture hang in the centre of the cavity. The drop of culture was examined under the microscope for movement.

Detection of motility in semi-solid nutrient agar

Inoculated semi solid nutrient agar with a straight wire making a single stab down the centre of the tube to about half the depth of the medium. Incubated the culture at 37^0C, for each day from one to 6 days. Non-motile bacteria generally gave growths that are confined to the stab line, have sharply defined margins and leave the surrounding medium clearly transparent. Motile bacteria typically gave diffuse, hazy growths that spread throughout the medium rendering it slightly opaque.

Andrade peptone water with meat extract (Hi-media)

Peptone	-	10g/l
Meat extract	-	3g/l
Sodium chloride	-	5g/l
Andrade's indicator	-	0.1g/l
Final p^H (at 25^0C)	-	7.1±0.2

Suspended 18 grams of the media in 1000ml distilled water. Heated to boiling to dissolve completely. Dispensed in tubes with inverted Durham tubes. Sterilized by autoclaving at 15 lbs pressure (121^0C) for 15 minutes. Cooled and aseptically added sterile solution of desired carbohydrate. Glucose, Lactose, Sucrose, Mannitol were used at final concentration of 1%, incubated for 24 hr Positive reactions gave reddish pink colour.

Amino acid decarboxylase Test

Composition of medium

Peptone	-	5g
Meat extract	-	5g
Glucose	-	0.5g
Pyridoxal	-	5mg
Bromocrescol purple (1 in 500 solution)	-	5ml
Crescol red (1 in 500 solution)	-	2.5ml
Distilled water	-	1 L

Dissolved the solids in water and adjusted the p^H to 6 before the addition of the indicators. This was the basal medium and to it was added the amino acid whose decarboxylation was to be tested. Divided the basal medium into four portions and treated as follows:-

(1) Added 1 percent L-lysine hydrochloride

(2) Added 1 percent L- ornithine hydrochloride

(3) Added 1 percent L-arginine hydrochloride

(4) No additions (control)

Readjusted the p^H to 6 when necessary. Distributed 1 ml quantities in small tubes containing sterile liquid paraffin to provide a layer about 5mm thick above the medium. Autoclaved at 121^0C for 15 mts.

Inoculated lightly through the paraffin layer with a straight wire. Incubated and read daily for 4 days. The medium first became yellow due to acid production during glucose fermentation, later if decarboxylation occured the medium became violet. The control was yellow.

Nitrate reduction test

Potassium nitrate	-	0.2g
Peptone	-	5g
Distilled water	-	1L

Distributed in 5ml tubes and autoclaved at 121^{0}C for 15 mts. Inoculated and incubated for 96 hr.

Test reagent

Solution A → Dissolved 8g of sulphanilic acid in 1L of 5N acetic acid.

Solution B → Dissolved 5g of α- naphthylamine in 1L of 5N acetic acid.

Immediately before use, mixed equal volumes of solutions A and B to give the test reagent.

Added 1ml of the test reagent to the test culture. A red colour developing within a few minutes indicated the presence of nitrite and hence the ability of the organism to reduce nitrate.

Production of Ammonia

To a peptone water culture grown for 5 days at 37^{0}C, Nessler's reagent was added. Brown colour was positive and faint yellow colour negative.

Oxidase Test

A strip of filter paper was soaked with a little freshly made 1% solution of tetramethyl –P- phenylene diamine dihydrochloride and then used once by rubbing a speck of culture on it with a platinum loop. A positive reaction was indicated by an intense deep – purple hue, appearing within 5-10 seconds.

Catalase Test

A small amount of the culture was picked from a nutrient agar slope, using a clear sterile platinum loop and inserted into hydrogen peroxide solution held in a small clean tube. The production of gas bubbles almost immediately from the surface of the solid culture indicated a positive reaction.

Indole production

Tryptone	-	20.0g
Sodium Chloride	-	5.0g
Distilled water	-	1L

Adjusted the p^H to 7.4. Sterilized by autoclaving at 121^0C for 15 mts.

Inoculated the medium and incubated for 48 hr at 37^0C. 2ml of Kovacs reagent was added and shaken gently. A red ring indicated a positive reaction.

Kovacs reagent

Amyl or isoamyl alcohol	-	150 ml
P- Dimethyl – aminobenzaldehyde	-	10g
Con. hydrochloric acid	-	50ml

Dissolved the aldehyde in the alcohol and slowly added the acid. Small quantities were prepared and stored in the refrigerator. Shaken gently for use.

Voges – Proskauer test

MR-. VP medium (Hi-media) was incubated with the culture. 0.6ml of a 5% solution of α - naphthol in ethanol and 0.2ml of 40% KOH were added to 1ml of the culture and incubated at room temperature for 72hr. In a positive reaction a pink colour appeared in 2-5 mts deepening to magenta or crimson in half an hour. In a negative reaction it remained colourless within half an hour.

Citrate utilization

Simmon's citrate (Hi-media) was sterilized by autoclaving and was poured as slopes. Inoculated from a saline suspension of the organism and Incubated for 96hr at 37^0C. The positive result was indicated as blue colour and streak of growth and original green colour and no growth as negative.

Starch Hydrolysis

Medium

Soluble starch	-	0.2%
Beef extract	-	0.3%
Agar	-	1.5%
Seawater	-	100 ml
P^H	-	7.2 sterilized at 15 lbs for 30 mts.

Inoculated starch agar and incubated plates at room temperature for 5 days. Flooded the plate with Lugol's iodine solution, the medium turned blue where starch had not been hydrolyzed while hydrolysis was indicated by clear colourless zones.

Gelatin liquefaction

Medium

Peptone	-	0.2%
Beef extract	-	0.2%
Gelatin	-	12%
Sea water	-	100 ml

Dissolved the ingredients by steaming (Tyndalization). Adjusted the p^H to 7.2, Filtered and steamed for 30 mts, for 3 consecutive days.

Inoculated the media with 24 hr broth culture. Incubated the tubes at room temperature for 5 days. Placed the tubes in the refrigerator and allowed them to remain for about 1 hr. At refrigeration temperature gelatin was solid in nature. Even after keeping the cultures at -4^0C if the gelatin remained solid it showed negative result. Gelatin was in liquid form if liquefaction had taken place.

Hydrolysis of Casein

Medium

Peptone	-	5.0g
Beef extract	-	1g
Sodium Chloride	-	5g
Agar Powder	-	30g
Distilled water	-	1000ml

Steamed to dissolve the ingredients in the distilled water. Filtered and adjusted the p^H to 7.5. This was dispensed, plugged and sterilized. Before use to the melted and cooled media 1000ml of filter sterilized fresh milk was aseptically added and mixed well before plating. The plates were streaked with the test culture and incubated for 24 hr. Positive cultures produce transparent areas around the culture indicating hydrolysis of casein.

Urease test (Christensen's medium)

Peptone	-	1g
Sodium Chloride	-	5g
Monopotassium dihydrogen phosphate	-	2g
Phenol red	-	6ml
		(1 in 500 aqueous solution)
Agar	-	20g
Distilled water	-	1L

The p^H was adjusted to 6.8-6.9 and the basal medium was sterilized by autoclaving at 121^{0}C for 15 minutes. When it cooled to about 50^{0}C a sterile solution of glucose was added to give a final concentration of 0.1% and 100 ml of a 20% solution of urea previously sterilized by filtration was added. The medium was tubed as deep slopes. Heavily inoculated agar slopes were incubated at 37^{0}C. Examined after 4 hr and after overnight incubation, no tube was treated negative until after 4 days incubation. Urease – positive cultures produced a purple pink colour due to a change in the colour of the indicator.

Aesculin hydrolysis

Medium

Peptone	-	1g
Sodium chloride	-	0.5g
D.W	-	100ml
Aesculin	-	0.1g
Ferric Citrate	-	0.05g

Sterilized the medium at 10 lb for 10 minutes

Incubated the medium for 2-7 days. Later poured as much of Benedicts' reagent as the culture medium and heated to boiling. A pinch of glucose was also added. Appearance of a red precipitate showed positive reaction.

Hydrogen sulphide production

Cystine broth

Peptone	-	1%
Beef extract	-	0.3%
Cystine	-	0.01%
Sodium chloride	-	0.5%
D.W	-	100ml
p^H	-	7.2

Sterilized at 15 lbs for 30 mts.

The organism was grown in cystine broth and a lead acetate paper was inserted between the plug and the tube. The blackening of the paper was observed daily for 7 days. Blackening of the lead acetate paper indicated the production of hydrogen sulphide.

Salt tolerance

Peptone broth	0% NaCl	6% NaCl	8% NaCl
Peptone -1%	"	"	"
Agar -2%	"	"	"
Ferric Phosphate - a pinch	"	"	"
D.W – 100 ml	-	+ 6% NaCl	+8% NaCl
P^H -7.2	"		

5ml of the medium was sterilized at 121^{0}C for 30 minutes. It was then inoculated and observed for 36 hr. The intensity in the growth of the culture medium indicated the salt tolerance.

Temperature tolerance

The culture was inoculated in 5ml of peptone broth and incubated for 36 hr at 4^{0}C and 40^{0}C. The intensity in the growth of the culture indicated the tolerance of the culture to the medium.

Sensitivity to 0/129 disc

0/129 (2-4-diamino 6-7 – diisopropyl pteridine) is a pteridine compound and is vibriostatic. Streaked the culture onto the agar plate and placed the disc (which contains 0/129 compound) over the culture. Incubated at 37^{0}C for 24 hours. Positive reactions, gave a clear zone around the disc.

Preparation of 0/129 discs

Whatman No.1 filter paper was made into discs. Sterilized the discs at 140^{0}C for 1 hour in hot air oven. Dissolved 30 mg of 0/129 pteridine compound in 2ml of acetone. This was sufficient for soaking 200 discs. Each disc contained about 150 microgram of pteridine compound. Mixed the discs with the pteridine compound solution in a sterile petriplate so that the compound was properly coated on all the discs. Kept the cover of the petridish half open and dried the discs in the incubator at 37^{0}C for 15 to 30 minutes.

4. RESULT

4.1 Quantification of total plate count and Vibrionaceae

4.2 Bacterial profile of water, sediment and *Oreochromis mossambicus* from culture ponds

The total plate count (TPC) of water from Station I (Valappu) ranged from a maximum value of 104.5x10^4/ml in the post monsoon season (1997-1998) to a minimum value of 9.5x10^4/ml during the monsoon season (1998). The total *Vibrio* count and the total aeromonad count also showed maximum values of 66.5 x10^4/ml and 25 x10^4/ml during the post monsoon season and the minimum values of 2.75x10^4/ml and 4 x10^4/ml during the monsoon season (Fig. 2). The TPC, TVC, and TAC of water from Station II (KVK) recorded the maximum and minimum values during the same seasons. The maximum value of TPC was 191 x10^4/ml during the post monsoon season (1997-98) and the minimum value was 7.25 x10^4/ml during the monsoon season. The maximum values of TVC and TAC were 23.75 x10^4/ml and 37.5x10^4/ml respectively while the minimum values were 2.25 x10^4/ml and 4.36x10^4/ml respectively (Fig. 7).

TPC of sediment from Station I recorded the maximum value during the pre monsoon season, 1999 (71.97x10^4/gm) and the minimum value during the monsoon season of 1998 (9.4 x10^4/gm). The maximum value of TVC was found to be 32.87 x10^4/gm which was recorded during the pre monsoon season (1998), while the minimum value was recorded during the monsoon season (1.54 x10^4/gm). TAC recorded the maximum value during the post monsoon season of 1998, (17.23 x10^4/gm) while the minimum value was recorded during the monsoon season (3.604 x10^4/gm) (Fig. 3).

At station II (KVK) the values showed variations from that of station I. The maximum TPC of sediment occurred during the post monsoon season of 1997-1998 (49.1 x10^4/gm) while the maximum TVC occurred during the post monsoon

season of 1998-1999 (9.29 $x10^4$/gm) and TAC during the post monsoon of 1997-98 (13.83 $x10^4$/gm). The minimum values of TPC and TAC occurred during the monsoon season (22.28 $x10^4$/gm and 3.375 $x10^4$/gm) whereas the minimum value of TVC was observed during the pre-monsoon season of 1998 (3.8 $x10^4$/gm) (Fig. 8).

At Station I, the maximum TPC of skin from *O.mossambicus* was recorded during the pre monsoon season, of 1998 (160.63 $x10^5$/gm) and the minimum value of TPC was recorded during the post monsoon season of 1998-1999 (45.56 $x10^5$/gm). The maximum TVC was recorded during the post monsoon season of 1997-98 (96.68 $x10^5$/gm) while the minimum value was recorded during the post monsoon season of 1998-1999 (29.577$x10^5$/gm). The maximum TAC recorded was 60.16$x10^5$/gm which occurred during the post monsoon of 1997-1998 while the minimum TAC was observed during the monsoon 9.56 $x10^5$/gm (Fig.4). At station II (KVK), the maximum TPC of skin of *O.mossambicus* was observed during the pre monsoon of 1999, (125.33$x10^5$/gm) and the minimum value was noted during the post monsoon season of 1998-1999 (101.58$x10^5$/gm). The maximum TVC of skin was recorded during the post monsoon of 1997-1998 (94$x10^5$/gm) and the minimum value was observed during the monsoon 67.41$x10^5$/gm. Maximum TAC of skin was observed during the pre monsoon season of 1999 (93.52 $x10^5$/gm) and the minimum during the monsoon season (48.211$x10^5$/gm) (Fig.9).

At station I, the maximum TPC and TVC of stomach of *O.mossambicus* was observed during the post monsoon season of 1997-1998 (93.68$x10^5$/gm and 54.74$x10^5$/gm) while minimum values were recorded during the post monsoon season of 1998-1999 (60.11$x10^5$/gm) and monsoon season respectively (36.38$x10^5$/gm). TAC recorded the maximum value during the pre monsoon season of 1999 (36.05$x10^5$/gm) and the minimum during the monsoon season (3.66$x10^5$/gm) (Fig.5). At Station II (KVK) the maximum values of TPC, TVC and TAC from the stomach region was observed during the post monsoon season of 1997-1998 (190.13$x10^5$/gm, 107.69$x10^5$/gm and 69.19 $x10^5$/gm) while the minimum TPC and TVC were observed during the post monsoon season of 1998-99 (38.78$x10^5$/gm and 4.309$x10^5$/gm) and TAC during the monsoon season (11.92$x10^5$/gm) (Fig.10).

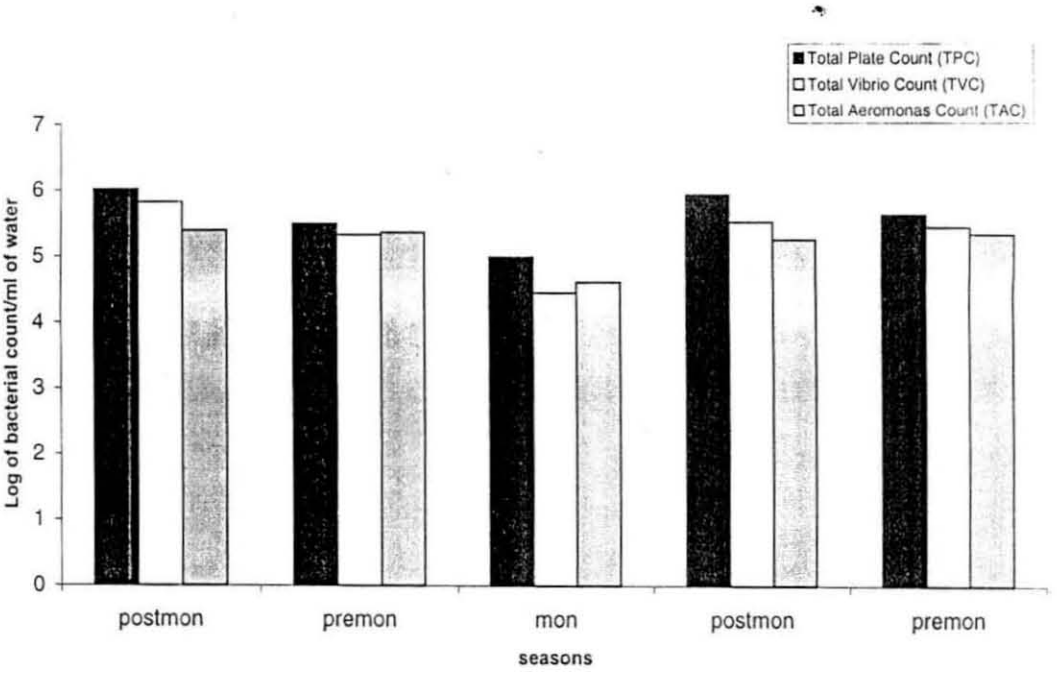

Fig.2 Bacterial profile of water at Station I

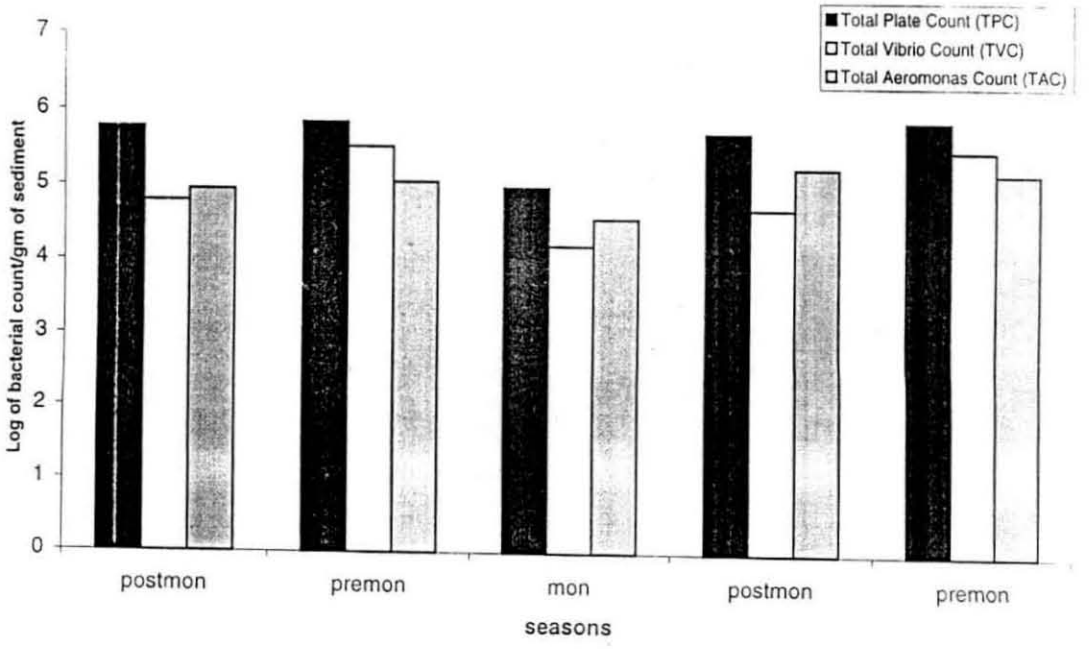

Fig.3 Bacterial profile of sediment at Station I

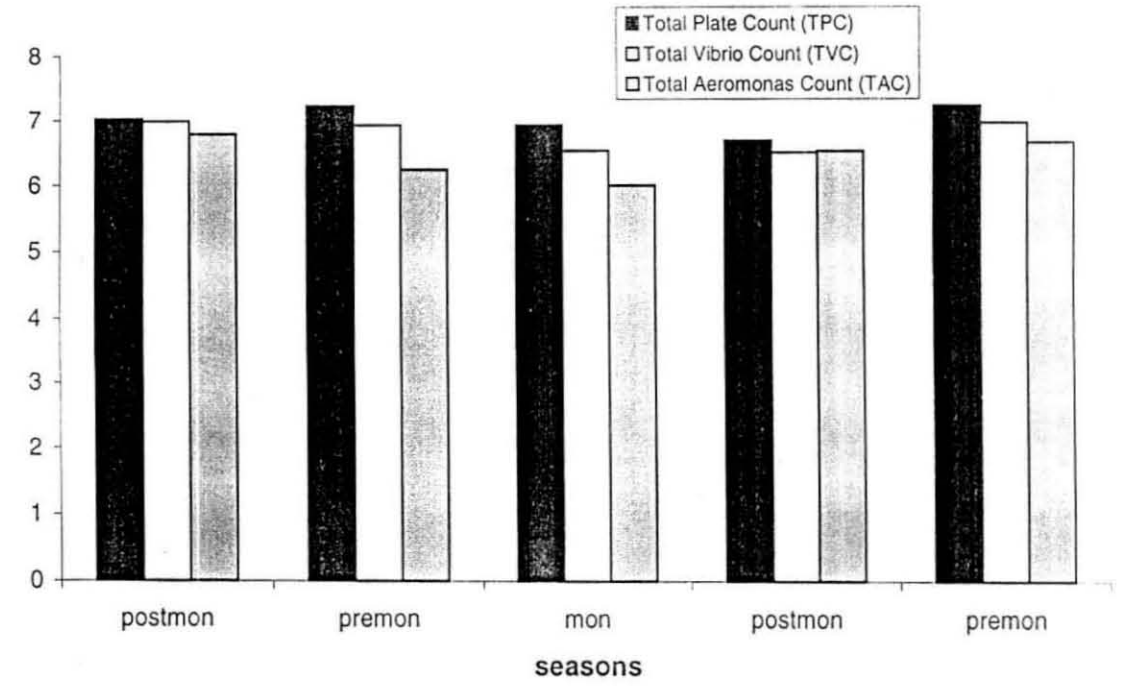

Fig.4 Bacterial profile of skin of *O. mossambicus* at Station I

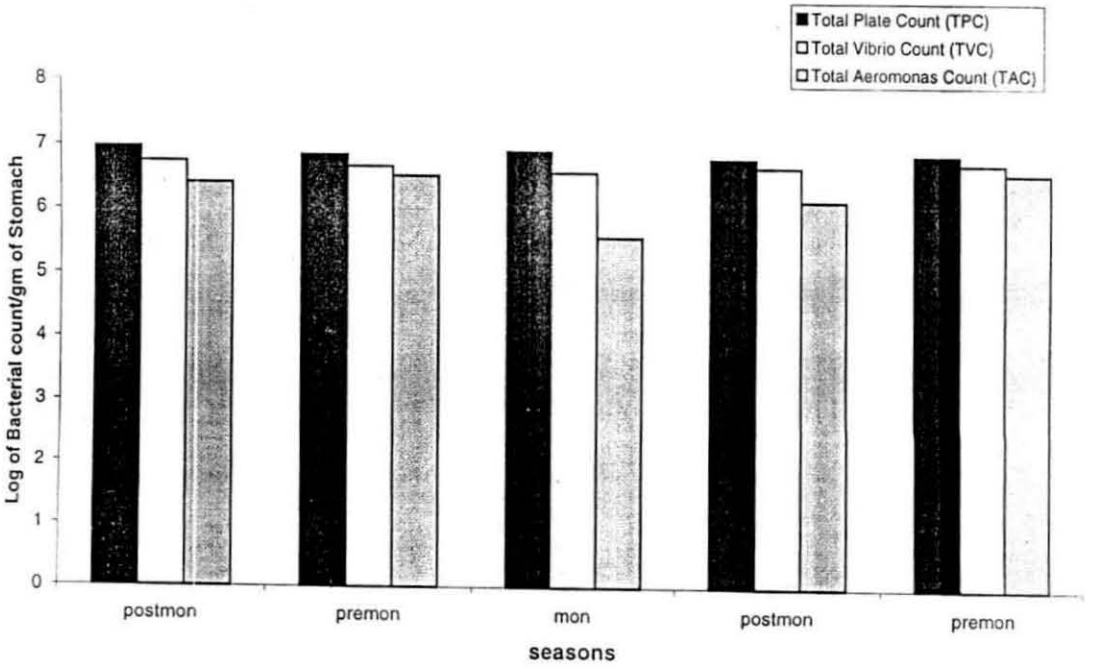

Fig.5 Bacterial profile of stomach of *O. mossambicus* at Station I

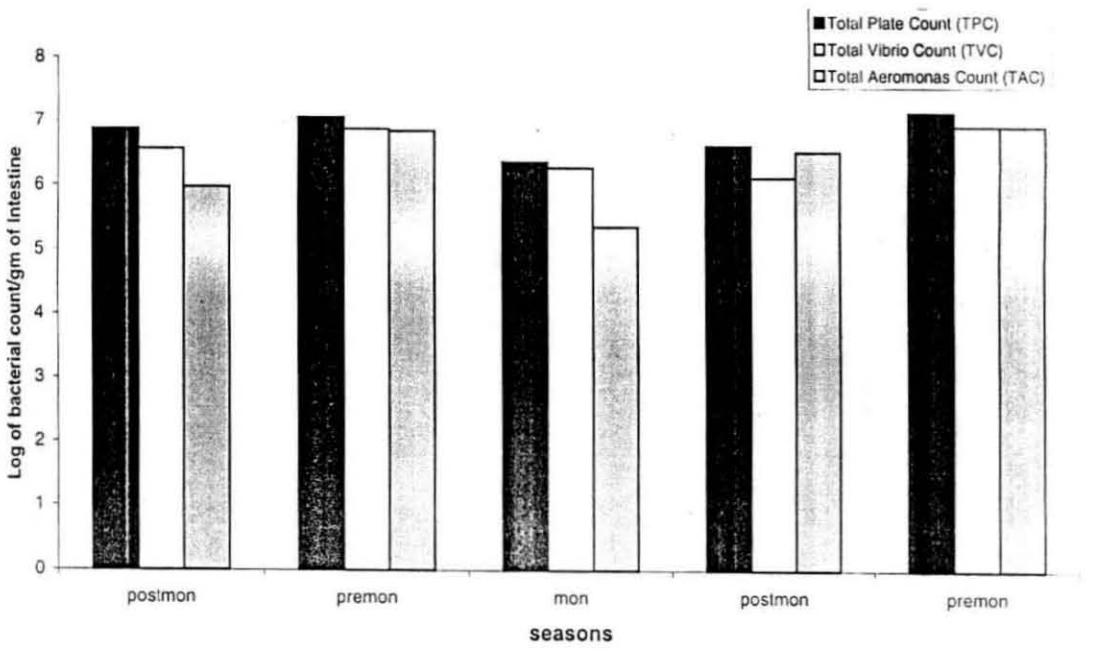

Fig.6 Bacterial profile of intestine of *O. mossambicus* at Station I

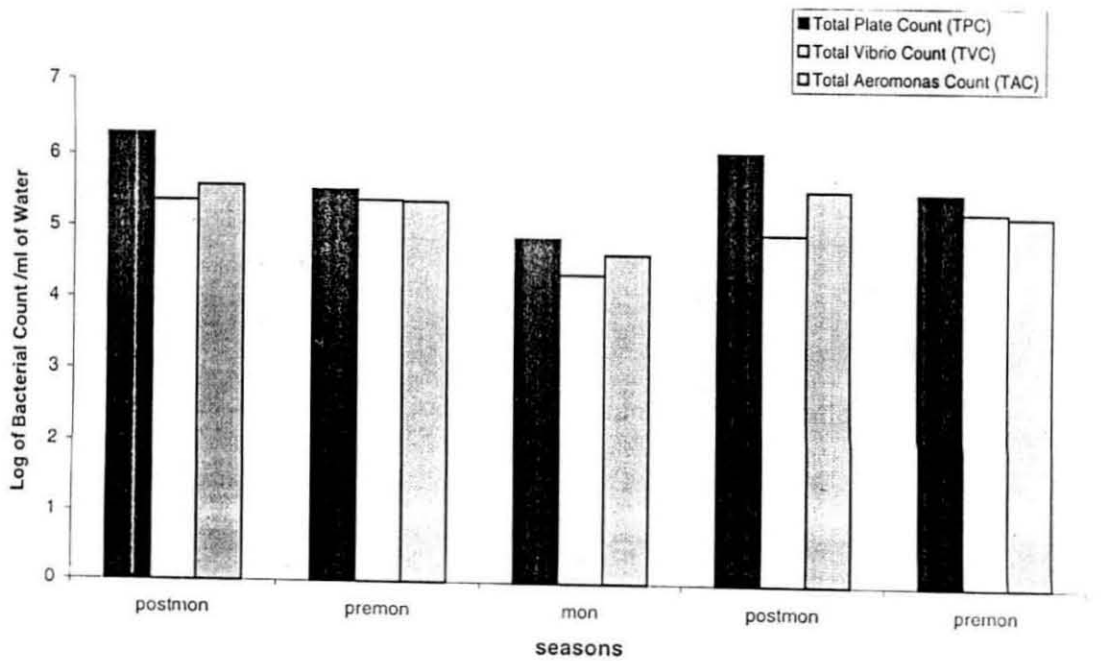

Fig.7 Bacterial profile of water at Station II

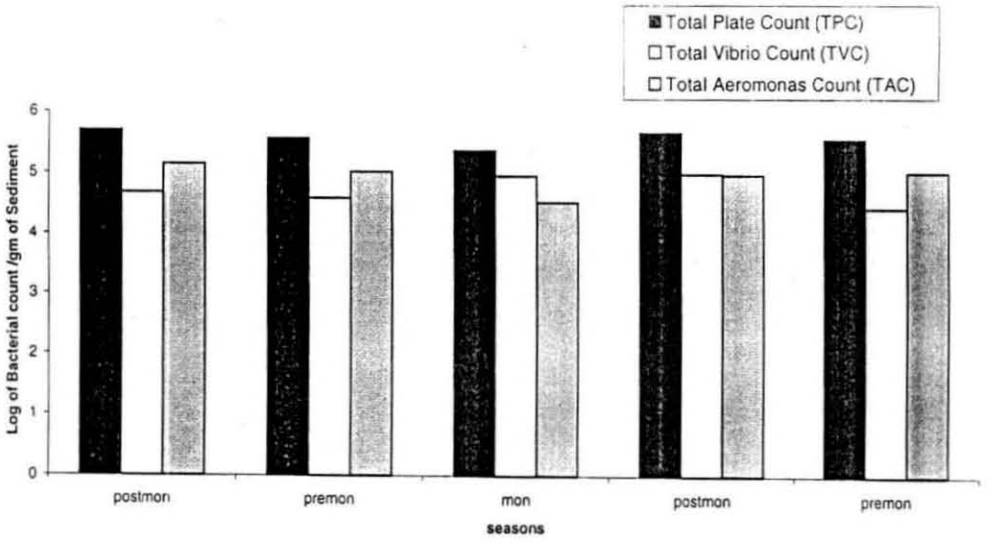

Fig. 8 Bacterial profile of sediment at Station II

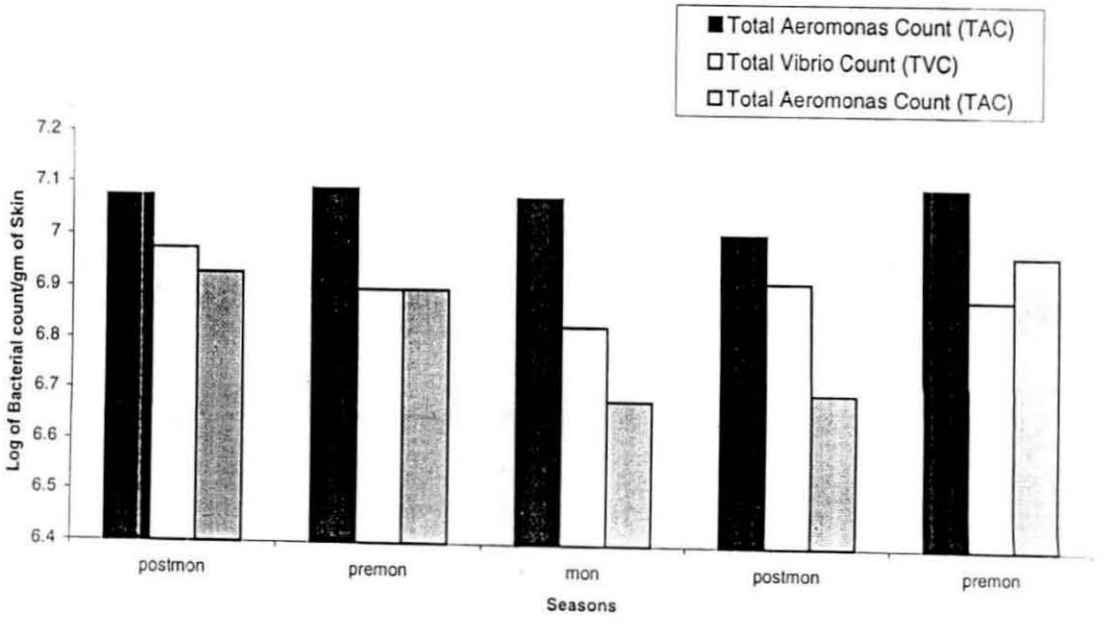

Fig.9 Bacterial profile of skin of *O. mossambicus* at Station II

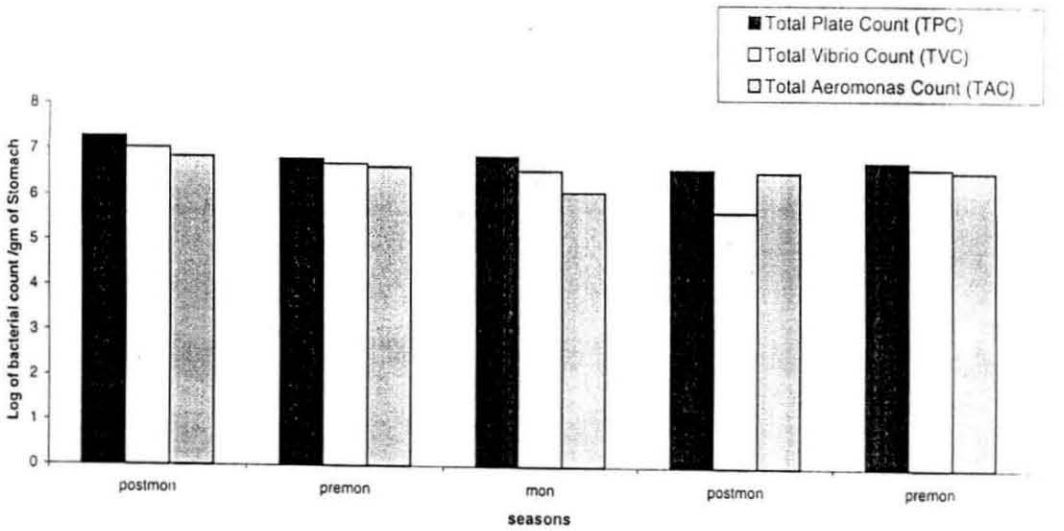

Fig.10 Bacterial profile of stomach of *O. mossambicus* at Station II

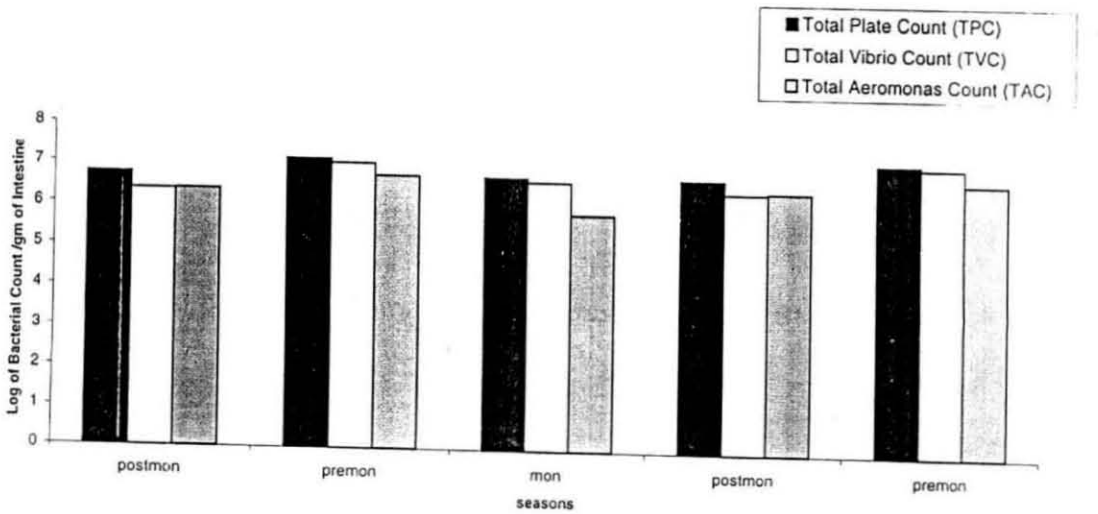

Fig.11 Bacterial profile of intestine of *O. mossambicus* at Station II

The maximum values of TPC, TVC and TAC from the intestine of *O.mossambicus* collected from Station I recorded maximum values during the pre monsoon season of 1999 (129.21 $x10^5$/gm, 77.74 $x10^5$/gm and 77.34 $x10^5$/gm). The minimum values of TPC and TAC occurred during the monsoon season (22.34 $x10^5$/gm and 2.21 $x10^5$/gm while the minimum TVC occurred during the post monsoon season of 1998-1999 (12.53 $x10^5$/gm) (Fig.6). The maximum values of TPC and TVC of the intestine of *O.mossambicus* from Station II were recorded during the pre monsoon season of 1999 (140.63 $x10^5$/gm and 118.38 $x10^5$/gm) while maximum value of TAC was noted during the pre monsoon season of 1998 (54.81 $x10^5$/gm). The minimum average value of TPC was recorded during the post-monsoon of 1998-1999 (51.22 x 10^5/gm) while that of TVC and TAC were recorded during the post monsoon of 1997-1998 (22.78 $x10^5$/gm and 23.78 $x10^5$/gm) (Fig.11).

4.3 Hydrobiological Parameters

At Station I (Valappu) temperature was found to be maximum in the month of April 1998 (35.7^{0}C) and the minimum was found to be 28^{0}C in the month of July 1998 (Fig.12). Salinity ranged from 1.66 ppt in the month of September 1998 to 20.77 ppt in March 1998 (Fig.13). Dissolved oxygen was found to be very high during June 1998 (9.75ml/l) and April 1999 (8.39ml/l) and lowest value was recorded during March 1999 (3.57ml/l) (Fig.14). pH was recorded maximum during June 1998 (9.3) and minimum during January 1998 (7.49) (Fig.15).

Temporal variations in nutrient values showed maximum nitrate values during March 1999 (5.7 μg at/l) and minimum during November 1998 (0.82μg at/l). Nitrite value was maximum during May 1998 (1.4μg at/l) and minimum during November 1998 (0.2μg at/l). In the case of ammonia maximum value was recorded during May 1998 (12.1μg at/l) and minimum value was recorded during February 1998 (2.6μg at/l). Phosphate was maximum during May1998 (7.5μg at/l) and minimum during January 1998 and January and March 1999 (1.1μg at/l) (Fig.16). Organic carbon was recorded maximum during November 1998 (1.82mg/g) and minimum during April 1999 (0.707mg/g) (Fig.17).

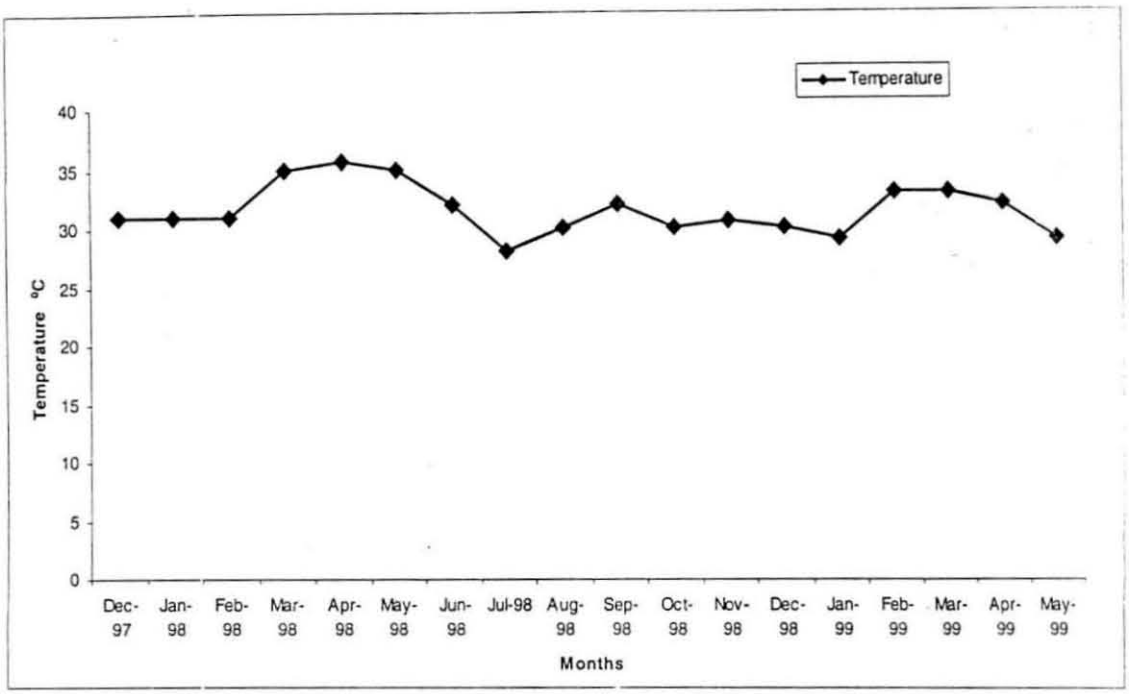

Fig.12 Temporal variations in temperature at Station I

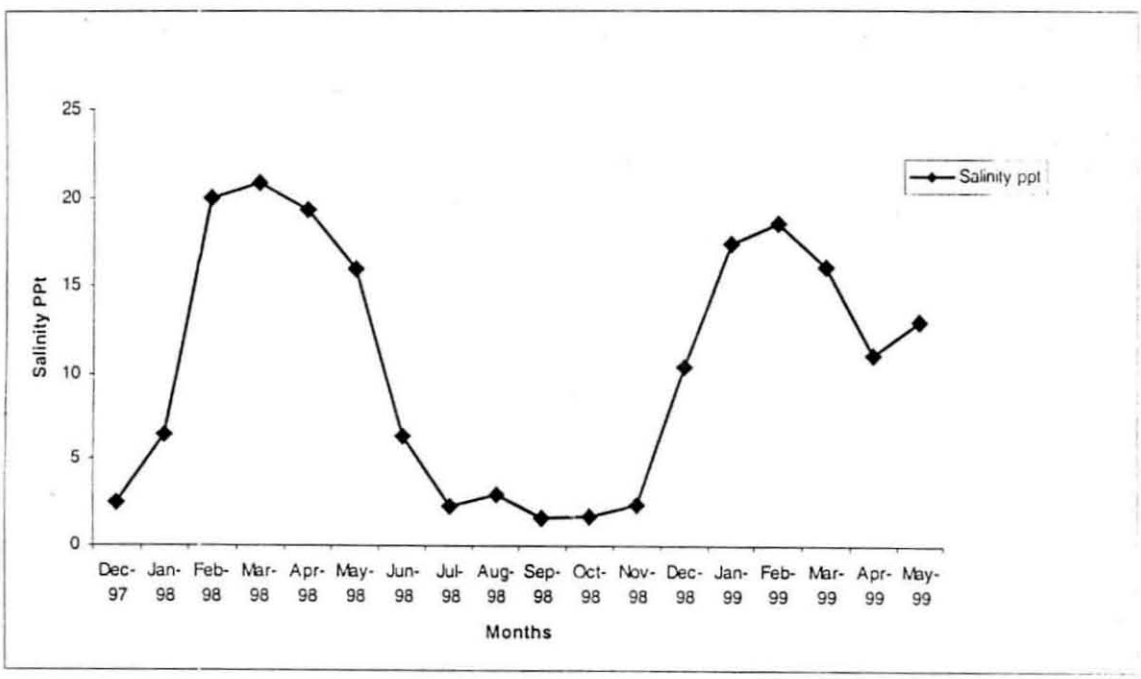

Fig.13 Temporal variations in salinity at Station I

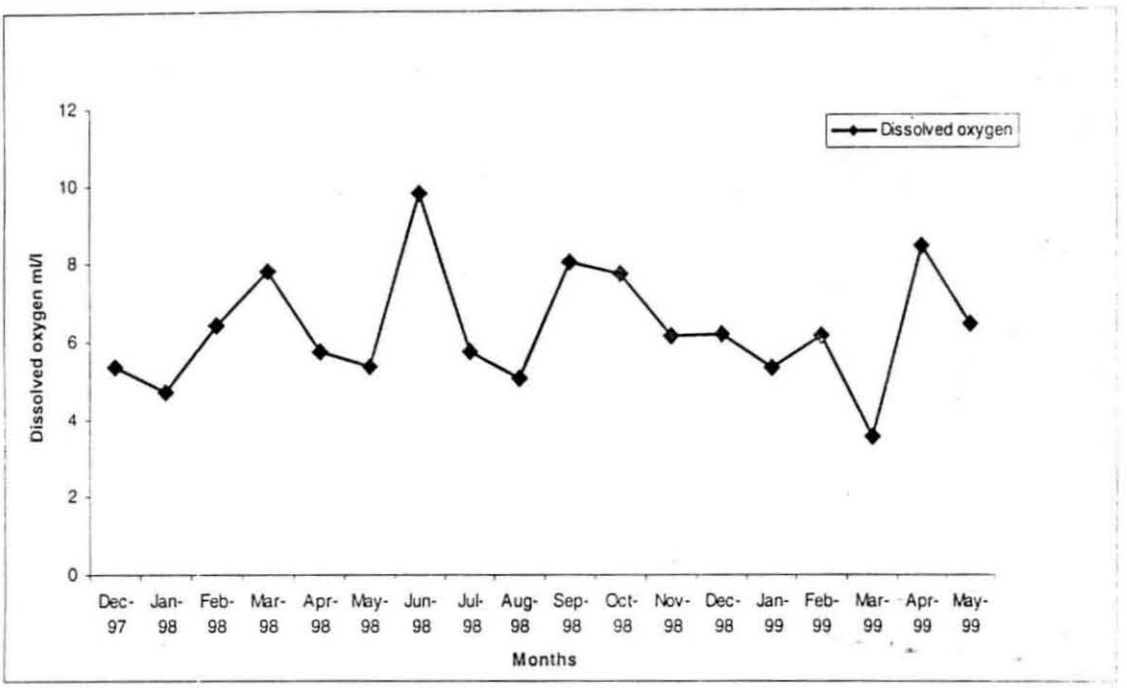

Fig.14 Temporal variations in dissolved oxygen at Station I

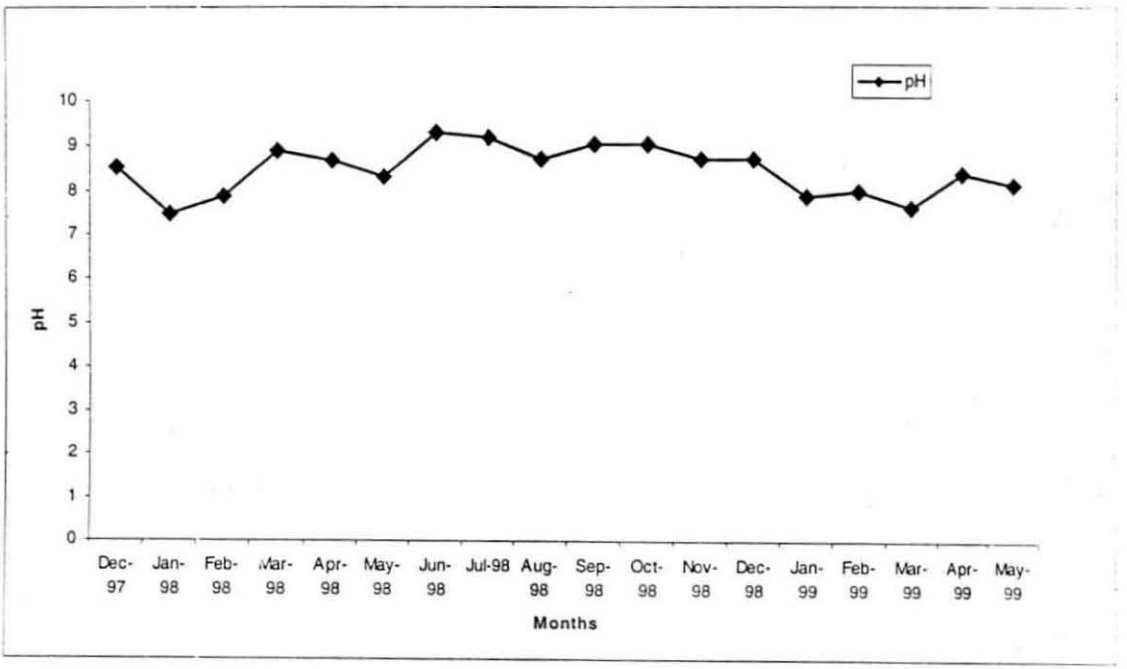

Fig.15 Temporal variations in pH at Station I

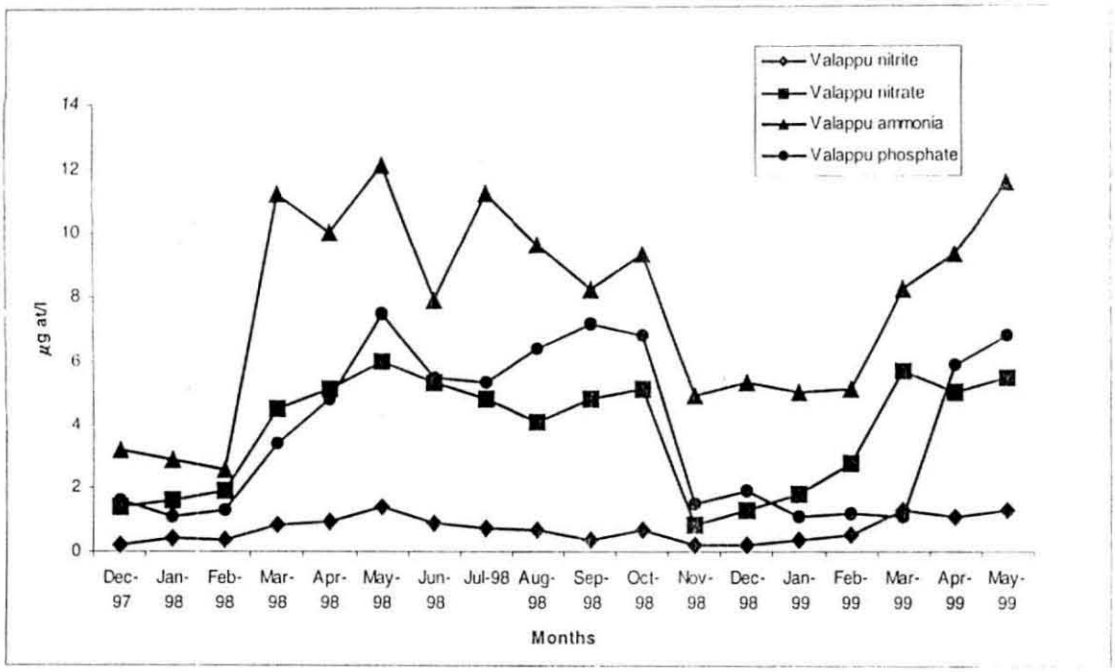

Fig.16 Temporal variations in nutrients at Station I

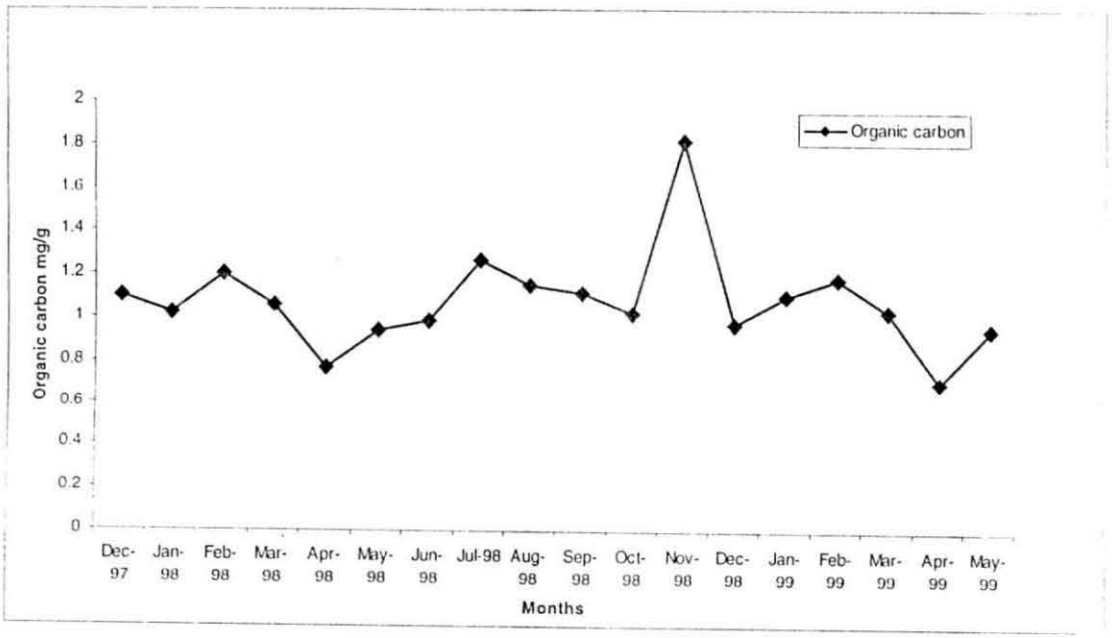

Fig.17 Temporal variations in organic carbon at Station I

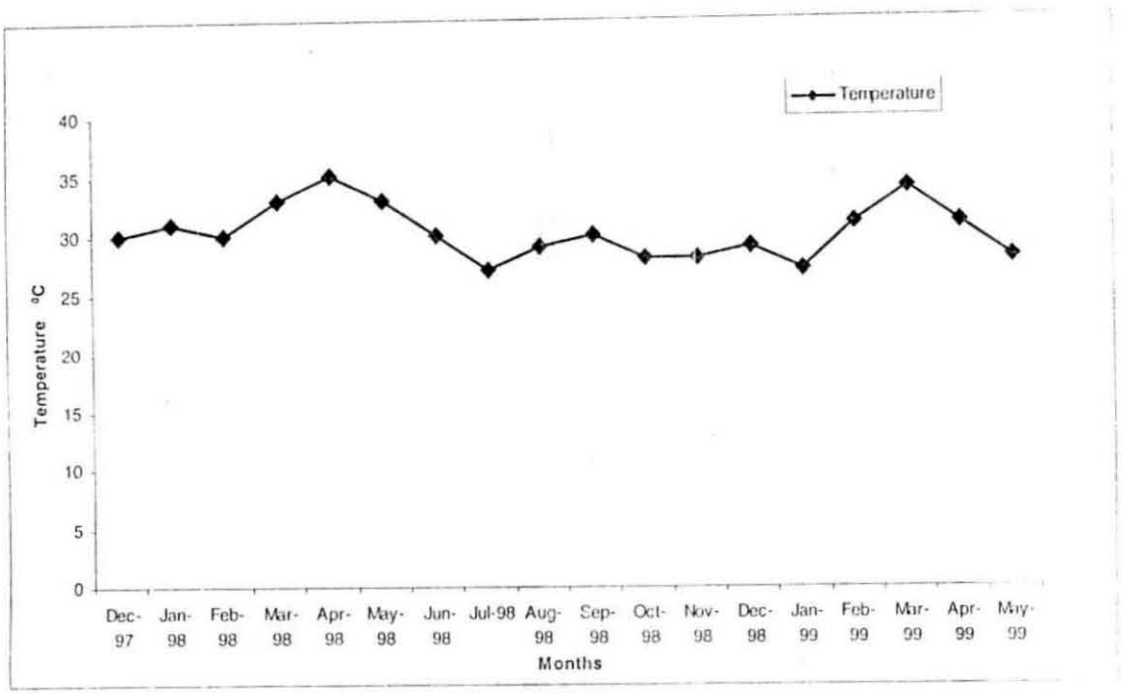

Fig.18 Temporal variations in temperature at Station II

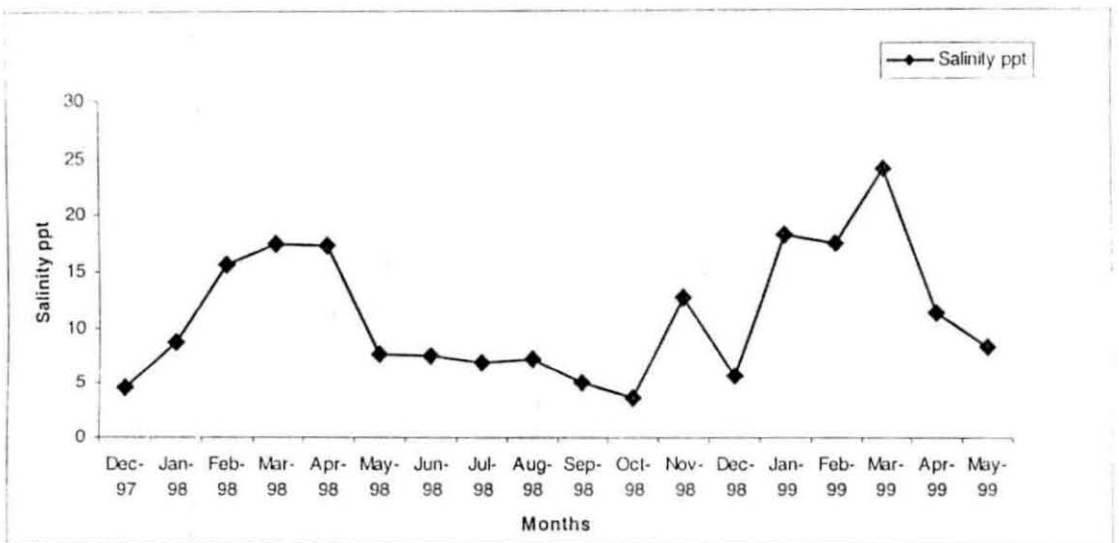

Fig.19 Temporal variations in salinity at Station II

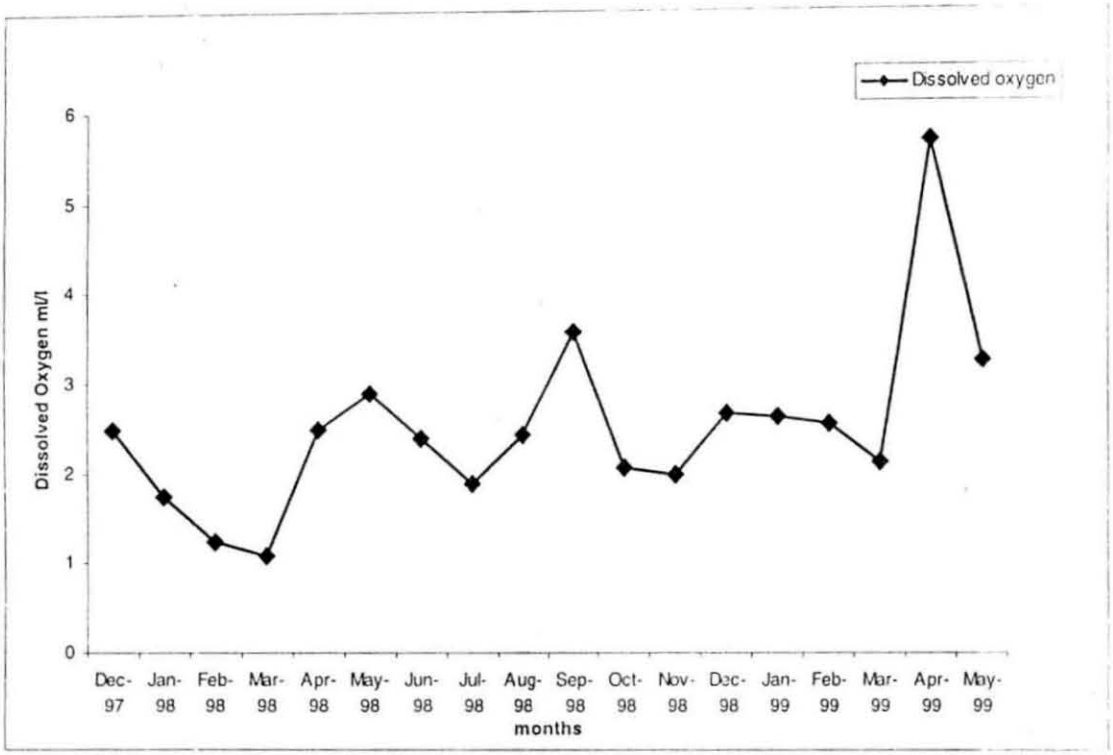

Fig.20 Temporal variations in dissolved oxygen at Station II

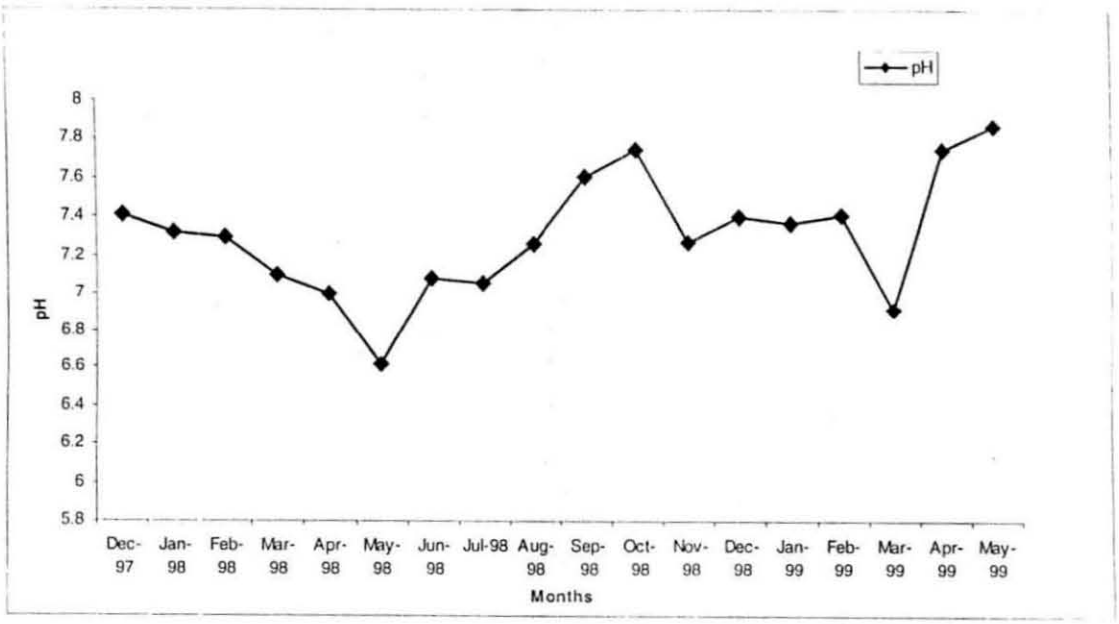

Fig.21 Temporal variations in pH at Station II

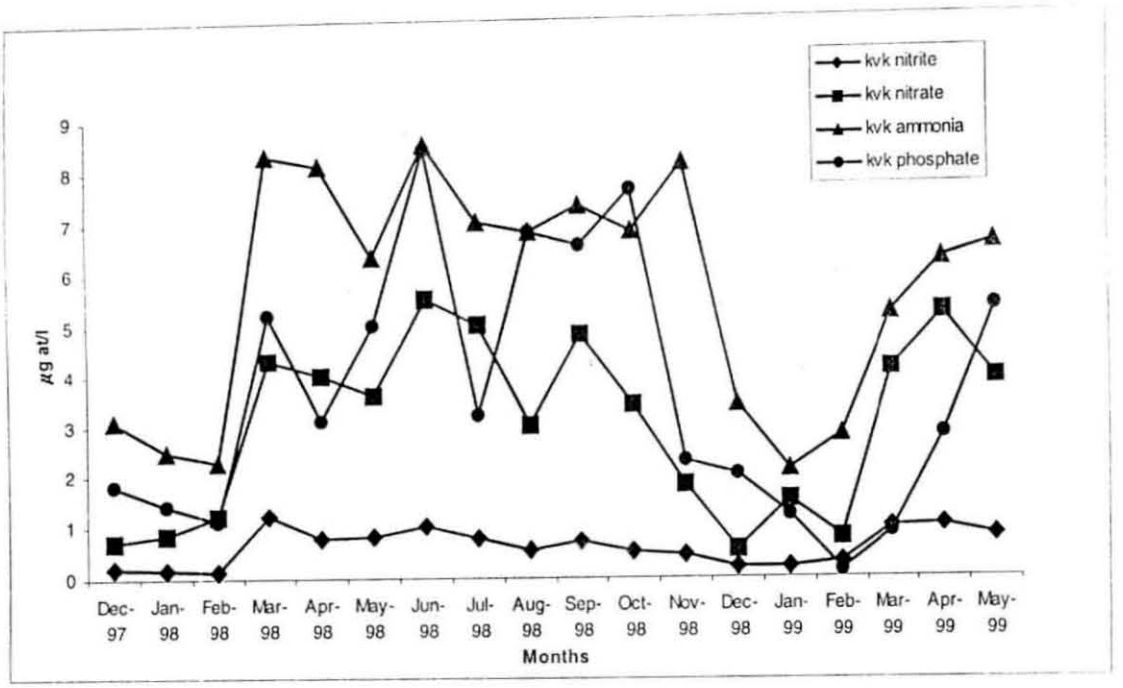

Fig.22 Temporal variations in nutrients at Station II

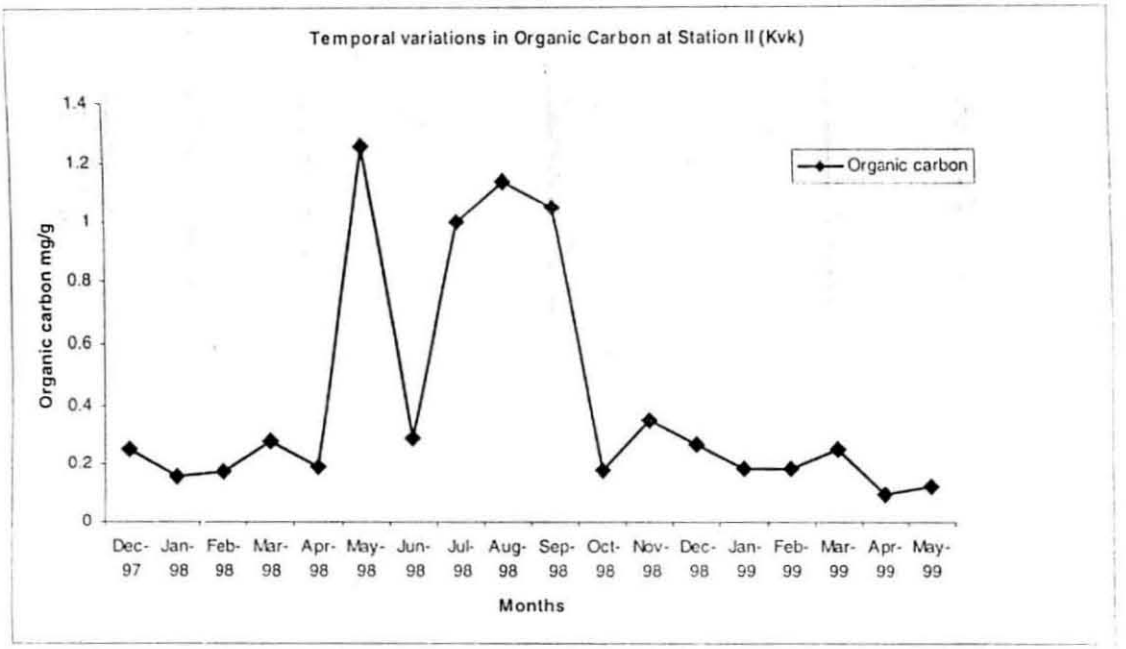

Fig.23 Temporal variations in organic carbon at Station II

In Station II (KVK) temperature was found to be maximum during the month of April 1998 (35.1^{0}C) and the minimum was recorded during July 1998 and January 1999 (27^{0}C) (Fig.18). Salinity recorded maximum values during March 1999 (24 ppt) and the minimum during October 1998 (3.6ppt) (fig.19). Dissolved oxygen was found to be maximum during April 1999 (5.67ml/l) and the minimum being recorded during March 1998 (1.08ml/l) (Fig.20). pH value was maximum during May 1999 (7.87) and minimum was observed during May 1998(6.62) (Fig.21).

The nitrate values at KVK ranged between 5.5μg at/l in the month of June 1998 to 0.53μg at/l in the month of December 1998. In the case of nitrite maximum values were recorded during March 1998 (1.2 μg at/l) and minimum during February 1998 (0.15μg at/l). Ammonia was recorded maximum during June 1998 (8.5μg at/l) and minimum during January 1999 (2.1μg at/l). Phosphate was found to be maximum during June 1998 (8.4μgat/l) and minimum during February1999 (0.13μg at/l) (Fig.22).

Organic carbon was found to be maximum during May 1998 (1.25mg/g) and minimum during April 1999 (.092mg/g) (Fig.23).

To study the relationship between TPC, TVC and TAC on each of the 9 variables at the 2 stations Valappu and KVK, multiple regression analysis was employed. The general multiple regression model used for the study was $Y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_4 x_4 + b_5 x_5 + b_6 x_6 + b_7 x_7 + b_8 x_8 + b_9 x_9$

Where Y is the bacterial count and x_1= temperature, x_2=pH, x_3=salinity, x_4= dissolved oxygen, x_5=nitrite, x_6=nitrate, x_7= ammonia x_8=phosphate and x_9=organic carbon.

The fitted regressions and the variability explained by the regression are presented in the following tables. The significance of the regression co-efficients were also tested using the 't' statistic. The matrix of correlation were formed to find the amount of interdependency of the variable cited above.

a = ($p<0.05$), b = ($p<0.01$), c = ($p<0.001$).

Table 1

ANOVA Table showing the significance of the fitted multiple regression of TPC of skin of *O.mossambicus* on the environmental parameters at Station I

Regression Statistics					
Multiple R				0.905067	
R Square				0.819146	
Adjusted R Square				0.615686	
Standard Error				0.300407	
Observations				18	
ANOVA					
	df	*SS*	*MS*	*F*	*Significance F*
Regression	9	3.269976503	0.363331	4.026076	0.031289075
Residual	8	0.72195495	0.090244		
Total	17	3.991931453			

Table 1 gives the multiple regression of total count of the skin of *O.mossambicus* on the 9 variables. For the purpose of analysis the bacterial counts and the parametric values were converted to their logarithmic values. The fitted regression for Station I Valappu is $Y = 9.2479\ x - 2.4919\ x_1 + 2.7447\ x_2 + 0.3285\ x_3 + .14013\ x_4 + 2.4156 x_5 - .62013 x_6 - .6678 x_7 - .3374 x_8 + .2330 x_9$.

The fitted regression explains 82% of the variability in the data. The ANOVA Table, showed that the fitted regression was significant ($P<.05$) indicating that the fitted regression is a good fit for the data.

Table 2

Matrix of correlation between TPC of skin of *O.mossambicus* and the environmental parameters studied from Station I

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	0.267841	1								
Column 3	-0.40208	-0.04205	1							
Column 4	b 0.647833	0.477579	-0.55237	1						
Column 5	-0.15692	0.049923	0.639921	-0.16118	1					
Column 6	c 0.740767	0.364579	-0.0321	0.377438	0.023215	1				
Column 7	a 0.477002	0.329512	0.251573	0.121023	0.209368	0.895392	1			
Column 8	0.293878	0.211529	0.485209	-0.00265	0.221444	0.747428	0.822616	1		
Column 9	0.023414	0.075329	0.664754	-0.29731	0.512442	0.537052	0.725038	0.797955	1	
Column 10	-0.40883	-0.37967	0.070087	-0.42273	-0.16569	-0.56791	-0.55172	-0.35041	-0.38391	1

Matrix of correlation between TPC of skin of *O.mossambicus* and the environmental parameters studied from Station I showed that the total bacterial count of skin of *O.mossambicus* was significantly positively correlated with salinity ($P<0.01$), with nitrite ($P<0.001$) and with nitrate ($P<.05$).

The multiple regression analysis of the total count of stomach of *O.mossambicus* with the 9 environmental parameters from Station I explained only 28.5% of the variability and was not significant at 5% level, indicating that the total bacterial count of stomach was not significantly influenced by these parameters.

The matrix of correlation between TPC of stomach of *O.mossambicus* and the environmental parameters at Station I showed that there was no significant correlation with any of the parameters.

The multiple regression analysis of the total count of intestine of *O.mossambicus* and the environmental parameters from Station I explained only 52.5% of the variability, which was not significant at 5% level. Therefore, the 9 parameters had little effect on total bacterial count in the intestine.

Table 3

Matrix of correlation between TPC of intestine of *O.mossambicus* and the environmental parameters studied from Station I

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	0.442196	1								
Column 3	-0.47681[a]	-0.04205	1							
Column 4	0.450697	0.477579	-0.55237	1						
Column 5	-0.14169	0.049923	0.639921	-0.16118	1					
Column 6	0.245445	0.364579	-0.0321	0.377438	0.023215	1				
Column 7	0.10497	0.329512	0.251573	0.121023	0.209368	0.895392	1			
Column 8	-0.11272	0.211529	0.485209	-0.00265	0.221444	0.747428	0.822616	1		
Column 9	-0.30715	0.075329	0.664754	-0.29731	0.512442	0.537052	0.725038	0.797955	1	
Column10	-0.20584	-0.37967	0.070087	-0.42273	-0.16569	-0.56791	-0.55172	-0.35041	-0.38391	1

Matrix of correlation between TPC of intestine of *O.mossambicus* and the environmental parameters studied from Station I showed that the total bacterial count in intestine was significantly negatively correlated with pH only ($P<0.05$) indicating that as pH increases the heterotrophic bacterial count decreases.

Table 4

ANOVA table showing the significance of the fitted multiple regression of TPC of water on the environmental parameters at Station I

Regression Statistics					
Multiple R					0.904397601
R Square					0.81793502
Adjusted R Square					0.613111918
Standard Error					0.342919901
Observations					18
ANOVA					
	df	*SS*	*MS*	*F*	*Significance F*
Regression	9	4.226372305	0.469597	3.993373	0.032011311
Residual	8	0.940752467	0.117594		
Total	17	5.167124772			

The regression analysis of the total count of water is given in table 4. The fitted regression explains 82% of the variability which was significant at 5% level.

Table 5

Matrix of correlation between TPC of water and the environmental parameters studied from Station I

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	0.026298	1								
	b									
Column 3	-0.62348	-0.04205	1							
Column 4	0.329924	0.477579	-0.55237	1						
Column 5	-0.22317	0.049923	0.639921	-0.16118	1					
Column 6	-0.41976	0.364579	-0.0321	0.377438	0.023215	1				
	a									
Column 7	-0.53343	0.329512	0.251573	0.121023	0.209368	0.895392	1			
	b									
Column 8	-0.63897	0.211529	0.485209	-0.00265	0.221444	0.747428	0.822616	1		
	c									
Column 9	-0.7268	0.075329	0.664754	-0.29731	0.512442	0.537052	0.725038	0.797955	1	
Column10	-0.02678	-0.37967	0.070087	-0.42273	-0.16569	-0.56791	-0.55172	-0.35041	-0.38391	1

Matrix of correlation between TPC of water and the environmental parameters studied from Station I showed that the total bacterial count of water was significantly negatively correlated with pH and ammonia ($P<.01$), with nitrite ($p<.05$) and with phosphate ($P<.001$).

The regression analysis of the total bacterial count of sediments with each of the environmental parameters explained only 47.5% of the variability which was not significant at 5% level.

Table 6

Matrix of correlation between TPC of sediment and the environmental parameters studied from Station I

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	a 0.471228	1								
Column 3	-0.32368	-0.04205	1							
Column 4	a 0.531123	0.477579	-0.55237	1						
Column 5	-0.05771	0.049923	0.639921	-0.16118	1					
Column 6	0.023134	0.364579	-0.0321	0.377438	0.023215	1				
Column 7	-0.14941	0.329512	0.251573	0.121023	0.209368	0.895392	1			
Column 8	-0.17379	0.211529	0.485209	-0.00265	0.221444	0.747428	0.822616	1		
Column 9	-0.30597	0.075329	0.664754	-0.29731	0.512442	0.537052	0.725038	0.797955	1	
Column10	-0.22419	-0.37967	0.070087	-0.42273	-0.16569	-0.56791	-0.55172	-0.35041	-0.38391	1

Matrix of correlation between TPC of sediment and the environmental parameters studied from Station I showed that the total count in sediment was significantly correlated ($P<.05$) with temperature and pH only.

The fitted regression of total bacterial count on skin with each of the environmental parameters at Station II (KVK) explained only 70% of the variability and was not found to be significant at 5% level.

Table 7

Matrix of correlation between TPC of skin of *O.mossambicus* and the environmental parameters studied from Station II

	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7	Column 8	Column 9	Column 10
Column 1	1									
Column 2	0.156184	1								
Column 3	-0.12529	-0.51836	1							
Column 4	0.207164	0.4423	-0.3394	1						
Column 5	-0.25505	-0.06401	0.37153	-0.25254	1					
Column 6	0.400944	0.329422	-0.2176	0.079505	0.235054	1				
	a									
Column 7	0.541197	0.169609	-0.14722	0.025605	0.192588	0.9085	1			
Column 8	0.212292	0.156259	-0.14899	-0.18005	0.137971	0.882879	0.814207	1		
Column 9	0.11467	-0.12986	0.023129	-0.54837	0.102599	0.524494	0.635219	0.711665	1	
Column 10	-0.0385	-0.06479	-0.49157	-0.34151	-0.04326	0.252078	0.268819	0.371115	0.37174	1

Matrix of correlation between TPC of skin of *O.mossambicus* and the environmental parameters studied from Station II showed that the total count was significantly positively correlated ($P<.05$) with nitrate only.

The fitted regression analysis of the bacterial count on stomach of *O.mossambicus* with the 9 environmental parameters studied at KVK explained only 42.7% of the variability which was not significant at 5% level.

Matrix of correlation between TPC of stomach of *O.mossambicus* and the environmental parameters studied from Station II was not found to be significantly correlated with any of the parameters.

Table 8

ANOVA table showing the significance of the fitted multiple regression of TPC of intestine of *O.mossambicus* with the environmental parameters at Station II

Regression Statistics					
Multiple R		0.891701092			
R Square		0.795130837			
Adjusted R Square		0.564653029			
Standard Error		0.208913518			
Observations		18			
ANOVA					
	df	*SS*	*MS*	*F*	*Significance F*
Regression	9	1.35514284	0.150571	3.449924	0.047670883
Residual	8	0.349158863	0.043645		
Total	17	1.704301704			

The regression analysis of the total bacterial count in the intestine of *O.mossambicus* with the 9 environmental parameters explained 80% of the variability in the data which was significant at 5% level.

Table 9

Matrix of correlation between TPC of intestine of *O.mossambicus* and the environmental parameters studied from Station II

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	a 0.49832	1								
Column 3	-0.23208	-0.51836	1							
Column 4	b 0.628557	0.4423	-0.3394	1						
Column 5	-0.15166	-0.06401	0.37153	-0.25254	1					
Column 6	0.163371	0.329422	-0.2176	0.079505	0.235054	1				
Column 7	0.051026	0.169609	-0.14722	0.025605	0.192588	0.9085	1			
Column 8	-0.12685	0.156259	-0.14899	-0.18005	0.137971	0.882879	0.814207	1		
Column 9	-0.44308	-0.12986	0.023129	-0.54837	0.102599	0.524494	0.635219	0.711665	1	
Column 10	a -0.53054	-0.06479	-0.49157	-0.34151	-0.04326	0.252078	0.268819	0.371115	0.37174	1

Matrix of correlation between TPC of intestine of *O.mossambicus* and the environmental parameters studied from Station II showed that the total count was significantly positively correlated with temperature ($P<.05$) and with salinity ($P<.01$) and significantly negatively correlated with organic carbon ($P<0.05$).

Table 10

ANOVA table showing the significance of the fitted multiple regression of TPC of water and the environmental parameters studied from Station II

Regression Statistics					
Multiple R			0.925019		
R Square			0.855661		
Adjusted R Square			0.693279		
Standard Error			0.370818		
Observations			18		
ANOVA					
	df	*SS*	*MS*	*F*	*Significance F*
Regression	9	6.521209909	0.724579	5.269444	0.014247338
Residual	8	1.100046121	0.137506		
Total	17	7.62125603			

The regression of total count in water with each of the 9 environmental parameters at KVK is given in Table 10. The fitted regression explained 86% of the variability in the data which was found significant at 5% level.

Table 11

Matrix of correlation between TPC of water and the environmental parameters studied from Station II

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	0.108004	1								
Column 3	0.128994	-0.51836	1							
Column 4	0.409409	0.4423	-0.3394	1						
Column 5	-0.13186	-0.06401	0.37153	-0.25254	1					
Column 6	-0.67142 b	0.329422	-0.2176	0.079505	0.235054	1				
Column 7	-0.73201 c	0.169609	-0.14722	0.025605	0.192588	0.9085	1			
Column 8	-0.69432 b	0.156259	-0.14899	-0.18005	0.137971	0.882879	0.814207	1		
Column 9	-0.59707 b	-0.12986	0.023129	-0.54837	0.102599	0.524494	0.635219	0.711665	1	
Column 10	-0.48372 a	-0.06479	-0.49157	-0.34151	-0.04326	0.252078	0.268819	0.371115	0.37174	1

Matrix of correlation between TPC of water and the environmental parameters studied from Station II showed that there was significant negative correlation of total count with nitrite ($P<0.01$), with nitrate ($P<0.001$), with ammonia ($P<0.01$), with phosphate ($P<0.01$) and with organic carbon ($P<0.05$).

The multiple regression of total count in sediments on each of the 9 environmental parameters at Station II explained only 47.4% of the variability which was not significant at 5% level.

The correlation between TPC of sediment and the environmental parameters from Station II showed that there was no significant correlation with any of the parameters.

The regression of *Vibrio* count on skin of *O.mossambicus* with each of the 9 environmental parameters at Station I (Valappu) though explained 71% of the variability in the data was not statistically significant.

Table 12

Matrix of correlation between TVC of skin of *O.mossambicus* and the environmental parameters studied from Station I

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	0.234055	1								
Column 3	-0.44272 b	-0.04205	1							
Column 4	0.631381	0.477579	-0.55237	1						
Column 5	-0.00297 a	0.049923	0.639921	-0.16118	1					
Column 6	0.526616	0.364579	-0.0321	0.377438	0.023215	1				
Column 7	0.335432	0.329512	0.251573	0.121023	0.209368	0.895392	1			
Column 8	0.059821	0.211529	0.485209	-0.00265	0.221444	0.747428	0.822616	1		
Column 9	0.052515 a	0.075329	0.664754	-0.29731	0.512442	0.537052	0.725038	0.797955	1	
Column10	-0.52428	-0.37967	0.070087	-0.42273	-0.16569	-0.56791	-0.55172	-0.35041	-0.38391	1

Matrix of correlation between TVC of skin of *O.mossambicus* and the environmental parameters studied from Station I showed that the total *Vibrio* count was positively correlated with salinity (P<.01) and with nitrate (P<0.05). TVC showed negative correlation with organic carbon (P<.05).

The regression analysis of vibrios in stomach of *O.mossambicus* at Station I with the parameters explained 52% of the variability, which was not significant at 5% level.

Matrix of correlation of vibrios from stomach of *O.mossambicus* from Station I with the environmental parameters showed that the total *Vibrio* count was not significantly correlated with any of the parameters.

The regression of vibrios in intestine of *O.mossambicus* from Station I with the parameters explained only 57% of the variability in the data, which was not significant at 5% level.

Table 13

Matrix of correlation between TVC of intestine of *O.mossambicus* and the environmental parameters studied from Station I

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	0.447229	1								
Column 3	-0.51297[a]	-0.04205	1							
Column 4	0.572106[a]	0.477579	-0.55237	1						
Column 5	-0.23355	0.049923	0.639921	-0.16118	1					
Column 6	0.266004	0.364579	-0.0321	0.377438	0.023215	1				
Column 7	0.07521	0.329512	0.251573	0.121023	0.209368	0.895392	1			
Column 8	-0.12125	0.211529	0.485209	-0.00265	0.221444	0.747428	0.822616	1		
Column 9	-0.34341	0.075329	0.664754	-0.29731	0.512442	0.537052	0.725038	0.797955	1	
Column 10	-0.05684	-0.37967	0.070087	-0.42273	-0.16569	-0.56791	-0.55172	-0.35041	-0.38391	1

Matrix of correlation between TVC of the intestine of *O.mossambicus* and the environmental parameters studied from Station I showed that the total *Vibrio* count in the intestine was significantly negatively correlated with pH ($P<0.05$) and significantly positively correlated with salinity ($P<.05$).

The regression analysis of the total count of *Vibrio* on water with the parameters from Station I was done. Nearly 63% of the variability was explained by the regression which was not statistically significant at 5% level.

Table 14

Matrix of correlation between TVC of water and the environmental parameters studied from Station I

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	0.265373	1								
Column 3	-0.64503[b]	-0.04205	1							
Column 4	0.539387[a]	0.477579	-0.55237	1						
Column 5	-0.20899	0.049923	0.639921	-0.16118	1					
Column 6	0.082533	0.364579	-0.0321	0.377438	0.023215	1				
Column 7	-0.0836	0.329512	0.251573	0.121023	0.209368	0.895392	1			
Column 8	-0.31152	0.211529	0.485209	-0.00265	0.221444	0.747428	0.822616	1		
Column 9	-0.48233[a]	0.075329	0.664754	-0.29731	0.512442	0.537052	0.725038	0.797955	1	
Column10	-0.28416	-0.37967	0.070087	-0.42273	-0.16569	-0.56791	-0.55172	-0.35041	-0.38391	1

Matrix of correlation between TVC of water and the environmental parameters studied from Station I showed that the total *Vibrio* count in water was significantly negatively correlated with pH ($P<0.01$), and with phosphate ($P<0.05$). It was significantly positively correlated with salinity ($P<.05$).

The regression of vibrios on sediments with the 9 environmental parameters at Station I explained 74% of the variability but it was not significant at 5% level.

Table 15

Matrix of correlation between TVC of sediment and the environmental parameters studied from Station I

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	0.269037	1								
Column 3	-0.5347[a]	-0.04205	1							
Column 4	0.562503[a]	0.477579	-0.55237	1						
Column 5	-0.13601	0.049923	0.639921	-0.16118	1					
Column 6	0.201119	0.364579	-0.0321	0.377438	0.023215	1				
Column 7	-0.01889	0.329512	0.251573	0.121023	0.209368	0.895392	1			
Column 8	-0.15008	0.211529	0.485209	-0.00265	0.221444	0.747428	0.822616	1		
Column 9	-0.13224	0.075329	0.664754	-0.29731	0.512442	0.537052	0.725038	0.797955	1	
Column10	-0.07954	-0.37967	0.070087	-0.42273	-0.16569	-0.56791	-0.55172	-0.35041	-0.38391	1

Matrix of correlation between TVC in sediment and the environmental parameters studied from Station I showed that the total *Vibrio* count was significantly negatively correlated with pH ($P<.05$) and positively correlated with salinity ($P<.05$).

The fitted multiple regression of vibrios in skin from *O.mossambicus* on the various parameters at KVK explained 67% of the variability which was not significant at 5% level.

Table 16

Matrix of correlation between TVC of skin of *O.mossambicus* and the environmental parameters studied from Station II

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	0.146615	1								
Column 3	-0.03757	-0.51836	1							
Column 4	0.316293	0.4423	-0.3394	1						
Column 5	-0.11093	-0.06401	0.37153	-0.25254	1					
Column 6	0.335645	0.329422	-0.2176	0.079505	0.235054	1				
Column 7	0.538577[a]	0.169609	-0.14722	0.025605	0.192588	0.9085	1			
Column 8	0.201311	0.156259	-0.14899	-0.18005	0.137971	0.882879	0.814207	1		
Column 9	0.069752	-0.12986	0.023129	-0.54837	0.102599	0.524494	0.635219	0.711665	1	
Column 10	-0.07932	-0.06479	-0.49157	-0.34151	-0.04326	0.252078	0.268819	0.371115	0.37174	1

Matrix of correlation between TVC of skin of *O.mossambicus* and the environmental parameters studied from Station II showed that the total *Vibrio* count was significantly positively correlated only with nitrate ($P<0.05$).

The regression analysis of vibrios in stomach of *O.mossambicus* on the various parameters at Station II was carried out. It explained only 25% of the variability in the data.

The correlation studies showed that the total *Vibrio* count of stomach was not significantly correlated with the any of the environmental parameters studied.

Table 17

ANOVA table showing the significance of the fitted multiple regression of the TVC of intestine of *O.mossambicus* with the environmental parameters at Station II

Regression Statistics					
Multiple R			0.908114		
R Square			0.824671		
Adjusted R Square			0.627425		
Standard Error			0.341419		
Observations			18		
ANOVA					
	df	*SS*	*MS*	*F*	*Significance F*
Regression	9	4.386229801	0.487359	4.180937	0.0281303
Residual	8	0.932535138	0.116567		
Total	17	5.318764939			

The regression of vibrios of *O.mossambicus* in the intestine with the 9 parameters at Station II explained 82.5% of the variability which was significant ($P<.05$).

Table 18

Matrix of correlation between TVC of intestine of *O.mossambicus* with the environmental parameters studied from Station II

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	0.372955	1								
Column 3	-0.18378 b	-0.51836	1							
Column 4	0.594564	0.4423	-0.3394	1						
Column 5	-0.09506	-0.06401	0.37153	-0.25254	1					
Column 6	0.411832 a	0.329422	-0.2176	0.079505	0.235054	1				
Column 7	0.482517	0.169609	-0.14722	0.025605	0.192588	0.9085	1			
Column 8	0.160366	0.156259	-0.14899	-0.18005	0.137971	0.882879	0.814207	1		
Column 9	-0.19036	-0.12986	0.023129	-0.54837	0.102599	0.524494	0.635219	0.711665	1	
Column 10	-0.2967	-0.06479	-0.49157	-0.34151	-0.04326	0.252078	0.268819	0.371115	0.37174	1

Matrix of correlation between TVC of intestine of *O.mossambicus* with the 9 environmental parameters studied from Station II showed that the total *Vibrio* count was significantly positively correlated with salinity ($P<0.01$) and with nitrate ($P<0.05$).

Table 20

Matrix of correlation between TVC of water and the environmental parameters studied from Station II

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
	a									
Column 2	0.4882	1								
Column 3	-0.17517	-0.51836	1							
	c									
Column 4	0.781131	0.4423	-0.3394	1						
Column 5	-0.34187	-0.06401	0.37153	-0.25254	1					
Column 6	-0.26011	0.329422	-0.2176	0.079505	0.235054	1				
Column 7	-0.2275	0.169609	-0.14722	0.025605	0.192588	0.9085	1			
	a									
Column 8	-0.49324	0.156259	-0.14899	-0.18005	0.137971	0.882879	0.814207	1		
	b									
Column 9	-0.62937	-0.12986	0.023129	-0.54837	0.102599	0.524494	0.635219	0.711665	1	
Column 10	-0.38858	-0.06479	-0.49157	-0.34151	-0.04326	0.252078	0.268819	0.371115	0.37174	1

Matrix of correlation between TVC of water and the environmental parameters studied from Station II showed that the total *Vibrio* count was positively correlated with temperature (P<.05) and with salinity (P<0.001). It was significantly negatively correlated with ammonia (P<.05) and with phosphate (P<.01).

Table 21

ANOVA table showing the significance of the fitted multiple regression of TVC of sediment and the environmental parameters at Station II

Regression Statistics					
Multiple R			0.936212		
R Square			0.876493		
Adjusted R Square			0.737547		
Standard Error			0.201692		
Observations			18		
ANOVA					
	df	*SS*	*MS*	*F*	*Significance F*
Regression	9	2.30953025	0.256614	6.30816921	0.00814582
Residual	8	0.325437652	0.04068		
Total	17	2.634967902			

The regression of vibrios in sediment with the environmental parameters explained 88% of the variability which was found highly significant ($P<0.01$).

Matrix of correlation between TVC of sediment and the environmental parameters isolated from Station II showed that the *Vibrio* count was not significantly positively correlated with any of the parameters.

The regression of total aeromonad count of the skin of *O.mossambicus* on the 9 parameters at Valappu explained nearly 70% of the variability in the data but it was not statistically significant.

Table 24

Matrix of correlation between TAC of water and the environmental parameters studied from Station I

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	0.061023	1								
Column 3	-0.46192	-0.04205	1							
Column 4	0.265518	0.477579	-0.55237	1						
Column 5	-0.17963	0.049923	0.639921	-0.16118	1					
Column 6	-0.41127	0.364579	-0.0321	0.377438	0.023215	1				
Column 7	a -0.53008	0.329512	0.251573	0.121023	0.209368	0.895392	1			
Column 8	a -0.58149	0.211529	0.485209	-0.00265	0.221444	0.747428	0.822616	1		
Column 9	b -0.61426	0.075329	0.664754	-0.29731	0.512442	0.537052	0.725038	0.797955	1	
Column 10	0.004217	-0.37967	0.070087	-0.42273	-0.16569	-0.56791	-0.55172	-0.35041	-0.38391	1

Matrix of correlation between TAC of water and the environmental parameters studied from Station I showed that the total aeromonads was significantly negatively correlated with nitrate and ammonia ($P<0.05$) and with phosphate ($P<.01$).

The regression analysis of aeromonads in sediments on the 9 parameters at Station I explained 47.5% of the variability which was not significant at 5%.

The matrix of correlation of TAC was not found to be significantly correlated with any of the environmental parameters.

The regression analysis of aeromonads in skin of *O.mossambicus* on various environmental parameters at KVK explained 62.6% of the variability which was not statistically significant.

Table 25

Matrix of correlation between TAC of skin of *O.mossambicus* and the environmental parameters studied from Station II

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	a 0.517349	1								
Column 3	0.054609	-0.51836	1							
Column 4	0.358451	0.4423	-0.3394	1						
Column 5	-0.01823	-0.06401	0.37153	-0.25254	1					
Column 6	0.092085	0.329422	-0.2176	0.079505	0.235054	1				
Column 7	0.102868	0.169609	-0.14722	0.025605	0.192588	0.9085	1			
Column 8	-0.14054	0.156259	-0.14899	-0.18005	0.137971	0.882879	0.814207	1		
Column 9	-0.18111	-0.12986	0.023129	-0.54837	0.102599	0.524494	0.635219	0.711665	1	
Column 10	a -0.48979	-0.06479	-0.49157	-0.34151	-0.04326	0.252078	0.268819	0.371115	0.37174	1

Matrix of correlation between TAC of skin of *O.mossambicus* and the environmental parameters studied from Station II showed that the total aeromonad count was significantly correlated ($P<.05$) with temperature and significantly negatively correlated with ($P<.05$) organic carbon.

The regression analysis of aeromonads in stomach of *O.mossambicus* on the 9 parameters explained only 45.8% of the variability which was not statistically significant.

The matrix of correlation between TAC of stomach of *O.mossambicus* and the environmental parameters showed that there was no significant correlation with any of the parameters.

Table 26

ANOVA table showing the significance of the fitted multiple regression of TAC of intestine of *O.mossambicus* on the environmental parameters at Station II

Regression Statistics					
Multiple R			0.894768		
R Square			0.800609		
Adjusted R Square			0.576294		
Standard Error			0.311432		
Observations			18		
ANOVA					
	df	*SS*	*MS*	*F*	*Significance F*
Regression	9	3.115534754	0.346171	3.569129	0.043543251
Residual	8	0.775921584	0.09699		
Total	17	3.891456338			

The regression analysis of aeromonads in intestine of *O.mossambicus* with the 9 environmental parameters explained 80% of the variability which was statistically significant ($P<0.05$).

Table 27

Matrix of correlation between TAC of intestine of *O.mossambicus* and the environmental parameters studied from Station II

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	0.434852	1								
Column 3	-0.12384	-0.51836	1							
Column 4	b 0.629967	0.4423	-0.3394	1						
Column 5	-0.13824	-0.06401	0.37153	-0.25254	1					
Column 6	0.145849	0.329422	-0.2176	0.079505	0.235054	1				
Column 7	0.136383	0.169609	-0.14722	0.025605	0.192588	0.9085	1			
Column 8	-0.08666	0.156259	-0.14899	-0.18005	0.137971	0.882879	0.814207	1		
Column 9	-0.24277	-0.12986	0.023129	-0.54837	0.102599	0.524494	0.635219	0.711665	1	
Column 10	b -0.65019	-0.06479	-0.49157	-0.34151	-0.04326	0.252078	0.268819	0.371115	0.37174	1

Matrix of correlation between TAC of intestine and the environmental parameters studied from Station II showed that the total aeromonads were significantly positively correlated ($P<.01$) with salinity and significantly negatively correlated with organic carbon.

Table 28

ANOVA table showing the significance of the fitted multiple regression of TAC of water on the environmental parameters at Station II

Regression Statistics					
Multiple R	0.902132				
R Square	0.813842				
Adjusted R Square	0.604414				
Standard Error	0.410023				
Observations	18				
ANOVA					
	Df	*SS*	*MS*	*F*	*Significance F*
Regression	9	5.879838	0.653315	3.88603	0.034531
Residual	8	1.344952	0.168119		
Total	17	7.22479			

The regression analysis of aeromonads in water with the 9 environmental parameters explained 81.4% of the variability which was significant ($P<0.05$).

Table 29

Matrix of correlation between TAC of water and the environmental parameters studied from Station II

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	0.269976	1								
Column 3	-0.05585	-0.51836	1							
Column 4	0.437363	0.4423	-0.3394	1						
Column 5	-0.33668	-0.06401	0.37153	-0.25254	1					
Column 6	a -0.58149	0.329422	-0.2176	0.079505	0.235054	1				
Column 7	b -0.68452	0.169609	-0.14722	0.025605	0.192588	0.9085	1			
Column 8	a -0.57251	0.156259	-0.14899	-0.18005	0.137971	0.882879	0.814207	1		
Column 9	a -0.57757	-0.12986	0.023129	-0.54837	0.102599	0.524494	0.635219	0.711665	1	
Column 10	-0.33061	-0.06479	-0.49157	-0.34151	-0.04326	0.252078	0.268819	0.371115	0.37174	1

Matrix of correlation between TAC of water and the environmental parameters studied from Station II showed that the total aeromonad count was significantly negatively correlated with nitrite, ammonia and phosphate ($P<.05$)and with nitrate ($P<.01$).

The regression analysis of aeromonads in sediment on the 9 parameters at Station II explained 71.3% of the variability which was not statistically significant.

Table 30

Matrix of correlation between TAC of sediment and the environmental parameters studied from Station II

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	0.308049	1								
Column 3	0.261879	-0.51836	1							
Column 4	0.372456	0.4423	-0.3394	1						
Column 5	-0.14347	-0.06401	0.37153	-0.25254	1					
Column 6	-0.39621	0.329422	-0.2176	0.079505	0.235054	1				
Column 7	-0.46443	0.169609	-0.14722	0.025605	0.192588	0.9085	1			
Column 8	-0.45876	0.156259	-0.14899	-0.18005	0.137971	0.882879	0.814207	1		
Column 9	a -0.52009	-0.12986	0.023129	-0.54837	0.102599	0.524494	0.635219	0.711665	1	
Column 10	b -0.62726	-0.06479	-0.49157	-0.34151	-0.04326	0.252078	0.268819	0.371115	0.37174	1

Matrix of correlation between TAC of sediment and the environmental parameters studied from Station II showed that the total aeromonad count was significantly negatively correlated with phosphate ($P<.05$) and with organic carbon ($P<.01$).

The *Vibrio*. spp isolated during the period of study were *V. anguillarum*, *V. parahaemolyticus*, *V. fischeri*, *V. furnissii*, *V. fluvialis*, *V. metschnikovii*, *V. campbellii*, *V. cincinnatiensis*, *V. harveyi* and *V. vulnificus*.

TABLE 31

BIO-CHEMICAL PROFILE OF VIBRIO SPECIES ISOLATED FROM STATION I (VALAPPU)

Biochemical Tests	Reactions of *V. anguillarum* (n=24)	%	Reactions of *V.parahaemolyticus* (n=17)	%	Reactions of *V fischeri* (n=11)	%	Reactions of *V.furnissii* n=8	%	Reactions of *V.fluvialis* (n=8)	%
Gram negative	-	100	-	100	-	100	-	100	-	100
Motility	+	100	+	100	+	100	+	100	+	100
Arginine Dihydrolase	+	100	-	100	-	100	+	100	+	100
Lysine Decarboxylase	-	100	+	100	+	100	-	100	-	100
Ornithine decarboxylase	-	100	+	100	(-)	100	-	100	-	100
Nitrate	+	100	+	76.47	+	81.82	+	75	+	100
Ammonia	+	100	+	100	+	100	+	87.5	+	100
Oxidase	+	100	+	70.58	+	72.73	+	100	+	100
Catalase	+	25	+	41.18	+	45.45	+	25	+	0
Acid from Lactose	-	100	-	100	-	100	-	100	-	100
Acid from Maltose	+	100	+	82.35	+	81.82	+	87.5	+	87.5
Acid from Mannitol	+	95.83	(+)	70.59	v	63.64	+	100	+	100
Acid from Sucrose	+	95.83	-	35.29	(-)	36.36	+	87.5	+	100
Acid from Glucose	+	100	+	100	v	100	+	100	+	100
Growth at 0% NaCl	-	58.33	-	70.59	-	72.73	d	100	v	87.5
Growth at 6% NaCl	d	20.83	+	82.34	d	100	+	75	+	100

Growth at 8% NaCl	-	37.5	+	58.82	-	90.91	+	87.5	(+)	87.5
Growth at 4^0C	d	95.83	-	100	(-)	90.91	-	100	-	100
Growth at 40^0C	-	58.33	+	82.34	-	100	+	87.5	+	87.5
Indole	+	0	+	0	-	100	(-)	100	(+)	12.5
Vogesproskauer	+	12.5	+	0	-	90.91	-	100	-	100
Citrate	+	33.33	+	35.29	v	81.82	+	62.5	+	12.5
Starch	+	100	+	64.71	-	45.45	+	62.5	+	100
Gelatinase	+	75	+	64.71	-	100	(+)	50	+	100
Caesin	+	62.5	v	58.82	+	81.82	+	50	+	75
Urease	(-)	91.67	-	94.12	+	54.55	-	100	-	87.5
Aesculin Hydrolysis	(-)	95.83	-	94.12	-	81.82	-	100	v	75
H_2S	-	20.83	-	47.05	-	45.45	-	37.5	-	0
Gas from Glucose	-	100	-	94.12	-	81.82	+	62.5	-	100
Resistance To 0/129	-	100	-	100	-	100	-	100	-	100

Biochemical Tests	Reactions of *V. harveyi* (n=5)	%	Reactions of *V.campbellii* (n=5)	%	Reactions of *V. metschnikovii* (n=5)	%	Reactions of *v. cincinnatiensis* (n=4)	%	Reactiions of *V.vulnificus* (n=1)	%
Gram Negative	-	100	-	100	-	100	-	100	-	100
Motility	+	100	+	100	+	100	+	100	+	100
Arginine Dihydrolase	-	100	-	100	d	100	-	100	-	100
Lysine Decarboxylase	+	100	d	100	v	100	(+)	100	+	100
Ornithine Decarboxylase	+	100	-	100	-	100	-	100	+	100
Nitrate	+	60	+	100	-	80	+	100	+	100
Ammonia	+	100	+	100	+	80	+	100	+	100
Oxidase	+	100	+	80	-	100	+	100	+	100
Catalase	+	40	+	0	+	80	+	50	+	0
Acid from Lactose	-	100	-	100	v	100	-	100	v	100
Acid from Maltose	+	100	+	40	+	80	+	100	+	100
Acid from Mannitol	+	100	v	100	(+)	60	+	100	(+)	100
Acid from Sucrose	v	100	-	80	+	80	+	100	(-)	0
Acid from Glucose	+	100	+	100	+	80	+	100	nd	100
Growth at 0% NaCl	-	80	-	60	d	80	-	25	-	100
Growth at 6% NaCl	(+)	80	d	80	(+)	80	+	75	d	100
Growth at 8% NaCl	(+)	100	v	60	(+)	80	+	75	-	0

Growth at 4^0C	-	100	-	100	d	80	-	100	-	100
Growth at 40^0C	v	80	-	80	+	100	nd	75	+	100
Indole	+	0	(+)	0	(-)	80	-	100	+	0
Vogesproskauer	-	100	-	100	(+)	40	d	50	-	100
Citrate	v	100	v	100	v	100	+	50	+	0
Starch	+	60	+	60	+	40	+	50	+	100
Gelatinase	+	60	+	100	+	40	-	100	+	100
Caesin	+	20	+	80	+	20	+	0	+	0
Urease	v	100	-	80	-	60	-	100	-	100
Aesculin Hydrolysis	(-)	80	v	60	v	60	nd	100	-	100
H_2S	-	20	-	40	-	80	nd	100	-	0
Gas from glucose	-	80	-	100	-	100	-	100	-	100
Resistance to 0/129	(-)	100	-	100	-	100	-	100	-	100

4.4 Bio-chemical reactions of *Vibrio* species isolated from station I (Valappu).

All the *Vibrio* strains isolated were actively motile gram negative rods. Except for 100% of *V.anguillarum*, *V.fluvialis* and *V.furnissii*, rest of the *Vibrio* strains isolated from Valappu could not decarboxylate arginine. 100% of *V.vulnificus*, *V.harveyi*, *V.campbellii*, *V.parahaemolyticus*, *V.fischeri*, *V.cincinnatiensis* and *V.metschnikovii* decarboxylated lysine while none of the strains of *V.anguillarum*, *V.fluvialis* and *V.furnissii* could utilize the amino acid. Except *V.vulnificus*, *V. parahaemolyticus* and *V.harveyi* rest of the strains could not metabolize ornithine.

Nitrate was actively reduced to nitrite by 100% of *V.anguillarum*, *V.vulnificus*, *V.campbellii*, *V.fluvialis* and *V.cincinnatiensis* followed by 81.82% of *V.fischeri*, 76.47% of *V.parahaemolyticus*, 75% of *V.furnissii*, 60% of *V.harveyi* and 20% of *V.metschnikovii*. All the *Vibrio* strains isolated from Valappu were found to produce ammonia except for 20% of *V.metschnikovii* and 12.5% of *V.furnissii*. Except for 100% of *V.metschnikovii*, 20% of *V.campbellii*, 27.27% of *V.fischeri* and 29.42% of *V.parahaemolyticus* all the vibrios isolated from Valappu produced cytochrome oxidase. Except 75% of *V.anguillarum*, 60% of *V.harveyi*, 58.82% of *V.parahaemolyticus*, 54.55% of *V.fischeri*, 50% of *V. cincinnatiensis*, 75% of *V.furnissii* and 20% of *V.metschnikovii* produced the enzyme catalase whereas none of the strains of *V. vulnificus*, *V.campbellii* and *V.fluvialis* could produce the enzyme.

Fermentation of carbohydrates

100% of *V. metschnikovii* and *V. vulnificus* fermented lactose, while lactose fermenting potential was absent in the rest of the strains isolated. Except 60% of *V.campbellii*, 17.65% of *V.parahaemolyticus*, 12.5% of *V.fluvialis*, 18.18% of *V. fischeri*, 20% of *V.metschnikovii* and 12.5% of *V.furnissii*, rest of the strains were found to be actively fermenting maltose. Except 4.17% of *V.anguillarum*, 100% of *V.campbellii*, 29.41% of *V. parahaemolyticus*, 36.36% of *V.fischeri* and 40% of *V.metschnikovii*, rest of the *Vibrio* strains isolated were found to be actively metabolising mannitol. 100% of *V.harveyi*, *V.fluvialis* and *V.cincinnatiensis* were able to ferment sucrose while 4.17% of *V.anguillarum*, 100% of *V.vulnificus*, 80% of

V.campbellii, 35.29% of *V.parahaemolyticus*, 36.36% of *V.fischeri*, 20% of *V.metschnikovii* and 12.5% of *V.furnissii* could not ferment the carbohydrate. Except 20% of *V.metschnikovii* rest of the *Vibrio* strains were found to ferment glucose actively.

Salinity Tolerance

41.67% of *V.anguillarum*, 20% of *V.harveyi*, 40% of *V.campbellii*, *29.41% of V.parahaemolyticus*, 87.5% of *V.fluvialis*, 80% of *V.metschnikovii*, 27.27% of *V.fischeri*, 75% of *V.cincinnatiensis* and 100% of *V.furnissii* could grow well in the absence of sodium chloride. 100% of *V.vulnificus*, *V.fluvialis* and *V. fischeri* could grow well in the presence of 6% sodium chloride followed by 80% of *V.harveyi*, *V.campbellii and V.metschnikovii*, 82.34% of *V.parahaemolyticus*, 75% of *V.cincinnatiensis* and *V.furnissii* and 79.17% of *V.anguillarum*. 100% of *V.harveyi* and 60% of *V.campbellii*, 58.82% of *V.parahaemolyticus*, 87.5% of *V.fluvialis* and *V.furnissii*, 75% of *V.cincinnatiensis* and 80% of *V.metschnikovii gave* good growth at 8% sodium chloride, whereas 37.5% of *V.anguillarum and* 90.91% of *V.fischeri* could not grow well at 8% sodium chloride.

Temperature Tolerance

Except for 4.17% of *V.anguillarum*, 9.09% of *V.fischeri* and 20% of *V.metschnikovii* none of the *Vibrio* strains isolated could tolerate 4^0C. 100% of *V.vulnificus*, and *V.metschnikovii*, 41.67% of *V.anguillarum*, 80% of *V.harveyi*, 20% of *V.campbellii*, 82.34% of *V.parahaemolyticus*, 87.5% of *V.fluvialis* and *V.furnissii* and 75% of *V.cincinnatiensis* grew well at 40^0C, whereas none of *V.fischeri* could grow at 40^0C. Except for 12.5% of *V.fluvialis* and 20% of *V.metschnikovii* rest of the vibrios failed to produce indole. Except 12.5% of *V.anguillarum*, 9.09% of *V.fischeri*, 50% of *V.cincinnatiensis* and 40% of *V.metschnikovii* rest of the *Vibrio* strains failed to produce acetyl methyl carbinol. 33.33% of *V.anguillarum*, 35.29% of *V.parahaemolyticus*, 12.5% of *V.fluvialis*, 18.18% of *V.fischeri*, 50% of *V.cincinnatiensis* and 62.5% of *V.furnissii* could utilize citrate as the sole source of carbon. None of the strains of *V.vulnificus*, *V.campbellii*, *V.harveyi* and *V.metschnikovii* could utilize citrate.

100% of *V.anguillarum*, *V.vulnificus* and *V.fluvialis* hydrolysed starch followed by 64.71% of *V.parahaemolyticus*, 62.5% of *V.furnissii*, 60% of *V.harveyi* and *V.campbellii*, 50% of *V.cincinnatiensis*, 54.55% *V.fischeri* and 40% *V.metschnikovii*. 100% of *V.vulnificus*, *V.campbellii* and *V.fluvialis* could liquefy gelatin followed by 75% of *V.anguillarum*, 60% of *V.harveyi*, 64.71% of *V.parahaemolyticus* 50% of *V.furnissii* and 40% of *V.metschnikovii*. All the strains of *V.fischeri* and *V.cincinnatiensis* isolated from Valappu failed to liquefy gelatin. 62.5% of *V.anguillarum*, 80% of *V.campbellii*, 58.82% of *V.parahaemolyticus*, 75% of *V.fluvialis* and 81.82% of *V.fischeri* hydrolyzed casein, while all the strains of *V.vulnificus and V.cincinnatiensis*, 80% of *V.harveyi*, *V.metschnikovii* and 50% of *V.furnissii* were unable to utilize casein. Except for 8.33% of *V.anguillarum*, 20% of *V.campbellii*, 5.88% of *V.parahaemolyticus*, 12.5% of *V.fluvialis*, 54.55% of *V.fischeri* and 40% of *V.metschnikovii* rest of the *Vibrio* strains failed to hydrolyze urea. None of the strains of *V.vulnificus*, *V.cincinnatiensis* and *V.furnissii* could lead to the hydrolysis of aesculin. 4.17% of *V.anguillarum*, 20% of *V.harveyi*, 40% of *V.campbellii* and *V.metschnikovii*, 5.88% of *V.parahaemolyticus*, 25% of *V.fluvialis* and 18.18% of *V.fischeri* actively hydrolyzed aesculin.

79.17% of *V.anguillarum*, 80% of *V.harveyi*, 60% of *V.campbellii*, 52.95% of *V.parahaemolyticus*, 54.55% of *V.fischeri*, 20% of *V.metschnikovii*, 62.5% of *V.furnissii* and 100% of *V.cincinnatiensis* actively produced hydrogen sulphide from L-cystine. All the *Vibrio* strains isolated were sensitive to O/129 (Plate 7).

4.5 Bio-chemical reactions of *Vibrio* spp. isolated from Station II (KVK)

All the *Vibrio* strains isolated were actively motile gram negative rods. 100% of *V.anguillarum*, *V.furnissii*, *V. fluvialis* and *V. metschnikovii*, could de-carboxylate arginine whereas rest of the vibrios could not utilize the aminoacid. 100% of *V.fischeri*, *V.parahaemolyticus*, *V.metschnikovii*, *V.harveyi*, *V.cincinnatiensis*, *V.campbellii* and *V.vulnificus*, decarboxylated lysine whereas none of the species like *V.anguillarum*, *V.furnissii* and *V.fluvialis* could de-carboxylate lysine. Except for *V.parahaemolyticus*, *V.harveyi* and *V.vulnificus* rest of the strains gave negative reaction to ornithine decarboxylation.

Nitrate was actively reduced to nitrite by 100% of *V.furnissii*, *V.fluvialis*, *V.campbellii*, *V.cincinnatiensis* and *V.vulnificus* followed by 95.12% of *V.anguillarum*, 95% of *V.fischeri*, 92.31% of *V.parahaemolyticus* and 75% of *V.harveyi* whereas 57.14% of *V. metschnikovii* could not reduce nitrate to nitrite. All the strains of vibrios isolated from KVK were found to produce ammonia except for 33.33% of *V.cincinnatiensis*, 14.29% of *V.metschnikovii*, and 4.88% of *V.anguillarum*. All the *Vibrio* strains produced cytochrome oxidase except 5% of *V.fischeri*, 7.69% of *V.parahaemolyticus* and 85.71% of *V.metschnikovii*. 83.33% of *V.furnissii*, 71.43% of *V.metschnikovii*, 50% of *V.vulnificus*, 35% of *V.fischeri*, 30.77% of *V.parahaemolyticus*, 25% of *V.harveyi* and 12.19% of *V.anguillarum* were found to produce the enzyme catalase.

Fermentation of carbohydrates

Except 100% of *V.vulnificus* and *V. metschnikovii* rest of the vibrios could not ferment lactose. Except 8.34% of *V.furnissii*, 7.69% of *V.parahaemolyticus* and 100% of *V.campbellii* all the other *Vibrio* species were found to be actively metabolizing maltose. Mannitol was actively fermented by 100% of *V.fluvialis*, *V.metschnikovii*, *V.harveyi* and *V.campbellii* followed by 92.31% of *V.parahaemolyticus*, 87.80% of *V.anguillarum*, 66.67% of *V.furnissii* and *V.cincinnatiensis* and 45% of *V.fischeri* while the two strains of *V.vulnificus* isolated could not ferment the sugar. Sucrose was fermented by 100% of *V.anguillarum*, *V. fluvialis*, *V.metschnikovii*, *V.harveyi* and *V.cincinnatiensis* followed by 91.66% of *V.furnissii*, 50% of *V.vulnificus*, 75% of *V.fischeri*, and 84.62% of *V.parahaemolyticus* whereas none of the strains of *V.campbellii* fermented sucrose. Glucose was actively fermented by all the *Vibrio* strains except 50% of *V.vulnificus*, 8.34% of *V.furnissii*, 7.69% of *V.parahaemolyticus* and 2.44% of *V.anguillarum*.

TABLE 32

BIO-CHEMICAL PROFILE OF *VIBRIO* SPECIES ISOLATED FROM STATION II (KVK)

Biochemical tests	Reactions of *V. anguillarum* (n=41)	%	*Reactions of V.fischeri* (n=20)	%	Reactions of *V. parahaemolyticus* (n=13)	%	Reactions of *V. furnissii* (n=12)	%	Reactions of *V. fluvialis* (n=11)	%
Gram negative	+	100	+	100	+	100	+	100	+	100
Motility	+	100	+	100	+	100	+	100	+	100
Arginine dihydrolase	+	100	-	100	-	100	+	100	+	100
Lysine decarboxylase	-	100	+	100	+	100	-	100	-	100
Ornithine decarboxylase	-	100	(-)	100	+	100	-	100	-	100
Nitrate	+	95.12	+	95	+	92.31	+	100	+	100
Ammonia	+	95.12	+	100	+	100	+	100	+	100
Oxidase	+	100	+	95	+	92.31	+	100	+	100
Catalase	+	12.19	+	35	+	30.77	+	83.33	+	0
Acid from Lactose	-	100	-	100	-	100	-	100	-	100

Contd....

Acid from Maltose	+	100	+	100	+	92.31	+	91.66	+	100
Acid from Mannitol	+	87.80	V	45	(+)	92.31	+	66.67	+	100
Acid from Sucrose	+	100	(-)	25	-	15.38	+	91.66	+	100
Acid from Glucose	+	97.56	V	100	+	92.31	+	91.66	+	100
Growth at 0% NaCl	-	65.85	-	70	-	46.15	d	83.33	V	100
Growth at 6% NaCl	d	85.37	d	80	+	92.31	+	100	+	100
Growth at 8% NaCl	-	48.78	-	80	+	53.85	+	75	(+)	100
Growth at 4^0C	d	85.37	(-)	25	-	53.85	-	91.66	-	100
Growth at 40^0C	-	95.12	-	45	+	92.31	+	100	+	100
Indole	+	0	-	100	+	0	(-)	100	(+)	0
Voges proskauer	+	2.44	-	100	+	0	-	100	-	100

Contd....

Citrate	+	41.46	v	95	+	23.08	+	41.67	+	0
Starch	+	85.37	-	65	+	84.62	+	91.66	+	100
Gelatinase	+	70.73	-	100	+	53.85	(+)	58.33	+	54.55
Caesin	+	60.98	+	45	V	38.46	+	50	+	54.55
Urease	-	100	+	65	-	92.31	-	75	-	100
Aesculin hydrolysis	(-)	95.12	-	95	-	92.31	-	91.66	V	54.55
H_2S	-	9.76	-	40	-	15.38	-	83.33	-	18.18
Gas from glucose	-	90.24	-	80	-	100	+	75	-	100
Resistance to 0/129	-	100	-	100	-	100	-	100	-	100

Biochemical tests	Reactions of *V. metschnikovii* (n=7)	%	Reactions of *V. harveyi* (n=4)	%	Reactions of *V. cincinnatiensis* (n=3)	%	Reactions of *V. campbellii* (n=3)	%	Reactions of *V. vulnificus* (n=2)	%
Gram negative	+	100	+	100	+	100	+	100	+	100
Motility	+	100	+	100	+	100	+	100	+	100
Arginine dihydrolase	d	100	-	100	-	100	-	100	-	100
Lysine decarboxylase	V	100	+	100	(+)	100	d	100	+	100
Ornithine decarboxylase	-	100	+	100	-	100	-	100	+	100
Nitrate	-	57.14	+	75	+	100	(+)	100	+	100
Ammonia	+	85.71	+	100	+	66.67	+	100	+	100
Oxidase	-	85.71	+	100	+	100	+	100	+	100
Catalase	+	71.43	+	25	+	0	+	0	+	50
Acid from Lactose	V	100	-	100	-	100	-	100	v	100
Acid from Maltose	+	100	+	100	+	100	+	0	+	100
Acid from Mannitol	(+)	100	+	100	+	66.67	V	100	(+)	0
Acid from Sucrose	+	100	V	100	+	100	-	100	(-)	50
Acid from Glucose	+	100	+	100	+	100	+	100	nd	50
Growth at 0% NaCl	d	85.71	-	100	-	66.67	-	100	-	100
Growth at 6% NaCl	(+)	100	(+)	75	+	33.33	d	100	d	100
Growth at 8% NaCl	(+)	71.43	(+)	100	+	66.67	V	100	-	50

Growth at 4^0C	d	57.14	-	100	-	66.67	-	100	-	100
Growth at 40^0C	+	85.71	V	100	nd	66.67	-	100	+	100
Indole	(-)	100	+	0	-	100	(+)	0	+	0
Voges proskauer	(+)	42.86	-	100	d	100	-	100	-	100
Citrate	v	100	v	25	+	0	v	100	+	100
Starch	+	85.71	+	75	+	100	+	100	+	100
Gelatinase	+	100	+	100	-	100	+	100	+	50
Caesin	+	57.14	+	75	+	66.67	+	0	+	50
Urease	-	100	V	100	-	66.67	-	100	-	50
Aesculin hydrolysis	V	85.71	(-)	100	nd	100	v	100	-	100
H_2S	-	71.43	-	0	-	33.33	-	0	-	50
Gas from glucose	-	100	-	75	-	100	-	100	-	100
Resistance to 0/129	-	100	(-)	100	-	100	-	100	-	100

Salt tolerance

None of the strains of *V.harveyi*, *V.campbellii* and *V.vulnificus* could grow in the absence of sodium chloride whereas 100% of *V.fluvialis*, 53.85% of *V.parahaemolyticus*, 33.33% of *V.cincinnatiensis*, 30% of *V.fischeri*, 83.33% of *V.furnissii*, 34.15% of *V.anguillarum* and 85.71% of *V.metschnikovii* could grow well in the absence of sodium chloride. 100% of *V.furnissii*, *V.fluvialis*, *V.metschnikovii*, *V.campbellii* and *V.vulnificus* gave good growth at 6% sodium chloride, whereas 14.63% of *V.anguillarum*, 20% of *V.fischeri*, 7.69% of *V.parahaemolyticus*, 25% of *V.harveyi and* 66.67% of *V.cincinnatiensis gave n*egative growth. 100% of *V.fluvialis*, *V. harveyi and V.campbellii*, 51.22% of *V.anguillarum*, 20% of *V.fischeri*, 75% of *V.furnissii*, 53.85% of *V.parahaemolyticus*, 71.43% of *V.metschnikovii*, 66.67% of *V.cincinnatiensis* and 50% of *V.vulnificus* gave good growth at 8% sodium chloride.

Temperature tolerance

None of the strains of *V.fluvialis*, *V.harveyi*, *V.campbellii* and *V.vulnificus* could grow at 4^0C whereas 14.63% of *V.anguillarum*, 75% of *V.fischeri*, 8.34% of *V.furnissii*, 46.15% of *V.parahaemolyticus*, 42.86% of *V.metschnikovii and* 33.33% of *V.cincinnatiensis* could grow at 4^0C. 100% of *V.furnissii*, *V.harveyi*, *V.fluvialis* and *V.vulnificus* could tolerate 40^0C whereas 95.12% of *V.anguillarum*, 45% of *V.fischeri*, 7.69% of *V.parahaemolyticus*, 14.29% of *V.metschnikovii*, 33.33% of *V.cincinnatiensis* and 100% of *V.campbellii* gave negative growth at 40^0C.

None of the *Vibrio* strains isolated produced indole. Except for 42.86% of *V.metschnikovii* and 2.44% of *V.anguillarum* none of the strains were able to produce acetyl methyl carbinol. 100% of *V.vulnificus*, 41.46% of *V.anguillarum*, 95% of *V.fischeri*, 41.67% of *V.furnissii*, 23.08% of *V.parahaemolyticus* and 25% of *V.harveyi* could utilize citrate as the sole source of carbon whereas none of the strains of *V.fluvialis*, *V.metschnikovii*, *V.cincinnatiensis* and *V.campbellii* utilized citrate.

100% of *V.cincinnatiensis*, *V.campbellii*, *V.vulnificus* and *V.fluvialis* hydrolysed starch followed by 85.37% of *V.anguillarum*, 91.66% of *V.furnissii*,

84.62% of *V.parahaemolyticus*, 85.71% of *V.metschnikovii*, 75% of *V.harveyi* and 35% of *V.fischeri*. 100% of *V.campbellii*, *V.harveyi* and *V.metschnikovii* were found to liquefy gelatin. Only 70.73% of *V.anguillarum*, 58.33% of *V.furnissii*, 53.85% of *V.parahaemolyticus*, 54.55% of *V.fluvialis* and 50% of *V.vulnificus* could produce gelatinase. None of the strains of *V.cincinnatiensis* and *V.fischeri* produced gelatinase. 60.98% of *V.anguillarum*, 45% of *V.fischeri*, 50% of *V.furnissii* and *V.vulnificus*, 38.46% of *V.parahaemolyticus*, 54.55% of *V.fluvialis*, 57.14% of *V.metschnikovii*, 75% of *V.harveyi* and 66.67% of *V.cincinnatiensis* hydrolysed casein. Except 65% of *V.fischeri*, 25% of *V.furnissii*, 7.69% of *V.parahaemolyticus*. 33.33% of *V.cincinnatiensis* and 50% of *V.vulnificus*, rest of the vibrios could not hydrolyse urea. Aesculin was hydrolyzed by 4.88% of *V.anguillarum*, 5% of *V.fischeri*, 8.34% of *V. furnissii*, 7.69% of *V. parahaemolyticus*, 45.45% of *V. fluvialis* and 85.71% of *V. metschnikovii*, while rest of the *Vibrio* strains could not hydrolyse aesculin. H_2S was produced by 90.24% of *V.anguillarum*, 60% of *V.fischeri*, 16.67% of *V.furnissii*, 84.62% of *V.parahaemolyticus*, 81.82% of *V.fluvialis*, 28.57% of *V.metschnikovii*, 66.67% of *V.cincinnatiensis*, 50% of *V.vulnificus* and 100% of *V.harveyi* and *V. campbellii*. Except 9.76% of *V.anguillarum*, 20% of *V.fischeri*, 75% of *V. furnissii* and 25% of *V. harveyi* none of the strains could produce gas from glucose. All the *Vibrio* spp. isolated in the present study were sensitive to O/129.

Zone size interpretative chart

Antibiotic	Symbol	Concentration mcg/disc	Inhibition zone		
			Resistant mm or less	Intermediate mm	Sensitivity mm or more
Ampicillin	A	10mcg	13	14-16	17
Tetracycline	T	30 mcg	14	15-18	19
Chloramphenicol	C	30 mcg	12	13-17	18
Erythromycin	E	15 mcg	13	14-22	23
Gentamycin	G	10 mcg	12	13-14	15
Penicillin	P	10 units	14	-	15
Streptomycin	S	10 mcg	11	12-14	15
Novobiocin	N	30 mcg	17	18-21	22

Table 33

Antibiogram of *Vibrio* spp. isolated from Station I (Valappu)

Species	Resistant	Intermediate	Sensitive
V.anguillarum (n=24)	A(24) P(17) G(16) N(15) E(13) T(10) S(9) C(7)	E(5) T(5) N(4) C(2)	G(8) C(15) S(15) T(9) P(7) E(6) N(5)
V.parahaemolyticus (n=17)	A(17) P(16) C(5) T(5) E(4) N(4) G(2)	N(11) E(10) T(5) C(4)	S(17) G(15) C(8) T(7) E(3) N(2) P(1)
V.fischeri (n=11)	P(11) A(11) E(2) T(2) G(2) C(2) N(1)	N(4) T(2) C(1) E(1)	S(11) G(9) E(8) C(8) T(7) N(6)
V. fluvialis (n=8)	A(8) P(5) C(2) T(2) N(2) E(1) G(1) S(1)	E(2) T(2) S(1)	G(7) C(6) N(6) S(6) E(5) T(4) P(3)
V.furnissii (n=8)	P(8) E(7) A(6) N(6) G(4) C(3)	T(5) A(2) N(1)	S(8) C(5) G(4) E(1) N(1) T(3)
V.campbellii (n=5)	A(5) N(4) P(3) E(3) C(2)	T(2) N(1)	S(5) G(5) T(3) C(3) E(2) P(2)
V.metschnikovii (n=5)	A(5) P(3) C(3) E(3) T(1)	N(2) E(2) T(2) C(1) S(1)	G(5) S(4) N(3) T(2) P(2) C(1)
V.harveyi (n=5)	A(5) P(4) N(4) T(4) C(3) S(1) G(1)	C(1) E(1) T(1) N(1)	S(4) G(4) E(4) P(1) C(1)
V. cincinnatiensis (n=4)	P(4) N(4) A(4) E(3) T(3) C(2)	S(1) C(1)	G(4) S(3)T(1) C(1) E(1)
V.vulnificus (n=1)	A(1) E(1) N(1) P(1)		C(1) G(1) T(1) S(1)

Table 34

Antibiotic sensitivity pattern of *Vibrio* spp. isolated from Station I

Antibiotics	*V. anguillarum* (n=24)			*V.parahaemolyticus* (n=17)			*V.fischeri* (n=11)		
	Resist-ance (%)	Inter-mediate (%)	Sen-sitive (%)	Resist-ance (%)	Inter-mediate (%)	Sen-sitive (%)	Resist-ance (%)	Inter -mediate (%)	Sen -sitive (%)
Ampicillin (10mcg)	100	0	0	100	0	0	100	0	0
Tetracycline (30mcg)	41.67	20.83	37.5	29.41	29.41	41.18	18.18	18.18	63.64
Chloramphenicol (30 mcg)	29.17	8.33	62.5	29.41	23.53	47.06	18.18	9.09	72.73
Erythromycin (15mcg)	54.17	20.83	25	23.53	58.82	17.65	18.18	9.09	72.73
Gentamycin (10mcg)	25	0	75	11.76	0	88.24	18.18	0	81.82
Penicillin (10 units)	70.83	0	29.17	94.12	0	5.88	100	0	0
Streptomycin (10mcg)	37.5	0	62.5	0	0	100	0	0	100
Novobiocin (30 mcg)	62.5	16.67	20.83	23.53	64.71	11.76	9.09	36.36	54.55

Antibiotics	*V.fluvialis (n=8)*			*V.metschnikovii(n=5)*			*V.harveyi(n=5)*		
	Resist-ance (%)	Inter-mediate (%)	Sen-sitive (%)	Resist-ance (%)	Inter-mediate (%)	Sen-sitive (%)	Resist-ance (%)	Inter-mediate (%)	Sen-sitive (%)
Ampicillin (10mcg)	100	0	0	100	0	0	100	0	0
Tetracycline (30mcg)	25	25	50	20	40	40	80	20	0
Chloramphenicol (30 mcg)	25	0	75	60	20	20	60	20	20
Erythromycin (15mcg)	12.5	25	62.5	60	40	0	0	20	80
Gentamycin (10mcg)	12.5	0	87.5	0	0	100	20	0	80
Penicillin (10 units)	62.5	0	37.5	60	0	40	80	0	20
Streptomycin(10mcg)	12.5	12.5	75	0	20	80	20	0	80
Novobiocin (30 mcg)	25	0	75	0	40	60	80	20	0

Antibiotics	*V.furnissii (n=8)*			*V.campbellii (n=5)*			*V.cincinnatiensis (n=4)*		
	Resist-ance (%)	Inter-mediate (%)	Sen-sitive (%)	Resist-ance (%)	Inter-mediate (%)	Sen-sitive (%)	Resist-ance (%)	Inter-mediate (%)	Sen-sitive (%)
Ampicillin (10mcg)	75	25	0	100	0	0	100	0	0
Tetracycline (30mcg)	37.5	62.5	0	0	40	60	75	0	25
Chloramphenicol (30 mcg)	37.5	0	62.5	40	0	60	50	25	25
Erythromycin (15mcg)	87.5	0	12.5	60	0	40	75	0	25
Gentamycin (10mcg)	50	0	50	0	0	100	0	0	100
Penicillin (10 units)	100	0	0	60	0	40	100	0	0
Streptomycin (10mcg)	0	0	100	0	0	100	0	25	75
Novobiocin (30 mcg)	75	12.5	0	80	20	0	100	0	0

V.vulnificus			
Antibiotics	**Resistance (%)**	**Intermediate (%)**	**Sensitive (%)**
Ampicillin (10mcg)	100	0	0
Tetracycline (30mcg)	0	0	100
Chloramphenicol (30 mcg)	0	0	100
Erythromycin (15mcg)	100	0	0
Gentamycin (10mcg)	0	0	100
Penicillin (10 units)	100	0	0
Streptomycin (10mcg)	0	0	100
Novobiocin (30 mcg)	100	0	0

4.6 Drug Sensitivity of *Vibrio* sp. isolated from Valappu

The pattern of drug sensitivity of *Vibrio* sp. from Valappu is given in Table 34. *V.anguillarum* was sensitive to chloramphenicol, gentamycin, streptomycin, erythromycin, tetracycline, penicillin and novobiocin of which novobiocin gave the lowest sensitivity (20.83%). 20.83% of the strains exhibited intermediate resistance

towards tetracycline and erythromycin. Maximum sensitivity was exhibited towards gentamycin 75%, chloramphenicol and streptomycin (62.5%). The multiple drug resistance (MDR) to antimicrobials was very frequent among the *Vibrio* strains tested. The most frequently observed resistotypes being A, P, N, E, T, S, C, G (Table 33).

A clear homogenity in the antimicrobial resistance pattern has been detected in the strains of several *Vibrio* spp. thus all the strains isolated from Valappu except *V.furnissii* were resistant to ampicillin.

All the *V.anguillarum* strains were resistant to ampicillin (100%), 70.83% to penicillin, 62.5% to novobiocin, 54.17% to erythromycin, 41.67% to tetracycline, 37.5% streptomycin and 29.17% of chloramphenicol.

17 strains of *V.parahaemolyticus* tested had a similar drug resistance pattern, they being resistant to ampicillin (100%), penicillin (94.12%), tetracycline and chloramphenicol (29.41%). 88.24% of *V.parahaemolyticus* strains were sensitive to gentamycin, 47.06% to chloramphenicol and 41.18% to tetracycline. All the *V.parahaemolyticus* strains were sensitive to streptomycin. 64.71% of *V. parahaemolyticus* and 58.82% of the strains showed intermediate resistance to novobiocin and erythromycin respectively.

The strains of *V.fischeri* showed variable resistance patterns to the antimicrobials tested, thus all strains (n=11) were resistant to ampicillin and penicillin while the percentage resistance to other antimicrobial agents were tetracycline, chloramphenicol, erythromycin and gentamycin (18.18%) followed by novobiocin (9.09%). All the strains were sensitive to streptomycin. The sensitivity pattern showed variability with 81.82% of *V.fischeri* being sensitive to gentamycin followed by chloramphenicol and erythromycin (72.73%), tetracycline (63.64%), and novobiocin (54.55%). Intermediate resistance was exhibited by 36.36% of the strains towards novobiocin followed by 18.18% towards tetracycline, 9.09% to chloramphenicol and erythromycin (Table 34).

The *V.fluvialis* strains isolated exhibited sensitiveness to the drugs tested than resistance. The strains showed 75% sensitivity to novobiocin and chloramphenicol, 87.5% to gentamycin 62.5% to erythromycin 50% to tetracycline

and 37.5% to penicillin. Maximum resistance was exhibited towards ampicillin (100%) and penicillin (62.5%). Except tetracycline, erythromycin and streptomycin none of the strains of *V. fluvialis* exhibited intermediate resistance to the drugs tested.

V.furnissii showed resistance to all the drugs tested than sensitivity, penicillin (100%), erythromycin (87.5%), novobiocin and ampicillin (75%), gentamycin (50%) and chloramphenicol (37.5%). The strains exhibited sensitivity towards streptomycin (100%), chloramphenicol (62.5%) and gentamycin (50%). Maximum intermediate resistance was exhibited towards tetracycline (62.5%).

All the strains of *V.campbellii* showed similar sensitivity pattern with 100% of the strains being sensitive to gentamycin and streptomycin, 60% of the isolates showed sensitivity to tetracycline and chloramphenicol and 40% to erythromycin and penicillin. None of the strains were resistant to tetracycline, gentamycin and streptomycin. Maximum resistance towards novobiocin was exhibited by 80% of the strains. 40% of *V.campbellii* showed intermediate resistance to tetracycline and 20% to novobiocin.

The resistotypes of *V.metschnikovii* were A, P, C, E, T (Table 33). Maximum resistance was exhibited to ampicillin (100%) followed by chloramphenicol erythromycin and penicillin (60%). All the strains were sensitive to gentamycin streptomycin (80%), novobiocin (60%) and penicillin and tetracycline (40%). 40% of the strains showed intermediate resistance to tetracycline erythromycin and novobiocin.

The luminescent bacteria *V. harveyi* was found to be resistant than sensitive to the drugs tested. The resistotype being P, A, T, C, N, G, S (Table 33). The strains being resistant to ampicillin (100%) tetracycline, penicillin, novobiocin (80%), chloramphenicol (60%) and streptomycin (20%). 80% of the strains were sensitive to erythromycin, gentamycin and streptomycin.

V. cincinnatiensis were resistant than sensitive to the antibiotics tested. The strains being resistant to ampicillin, penicillin and novobiocin (100%), tetracycline and erythromycin (75%) and chloramphenicol (50%). Maximum sensitivity was exhibited towards gentamycin (100%) and streptomycin (75%). Except for

chloramphenicol and streptomycin (25%) none of the strains showed intermediate resistance to *V.cincinnatiensis*. The sole strain of *V.vulnificus* showed resistance to ampicillin, erythromycin, penicillin and novobiocin, while they were sensitive to tetracycline, chloramphenicol, gentamycin and streptomycin.

Table 35

Antibiogram of *Vibrio* spp. isolated from Station II (KVK)

Species	Resistant	Intermediate	Sensitive
Vibrio anguillarum (n=41)	A(37) P (34) N(28) E (21) G (18) T(12) S (11) C(10)	T(9) E(8) C(7) N(3) A(1) S(1)	S(29) C(24) G(23) T(20) E(12) N(10) A(3) P(7)
Vibrio fischeri (n=20)	A(18) P(17) G(16) E(12) T(2) S(3) N(3) C(1)	T(7) C(6) N(5) E(3)	S(17) G(14)C(13) N(12) T(11) E(5) P(3) A(2)
Vibrio parahaemolyticus (n=13)	A(12) P(12) E(7) G(5) N(5) S(2) T(2) C(1)	T(4) N(4) E(4) C(3) A(1)	S(11) C(9) G(8) T(7) N(4) E(3) P(1)
Vibrio furnissii (n=12)	A(12) P(12) N(9) E(8) G(3) T(3) C(3) S(1)	T(5) E(4) N(3) C(3)	S(11)G(9) C(6) T(4)
Vibrio fluvialis (n=11)	A(11) P(6) E(6) G(2) N(1) S(1) C(1)	T(4) E(4) E(2) N(3)	S(10) G(9) E(8) T(7) N(7) P(5) E(1)
Vibrio metschnikovii (n=7)	P(7) A(7) C(4) E(4) T(3) G(2)	C(1)	S(7) N(7) G(5) T(4) E(3) C(2)
Vibrio harveyi (n=4)	P(4) A(4) T(4) N(4) E(3) C(2)	E(1)	S(4) G(4) C(2)
Vibrio campbellii (n=3)	A(3) P(3) E(3)	C(3) N(3)	G(3) T(3) S(3)
Vibrio cincinnatiensis (n=3)	A(3) E(3)	G(3) N(2)	T(3) C(3) P(3) S(3) N(1)
Vibrio vulnificus (n=2)	E(2) P(1) T(1) N(1) A(1) C(1) G(1)	C(1) T(1)	S(2) A(1) G(1) N(1) P(1)

Table 36

Antibiotic sensitivity pattern of *Vibrio* spp. isolated from Station II (KVK)

Antibiotics	*V.anguillarum* (n=41)			*V.parahaemolyticus* (n=13)			*V.fischeri* (n=20)		
	Resist-ance (%)	Inter-mediate (%)	Sens-itive (%)	Resist-ance (%)	Inter-mediate (%)	Sens-itive (%)	Resist-ance (%)	Inter-mediate (%)	Sen-sitive (%)
Ampicillin (10mcg)	90.24	2.44	7.32	92.31	7.69	0	90	0	10
Tetracycline (30mcg)	29.27	21.95	48.78	15.38	30.77	53.85	10	35	55
Chloramphenicol (30 mcg)	24.39	17.07	58.54	7.69	23.08	69.23	5	30	65
Erythromycin (15mcg)	51.22	19.51	29.27	46.15	30.77	23.08	60	15	25
Gentamycin (10mcg)	43.90	0	56.10	38.46	0	61.54	30	0	70
Penicillin (10 units)	82.93	0	17.07	92.31	0	7.69	85	0	15
Streptomycin (10mcg)	26.83	2.44	70.73	15.38	0	84.62	15	0	85
Novobiocin (30 mcg)	68.29	7.32	24.39	38.46	30.77	30.77	15	25	60

Antibiotics	*V.furnissii* (n=12)			*V.fluvialis* (n=11)			*V. metschnikovii* (n=7)		
	Resistance (%)	Intermediate (%)	Sensitive (%)	Resistance (%)	Intermediate (%)	Sensitive (%)	Resistance (%)	Intermediate (%)	Sensitive (%)
Ampicillin (10mcg)	100	0	0	100	0	0	100	0	0
Tetracycline (30mcg)	25	41.67	33.33	0	36.36	63.64	42.86	0	57.14
Chloramphenicol (30 mcg)	25	25	50	9.09	18.18	72.73	57.14	14.29	28.57
Erythromycin (15mcg)	66.67	33.33	0	54.55	36.36	9.09	57.14	0	42.86
Gentamycin (10mcg)	25	0	75	18.18	0	81.82	28.57	0	71.43
Penicillin (10 units)	100	0	0	54.55	0	45.45	100	0	0
Streptomycin (10mcg)	8.33	0	91.67	9.09	0	90.91	0	0	100
Novobiocin (30 mcg)	75	25	0	9.09	27.27	63.64	0	0	100

Antibiotics	*V.harveyi* (n=4)			*V.campbellii* (n=3)			*V.cincinnatiensis* (n=3)		
	Resistance (%)	Intermediate (%)	Sensitive (%)	Resistance (%)	Intermediate (%)	Sensitive (%)	Resistance (%)	Intermediate (%)	Sensitive (%)
Ampicillin (10mcg)	100	0	0	100	0	0	100	0	0
Tetracycline (30mcg)	100	0	0	0	0	100	0	0	100
Chloramphenicol (30 mcg)	50	0	50	0	100	0	0	100	0
Erythromycin (15mcg)	75	25	0	100	0	0	100	0	0
Gentamycin (10mcg)	0	0	100	0	0	100	0	0	100
Penicillin (10 units)	100	0	0	100	0	0	100	0	0
Streptomycin (10mcg)	0	0	100	0	0	100	0	0	100
Novobiocin (30 mcg)	100	0	0	0	100	0	0	100	0

V.vulnificus			
Antibiotics	**Resistance (%)**	**Intermediate (%)**	**Sensitive (%)**
Ampicillin (10mcg)	50	0	50
Tetracycline (30mcg)	50	50	0
Chloramphenicol (30 mcg)	50	50	0
Erythromycin (15mcg)	100	0	0
Gentamycin (10mcg)	50	0	50
Penicillin (10 units)	50	0	50
Streptomycin (10mcg)	0	0	100
Novobiocin (30 mcg)	50	0	50

4.7 Drug Sensitivity of *Vibrio* sp. isolated from KVK

V. anguillarum the most frequently isolated *Vibrio* sp. was found to exhibit 90.24% resistance to ampicillin (Table 36), 82.93% to penicillin, and 68.29% to novobiocin. The most frequently observed resistotype of the species was A P, N, E, G, T, S, C (Table 35). Maximum sensitivity was exhibited towards streptomycin 70.73%. 21.95% of the species was found to exhibit intermediate resistance to tetracycline.

A clear homogenicity in the antimicrobial resistance pattern has been detected in the strains of several *Vibrio* spp. Thus all the strains isolated from KVK except *V. anguillarum* (7.32%), *V. fischeri* (10%), and *V.vulnificus* (50%) were sensitive to ampicillin. The resistance pattern exhibited by the strains of vibrios at Valappu were different. Only two strains of *V. furnissii* isolated from Valappu exhibited intermediate resistance to ampicillin while rest of the 6 spp. were ampicillin resistant.

No clear-cut homogenicity in the sensitivity pattern or resistance could be observed in the antibiogram of *V. fischeri*, the second most frequently isolated *Vibrio* species. The resistotype being A, P, G, E (Table 35). Most of the *V.fischeri* was found sensitive towards streptomycin (10 mcg). The most resistant was ampicillin (10mcg) followed by penicillin (10units). Maximum intermediate resistance was exhibited towards tetracycline (30 mcg).

Food borne human pathogen *V. parahaemolyticus* exhibited multiple drug resistance indicating the presence of *R plasmids*. In addition to ampicillin and penicillin (92.31%), *V. parahaemolyticus* exhibited resistance towards erythromycin (46.15%), gentamycin and novobiocin (38.46%). Maximum sensitivity was exhibited towards streptomycin (84.62%) followed by 61.54% to gentamycin and 69.23% to chloramphenicol. 30.77% of the strains showed intermediate resistance to tetracycline, erythromycin and novobiocin.

V. furnissii showed greater resistance to the drugs tested than sensitiveness. The resistotypes being A, P, N, E, T, G, C, S (Table 35). Sensitivity was exhibited by 91.67% of the strains to streptomycin, 75% to gentamycin, 50% to

chloramphenicol and 33.33% to tetracycline. Maximum resistance was exhibited to erythromycin (66.67%) in addition to ampicillin and penicillin (100%). Maximum intermediate resistance was noted towards tetracycline (41.67%).

11 strains of *V. fluvialis* exhibited drug resistance to ampicillin (100%), erythromycin and penicillin (54.55%) and gentamycin (18.18%). Maximum sensitivity was shown towards streptomycin (90.91%) followed by gentamycin (81.82%) and chloramphenicol (72.73%). 36.36% of the strains exhibited intermediate resistance to tetracycline and erythromycin.

The lactose fermenting *V. metschnikovii* exhibited 100% resistance to ampicillin and penicillin where as all the strains were sensitive towards streptomycin and novobiocin. 57.14% of the strains were resistant to chloramphenicol and erythromycin. 71.43% and 57.14% of the strains were sensitive towards gentamycin (10mcg) and tetracycline (30 mcg) respectively. One strain exhibited intermediate resistance to chloramphenicol.

The luminescent bacteria *V. harveyi* showed 100% resistance to ampicillin, penicillin, tetracycline, and novobiocin whereas all the strains were sensitive to streptomycin and gentamycin. Only one strain showed sensitivity to erythromycin.

All the three isolates of *V.campbellii* showed similar drug resistance as well as drug sensitivity pattern. All of them were being sensitive to gentamycin, tetracycline and streptomycin while being resistant to ampicillin, penicillin and erythromycin. Intermediate resistance was exhibited towards chloramphenicol and novobiocin.

The three strains of *V. cincinnatiensis* were resistant to penicillin, ampicillin and erythromycin and sensitive to tetracycline, gentamycin and streptomycin. Chloramphenicol and novobiocin showed intermediate resistance.

V. vulnificus showed stronger drug resistance pattern than sensitivity, the pattern being E, P, T, N, A, C, G (Table 35). Of the limited strains studied the sensitivity pattern showed variance with some of the strains being sensitive towards

ampicillin, gentamycin, novobiocin and penicillin, whereas other strains were resistant. One strain exhibited intermediate resistance to chloramphenicol and tetracycline.

Table 37

Percentage composition of *Vibrio* spp. isolated from the sampling sites.

Species	Total isolates (n= 204)	Percentage
V.anguillarum	65	31.86
V.fischeri	31	15.19
V.parahaemolyticus	30	14.71
V.furnissii	20	9.80
V.fluvialis	19	9 31
V.metschnikovii	12	5.88
V.harveyi	9	4.41
V.campbellii	8	3.92
V.cincinnatiensis	7	3.43
V.vulnificus	3	1.47

Table 38

Percentage composition of *Vibrio* spp. isolated from Station I

Species	Total no. of isolates (n=88)	Percentage
V.anguillarum	24	27.27
V.parahaemolyticus	17	19.32
V.fischeri	11	12.5
V.fluvialis	8	9.09
V.furnissii	8	9.09
V.harveyi	5	5.68
V. campbellii	5	5.68
V.metschnikovii	5	5.68
V.cincinnatiensis	4	4.55
V.vulnificus	1	1.14

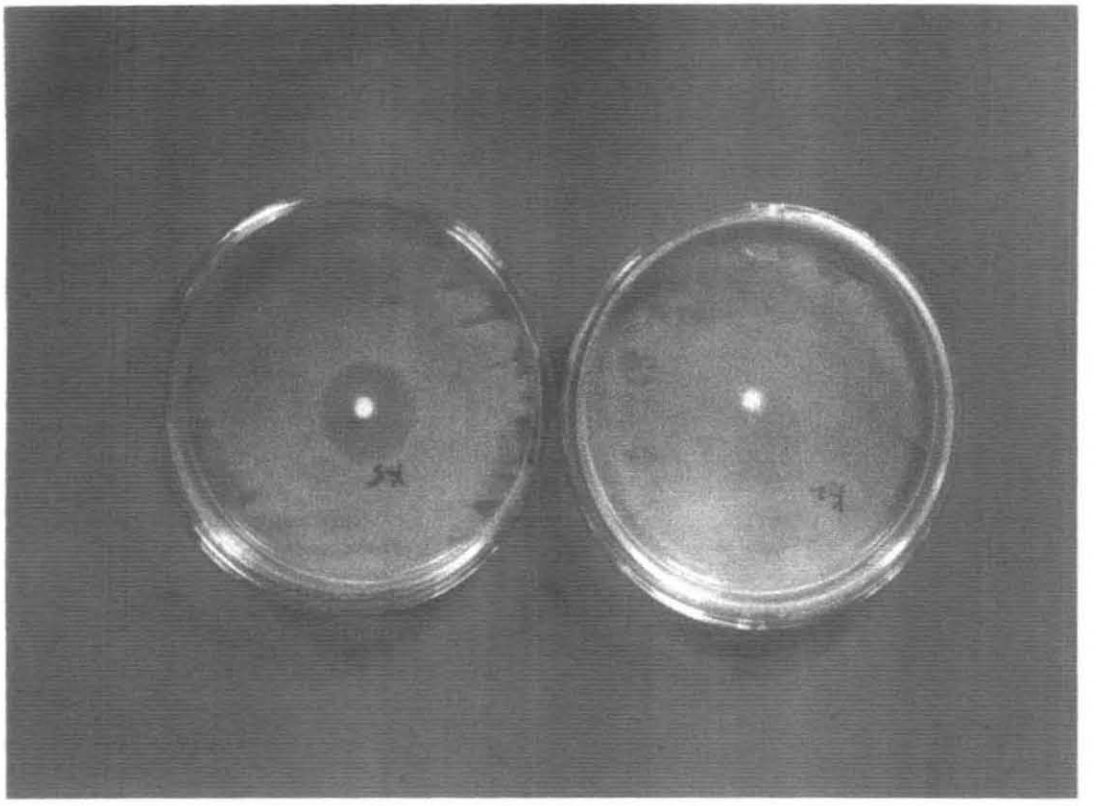

Plate 7 Vibriostatic activity (150μg) exhibited *by* *Vibrio* sp. and *Aeromonas* sp.

Valappu, while *V.vulnificus* formed 1.72% at KVK and 1.14% at Valappu. Luminescent forms such as *V. fischeri* (17.24%) at KVK and (12.5%) at Valappu and *V.harveyi* 3.45% at KVK and 5.68% at Valappu were observed through out the period of the study (Plate 8). *V.cincinnatiensiss* contributed 2.59% at KVK and 4.55% at Valappu while *V.campbellii* contributed 2.59% at KVK (Table 39) and 5.68% at Valappu (Table 38).

The multiple regression of the ten *Vibrio* spp. on the 9 environmental parameters studied from both the stations showed that except *V.campbellii* from Station I (Valappu), none of the fitted regressions were found to be significant in the present study.

Table 40

ANOVA table showing the significance of the fitted multiple regression of *Vibrio campbellii* on the environmental parameters at Station I.

SUMMARY OUTPUT					
Regression Statistics					
Multiple R	0.924748				
R Square	0.855158				
Adjusted R Square	0.692211				
Standard Error	1.219382				
Observations	18				
ANOVA					
	df	*SS*	*MS*	*F*	*Significance F*
Regression	9	70.22987	7.803319	5.248075	0.014424
Residual	8	11.89513	1.486892		
Total	17	82.125			

Table 40 gives the regression analysis of the total count of *V.campbellii* on the 9 environmental parameters. *V.campbellii* in Valappu showed a significant

regression on the listed 9 environmental parameters. The fitted regression explains $Y= -5.41917 x +11.64124 x_1 -29.8959 x_2 + .204935 x_3 + 8.590076 x_4 - 5.59219 x_5 + 8.417095 x_6 + 10.64901 x_7 - 10.0486 x_8 + 5.705466 x_9$ where y is the count of *V.campbellii*, x_1 = temperature, x_2 = pH, x_3 = salinity, x_4 = dissolved oxygen, x_5 = nitrite, x_6 = nitrate, x_7 = ammonia, x_8 = phosphate and x_9 = organic carbon. The regression was found significant ($p<.05$) indicating that the fitted regression explains a significant part (85.5%) of the variability in the data.

Table 41

Matrix of correlation between *Vibrio anguillarum* and environmental parameters studied from Valappu (Station I).

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	a 0.466594	1								
Column 3	-0.10025	-0.04205	1							
Column 4	0.092903	0.477579	-0.55237	1						
Column 5	0.068809	0.049922	0.63992	-0.16118	1					
Column 6	0.373477	0.364577	-0.0321	0.377438	0.023215	1				
Column 7	0.428216	0.329509	0.251576	0.121024	0.209368	0.895392	1			
Column 8	0.151038	0.211525	0.485211	-0.00265	0.221444	0.747428	0.822616	1		
Column 9	0.25513	0.075325	0.664754	-0.29731	0.512442	0.537052	0.725037	0.797955	1	
Column 10	-0.18544	-0.37967	0.070086	-0.42273	-0.16569	-0.56791	-0.55172	-0.35041	-0.38391	1

The matrix of correlation of *V.anguillarum* with the 9 environmental parameters is given in Table 41. Here *V anguillarum* was found to be positively correlated with temperature at 5% level.

Table 42

Matrix of correlation between *Vibrio fischeri* and environmental parameters studied from Station I.

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	b 0.590429	1								
Column 3	-0.14502	-0.04205	1							
Column 4	0.409883	0.477579	-0.55237	1						
Column 5	0.125223	0.049922	0.63992	-0.16118	1					
Column 6	0.091798	0.364577	-0.0321	0.377438	0.023215	1				
Column 7	-0.06991	0.329509	0.251576	0.121024	0.209368	0.895392	1			
Column 8	-0.13761	0.211525	0.485211	-0.00265	0.221444	0.747428	0.822616	1		
Column 9	-0.19829	0.075325	0.664754	-0.29731	0.512442	0.537052	0.725037	0.797955	1	
Column 10	0.114289	-0.37967	0.070086	-0.42273	-0.16569	-0.56791	-0.55172	-0.35041	-0.38391	1

The matrix of correlation of *V.fischeri* on the 9 environmental parameters showed significant positive correlation ($p<0.01$) with temperature only.

Table 43

Matrix of correlation between *Vibrio furnissii* and environmental parameters studied from Station I.

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Colun 10*
Column 1	1									
Column 2	-0.07962	1								
Column 3	0.187156	-0.04205	1							
Column 4	-0.2229	0.477579	-0.55237	1						
Column 5	-0.13752	0.049922	0.63992	-0.16118	1					
Column 6	0.361614	0.364577	-0.0321	0.377438	0.023215	1				
Column 7	0.326783	0.329509	0.251576	0.121024	0.209368	0.895392	1			
Column 8	a 0.489733	0.211525	0.485211	-0.00265	0.221444	0.747428	0.822616	1		
Column 9	a 0.546213	0.075325	0.664754	-0.29731	0.512442	0.537052	0.725037	0.797955	1	
Column 10	0.087537	-0.37967	0.070086	-0.42273	-0.16569	-0.56791	-0.55172	-0.35041	-0.38391	1

The matrix of correlation of *V.furnissii* on the 9 parameters showed significant positive correlation with ammonia and phosphate at 5% level.

Table 44

Matrix of correlation between *Vibrio campbellii* and environmental parameters studied from Station I

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	0.32145	1								
Column 3	-0.38078	-0.04205	1							
Column 4	a 0.489694	0.477579	-0.55237	1						
Column 5	-0.24205	0.049922	0.63992	-0.16118	1					
Column 6	0.149642	0.364577	-0.0321	0.377438	0.023215	1				
Column 7	0.0733	0.329509	0.251576	0.121024	0.209368	0.895392	1			
Column 8	-0.00086	0.211525	0.485211	-0.00265	0.221444	0.747428	0.822616	1		
Column 9	-0.4749	0.075325	0.664754	-0.29731	0.512442	0.537052	0.725037	0.797955	1	
Column 10	0.105227	-0.37967	0.070086	-0.42273	-0.16569	-0.56791	-0.55172	-0.35041	-0.38391	1

Table 44 gives the correlation of *V.campbellii* on the 9 environmental parameters. The species was found to be significantly positively correlated with salinity at 5% level.

The matrix of correlation between *V.parahaemolyticus*, *V.fluvialis*, *V. harveyi*, *V.metschnikovii*, *V.cincinnatiensis and V.vulnificus* isolated from Station I and the 9 environmental parameters was not found to be significantly correlated, showing that none of the environmental parameters had any influence over the density and distribution of the above mentioned *Vibrio* spp.

Table 45

Matrix of correlation between *Vibrio furnissii* and environmental parameters studied from Station II (KVK).

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	-0.19718	1								
Column 3	0.320676	-0.51836	1							
Column 4	-0.29874	0.442302	-0.33941	1						
Column 5	a 0.559998	-0.06401	0.371533	-0.25254	1					
Column 6	0.275834	0.329422	-0.2176	0.079505	0.235054	1				
Column 7	0.216496	0.169609	-0.14722	0.025605	0.192588	0.908501	1			
Column 8	0.226982	0.156257	-0.14899	-0.18005	0.137971	0.882879	0.814207	1		
Column 9	0.276684	-0.12986	0.023134	-0.54837	0.102599	0.524494	0.635218	0.711665	1	
Column 10	-0.11477	-0.06479	-0.49156	-0.34151	-0.04326	0.252078	0.268819	0.371115	0.371739	1

The matrix of correlation of *V.furnissii* on the 9 environmental parameters showed significant positive correlation ($P<0.05$) with dissolved oxygen only.

Table 46

Matrix of correlation between *Vibrio metschnikovii* and environmental parameters studied from Station II.

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	0.084378	1								
Column 3	-0.13464	-0.51836	1							
Column 4	a 0.5125	0.442302	-0.33941	1						
Column 5	-0.31574	-0.06401	0.371533	-0.25254	1					
Column 6	0.238346	0.329422	-0.2176	0.079505	0.235054	1				
Column 7	0.1437	0.169609	-0.14722	0.025605	0.192588	0.908501	1			
Column 8	0.1871	0.156257	-0.14899	-0.18005	0.137971	0.882879	0.814207	1		
Column 9	-0.13386	-0.12986	0.023134	-0.54837	0.102599	0.524494	0.635218	0.711665	1	
Column 10	-0.12114	-0.06479	-0.49156	-0.34151	-0.04326	0.252078	0.268819	0.371115	0.371739	1

Table 46 gives the correlation of *V.metschnikovii* with the 9 environmental parameters. Here the species was found to be significantly positively correlated ($p<0.05$) with salinity.

Table 47

Matrix of correlation between *Vibrio campbellii* and environmental parameters studied from Station II.

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	0.281296	1								
Column 3	c -0.63125	-0.51836	1							
Column 4	-0.00955	0.442302	-0.33941	1						
Column 5	0.036339	-0.06401	0.371533	-0.25254	1					
Column 6	0.311033	0.329422	-0.2176	0.079505	0.235054	1				
Column 7	0.320156	0.169609	-0.14722	0.025605	0.192588	0.908501	1			
Column 8	0.30166	0.156257	-0.14899	-0.18005	0.137971	0.882879	0.814207	1		
Column 9	0.174328	-0.12986	0.023134	-0.54837	0.102599	0.524494	0.635218	0.711665	1	
Column 10	a 0.45373	-0.06479	-0.49156	-0.34151	-0.04326	0.252078	0.268819	0.371115	0.371739	1

Table 47 gives the correlation of *V.campbellii* on the 9 environmental parameters. The species was found to be significantly positively correlated with pH and organic carbon at 1% and 5% level respectively.

The matrix of correlation between *V.anguillarum, V.parahaemolyticus, V.fluvialis, V.fischeri, V. harveyi, V. cincinnatiensis* and *V. vulnificus* isolated from Station II and the environmental parameters was not found to be significantly correlated showing that none of the parameters had any significant influence over the density and distribution of the *Vibrio* spp.

4.9. Seasonal Distribution of *Vibrio* spp. Isolated from Station 1

The predominant flora *V.anguillarum* recorded throughout the study period was found dominating the premonsoon season (33.96%) and monsoon season (33.33%), whereas the second predominant flora V.parahaemolyticus was found dominating during the post monsoon season (30.43%), the lowest count was observed during the pre monsoon season (13.21%) (Table 48). Maximum bacterial diversity was observed during the premonsoon season. (10strains) (Table 48).

V. fischeri was found to occur throughtout the period of study, post monsoon (8.70%) and monsoon (8.33%) (Table 48) with their maximum numbers occurring during the pre monsoon season (15.09%). The diversity of *Vibrio* species was found to be very low during the monsoon season when compared to pre and post monsoon seasons (Table 48). *V.fluvialis* was found to predominate during the premonsoon season while during the post monsoon season only one isolate was observed.

V. harveyi. V. metchnikovii, V.campbellii, V.cincinnatiensis and *V.vulnificus* were absent during the monsoon season (Table 48). *V. vulnificus* was isolated only during the pre monsoon season (n=1). *V. campbellii* dominated during the pre monsoon season (n=4).

Table 48

Seasonal distribution of *Vibrio* spp. Isolated from Station I

Post monsoon (n=23)			Pre monsoon (n=53)		
Species	No. of strains isolated	%	Species	No. of strains isolated	%
V.parahaemolyticus	7	30.43	*V. anguillarum*	18	33.96
V. harveyi	3	13.04	*V.fischeri*	8	15.09
V.cincinnatiensis	3	13.04	*V.parahaemolyticus*	7	13.21
V.anguillarum	2	8.70	*V.fluvialis*	6	11.32
V.furnissii	2	8.70	*V.campbellii*	4	7.55
V.fischeri	2	8.70	*V.furnissii*	3	5.66
V. metschnikovii	2	8.70	*V. metschnikovii*	3	5.66
V.fluvialis	1	4.35	*V.harveyi*	2	3.77
V.campbellii	1	4.35	*V.cincinnatiensis*	1	1.89
			V.vulnificus	1	1.89

Monsoon (n=12)		
Species	**No. of strains isolated**	**%**
V. anguillarum	4	33.33
V. furnissii	3	25
V. parahaemolyticus	3	25
V fischeri	1	8.33
V fluvialis	1	8.33

Table 49

Two way ANOVA of *Vibrio* with seasons at Station I

SUMMARY	*Count*	*Sum*	*Average*	*Variance*		
Row 1	10	57.4985	5.74985	5.657315		
Row 2	10	22.667	2.2667	6.192844		
Row 3	10	37.334	3.7334	3.205002		
Column 1	3	18.7495	6.249833	0.465223		
Column 2	3	15	5	1.776889		
Column 3	3	14.583	4.861	0.640963		
Column 4	3	7	2.333333	4.333333		
Column 5	3	15	5	7		
Column 6	3	14	4.666667	20.33333		
Column 7	3	9	3	9		
Column 8	3	10.667	3.555667	9.926296		
Column 9	3	7.5	2.5	5.25		
Column 10	3	6	2	12		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	61.16538	2	30.58269	6.856533	0.006114	3.554561
Columns	55.20976	9	6.134418	1.375315	0.269351	2.456282
Error	80.2867	18	4.460372			
Total	196.6618	29				

The table 49 gives the two way ANOVA of *Vibrio* spp with seasons at Station I. There was significant difference between seasons as far as *Vibrio* spp. was concerned ($P<0.01$). Pre monsoon season showed higher count and monsoon showed significantly lower counts.

4.10 Seasonal distribution of *Vibrio* spp. isolated from Station II (KVK).

V. anguillarum was the most frequently isolated *Vibrio* spp. With the maximum density being observed during the post monsoon season (44.44%). Unlike Valappu, *V. fischeri* was the second predominant *Vibrio* spp. isolated from KVK. It occurred throughout the period of study with the maximum count occurring during the pre monsoon and monsoon seasons (Table 50).

Maximum density in the *Vibrio sp.* was observed during the pre monsoon season (n=10). Food borne human pathogen, *V.parahaemolyticus* was observed during the three seasons with their maximum density during the post monsoon season (13.89%). The human pathogenic forms such as *V. fluvialis* and *V. furnissii* were observed through out the study period with *V. furnissii* dominating during the monsoon season (22.22%) (Table 50).

The lactose fermenting species of *Vibrio*, *V. vulnificus* was absent during the post monsoon season, while one isolate each was encountered during the other two seasons. Premonsoon season recorded high values for *V.metschnikovii* (9.09%). *V.cincinnatiensis* was observed exclusively during the premonsoon season (Table 50). *V. campbellii* and *V. harveyi* were absent during the post monsoon season and monsoon season respectively.

Table 50

Seasonal distribution of *Vibrio* spp. isolated from Station II

Post monsoon (n=36)			Pre monsoon (n=44)		
Species	No. of strains isolated	%	Species	No. of strains Isolated	%
V. anguillarum	16	44.44	*V. anguillarum*	11	25
V. parahaemolyticus	5	13.89	*V. fischeri*	9	20.45
V. fischeri	4	11.11	*V. fluvialis*	6	13.64
V. fluvialis	4	11.11	*V. parahaemolyticus*	5	11.36
V. harveyi	3	8.33	*V. metschnikovii*	4	9.09
V. furnissii	2	5.56	*V. cincinnatiensis*	3	6.82
V. metschnikovii	2	5.56	*V. furnissii*	2	4.55
			V. campbellii	2	4.55
			V. vulnificus	1	2.27
			V. harveyi	1	2.27

Monsoon (n=36)		
Species	No. of strains isolated	%
V. anguillarum	14	38.89
V. furnissii	8	22.22.
V. fischeri	7	19.44
V. parahaemolyticus	3	8.33
V. campbellii	1	2.78
V. vulnificus	1	2.78
V. metschnikovii	1	2.78
V.fluvialis	1	2.78

Table 51

Two-way ANOVA of *Vibrio* with seasons at Station II

SUMMARY	*Count*	*Sum*	*Average*	*Variance*		
Row 1	10	46.5	4.65	7.947222		
Row 2	10	34.179	3.4179	3.828875		
Row 3	10	26.437	2.6437	5.725366		
Column 1	3	12.145	4.048333	0.317008		
Column 2	3	15.334	5.111333	2.925296		
Column 3	3	16.5	5.5	0.75		
Column 4	3	13.25	4.416667	5.020833		
Column 5	3	9.887	3.295667	9.262256		
Column 6	3	17.5	5.833333	3.583333		
Column 7	3	0	0	0		
Column 8	3	5	1.666667	8.333333		
Column 9	3	10.5	3.5	9.25		
Column 10	3	7	2.333333	4.333333		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	20.47565	2	10.23783	2.74738	0.090931	3.554561
Columns	90.43803	9	10.04867	2.696619	0.034994	2.456282
Error	67.07514	18	3.726396			
Total	177.9888	29				

To study the seasonal variation of *Vibrio*. spp from Station II (KVK) two way ANOVA was carried out and it is presented in Table 51. There was significant difference between the count of bacterial species ($P<0.05$) but there was no significant difference between seasons. Significant higher counts of *V.metschnikovii* was noted followed by *V.parahaemolyticus*.

The *Aeromonas* spp. isolated during the period of study were *A. hydrophila*, *A.caviae*, and *A.sobria*.

Table 52

Biochemical reactions of *Aeromonas* and *Plesiomonas* spp. isolated from Station I

Biochemical tests	Reactions of *A.hydrophila* (n = 33)	%	Reactions of *A. caviae* (n=17)	%	Reactions of *A.sobria* (n = 13)	%	Reactions of *P.shigelloides* (n=4)	%
Gram stain	-	100	-	100	-	100	-	100
Motility	+	100	+	100	+	100	+	100
Brown pigment	-	100	-	100	-	100	-	100
Arginine dihydrolase	+	100	+	100	-	100	+	100
Lysine decarboxylase	d	84.85	d	88.24	d	76.92	+	100
Ornithine Decarboxylase	-	100	-	100	-	100	+	100
Indole	+	0	+	0	+	7.69	+	0
NO_3	+	78.79		76.47	+	76.92	+	75
Ammonia	+	90.91	+	100	+	100	+	75
Catalase	+	45.45	+	52.94	+	53.85	+	100
Oxidase	+	100	+	100	+	100	+	100
Acid from Lactose	d	96.97	d	76.47	d	100	d	100
Acid from Maltose	+	75.76	+	82.35	+	69.23	+	100
Acid from Mannitol	+	66.67	+	58.82	+	38.46	-	100
Acid from Sucrose	+	72.73	+	100	+	61.54	-	100
Acid from Glucose	+	100	+	100	+	100	+	100
Growth at 37°C	+	84.85	+	70.58	+	76.92	+	0
Voges Proskauer	+	18.18	-	100	d	30.77	-	100
Gas from glucose	+	12.12	-	100	+	15.38	-	100
H_2S	+	57.57	-	100	+	69.23	-	100
Urea se	-	100	-	100	-	69.23	-	100
Starch hydrolysis	+	60.61	+	100	+	46.15	-	50
Gelatinase	+	60.61	+	41.18	+	53.85	-	100
Citrate	d	36.36	d	94.12	d	46.15	-	100
Growth at 0% NaCl	+	100	+	100	+	100	+	100
Growth at 5% NaCl	-	78.79	-	29.41	-	38.46	-	100
Growth at 7% NaCl	-	66.67	-	47.05	-	46.15	-	100
Caesin	-	54.55	-	76.47	-	61.54	-	100
Resistance to 0/129	+	100	+	100	+	100	-	100
Aesculin hydrolysis	+	100	+	100	-	100	-	100

4.11 Biochemical reactions of *Aeromonas* isolated from Station 1

All the *Aeromonas* strains isolated were motile gram-negative rods, which were unable to produce brown pigment. All the strains of *A. hydrophila* and *A. caviae* hydrolysed arginine while all the thirteen strains of *A. sobria* failed to hydrolyse arginine. 84.85% of *A. hydrophila* and 76.92% of *A. sobria* hydrolysed

lysine while 88.24% of *A.caviae* failed to hydrolyse lysine. Ornithine was not hydrolysed by any of the strains.

All the *Aeromonas* spp. isolated were capable of producing cytochrome oxidase. Except 7.69% of *A. sobria* isolated from Valappu rest of the strains were unable to produce indole. 78.79% of *A. hydrophila*, 76.92% of *A. sobria* and 76.47% of *A. caviae* prouced nitrate. 100% of *A. caviae* and *A.sobria* produced ammonia while only 90.91% of *A. hydrophila* produced ammonia. 53.85% of *A. sobria*, 52.94% of *A. caviae* and 45.45% of *A.hydrophila* produced catalase (Table 52).

Fermentation of carbohydrates

100% of *A. sobria*, 96.97% of *A. hydrophila* and 76.47% of *A. caviae* failed to ferment lactose actively. 82.35% of *A.caviae*, 75.76% of *A. hydrophila* and 69.23% of *A. sobria* fermented maltose. Mannitol was fermented by 66.67% of *A.hydrophila*, 58.82% of *A. caviae* and 38.46% of *A. sobria*. All the strains of *A. caviae*, 72.73% of *A. hydrophila* and 61.54% of *A. sobria* fermented sucrose. All the 63 strains of *Aeromonas* spp. isolated fermented glucose without gas.

Temperature Tolerance

84.85% of *A. hydrophila* and 76.92% of *A. sobria* gave good growth at 37^0C while only 70.58% of *A. caviae* gave good growth at the same temperature. 30.77% of *A. sobria* and 18.18% of *A. hydrophila* produced acetyl methyl carbinol, whereas none of the strains of *A. caviae* could produce the same. 15.38% of *A. sobria* and 12.12% of *A. hydrophila* liberated gas from glucose whereas *A. caviae* strains were unable to do so.

69.23% of *A. sobria* and 57.57% *A.hydrophila* liberated hydrogen sulphide from L-cystine while none of *A. caviae* were able to produce hydrogen sulphide. Except 30.77% of *A.sobria* rest of the *Aeromonas* strains isolated from Valappu failed to hydrolyse urea. All the *A. caviae* strains hydrolysed starch whereas 39.39% of *A. hydrophila* and 53.85% of *A. sobria* failed to do so. 60.61% of *A. hydrophila*, 53.85% of *A. sobria* and 41.18% of *A. caviae* liquefied gelatin. 94.12% of

A.caviae failed to utilize citrate as sole carbon source while 46.15% of *A. sobria* and 36.36% of *A. hydrophila* utilized the same (Table 52).

Tolerance to salinity

All the 63 strains of *Aeromonas spp.* isolated from Valappu gave very good growth in the absence of sodium chloride. 61.54% of *A. sobria* could grow at 5% sodium chloride. 46.15% of *A. sobria* and 47.05% of *A. caviae* failed to grow at 7% sodium chloride.

Caesin hydrolysis was performed by 45.45% of *A. hydrophila*, 23.53% of *A. caviae* and 38.46% *A. sobria*. All the strains of *A. hydrophila* and *A. caviae* isolated in the present study hydrolysed aesculin. All the *Aeromonas* strains isolated were resistant to O/129.

Table 53

Antibiogram of *Aeromonas* spp. isolated from Station I

Species	Resistant	Intermediate	Sensitive
A.hydrophila (n=33)	A(29) P(29) N(23) E (14) C(10) S(7) T(7) G(3)	E(14) S(4) T(4) C(4) G(1) N(1)	G(29) S(22) C(19) T(16) N(9) E(5) P(4) A(4)
A.caviae (n=17)	A(15) P(14) T(8) C(7) N(7) S(5) E(3) G(1)	E(8) S(3) T(1) G(1) C(1)	G(15) N(10) S(9) C(9) T(8) E(6) P(3) A(2)
A.sobria (n=13)	P(12) A(11) N(10) T(5) S(4) E(4) C(3) G(1)	T(3) E(3) C(1)	G(12) S(9) C(9) E(6) T(5) N(3) A(2) P(1)

Table 54

Antibiotic sensitivity pattern of *Aeromonas* spp. isolated from Station I

Antibiotics	*A.hydrophila*			*A.caviae*			*A.sobria*		
	Resis-tance (%)	Interm-ediate (%)	Sens-itive (%)	Resis-tance (%)	Interm-ediate (%)	Sens-itive (%)	Resis-tance (%)	Interm-ediate (%)	Sens-itive (%)
Ampicillin (10mcg)	87.88	0	12.12	88.24	0	11.76	84.62	0	15.38
Tetracycline (30mcg)	21.21	12.12	66.67	47.06	5.88	47.06	38.46	23.08	38.46
Chloramphenicol (30 mcg)	30.30	12.12	57.58	41.18	5.88	52.94	23.08	7.69	69.23
Erythromycin (15mcg)	42.42	42.42	15.16	17.65	47.06	35.29	30.77	23.08	46.15
Gentamycin (10mcg)	9.09	3.03	87.88	5.88	5.88	88.24	7.69	0	92.31
Penicillin (10 units)	87.88	0	12.12	82.35	0	17.65	92.31	0	7.69
Streptomycin (10mcg)	21.21	12.12	66.67	29.41	17.65	52.94	30.77	0	69.23
Novobiocin (30 mcg)	69.70	3.03	27.27	41.18	0	58.82	76.92	0	23.08

The resisto types of *A. hydrophila*, the most frequently isolated *Aeromonas* sp. were A, P, N, E, C (Table 53). The species was found to be sensitive towards streptomycin and tetracycline (66.67%), gentamycin (87.88%) and chloramphenicol (57.58%). 42.42% of the strains exhibited intermediate resistance towards erythromycin. A clear homogeneity in the antimicrobial resistance pattern has been detected with a high percentage of the *Aeromonas sp.* exhibiting resistance towards ampicillin and penicillin (Table 54).

Susceptibility of *A. caviae* to eight antibiotics were tested. The resistance pattern was A, P, T, C, N. 88.24% of *A. caviae* were resistant to ampicillin (10mcg) while only 82.35% were resistant to penicillin, followed by 47.06% to tetracycline, 41.18% to chloramphenicol and novobiocin. Maximum sensitivity was exhibited towards gentamycin (88.24%). 47.06% of the strains showed intermediate resistance to erythromycin.

The thirteen strains of the fish pathogen *A. sobria* gave the following resistotypes, P, A, N. Maximum resistance was exhibited towards penicillin (92.31%) followed by ampicillin (84.62%) and novobiocin (76.92%). 23.08% of the strains showed intermediate resistance to erythromycin and tetracycline. Maximum sensitivity was found towards gentamycin (92.31%) and the least towards penicillin (7.69%) (Table 55).

Table 55

Biochemical reactions of *Aeromonas* and *Plesiomonas* spp. isolated from Station II

Biochemical tests	Reactions of *A.hydrophila* (n=30)	%	Reactions of *A. caviae* (n=18)	%	Reactions of *A.sobria* (n=12)	%	Reactions of *P.shigelloides* (n=3)	%
Gram stain	-	100	-	100	-	100	-	100
Motility	+	100	+	100	+	100	+	100
Brown pigment	-	100	-	100	-	100	-	100
Arginine dihydrolase	+	100	+	100	-	100	+	100
Lysine decarboxylase	+d	93.33	d+	88.88	+d	83.33	+	100
Ornithine Decarboxylase	-	100	-	100	-	100	+	100
Oxidase	+	100	+	100	+	100	+	100
Indole	+	0	+	5.56	+	8.33	+	0
Nitrite	+	83.33	+	55.56	+	83.33	+	100
Ammonia	+	96.67	+	94.44	+	100	+	100
Catalase	+	20	+	72.22	+	58.33	+	66.67

Acid from Lactose	d-	93.33	d+	5.56	d+	8.33	d-	100
Acid from Maltose	+	86.67	+	77.78	+	83.33	+	100
Acid from Mannitol	+	80	+	55.56	+	83.33	-	100
Acid from Sucrose	+	90	+	66.67	+	91.67	-	100
Acid from Glucose	+	90	+	88.89	+	91.67	+	100
Growth at 37^0C	+	90	+	72.22	+	83.33	+	66.67
Voges-proskauer	+	10	-	94.44	d+	8.33	-	100
Gas from glucose	+	20	-	100	+	0	-	100
H_2S	+	86.67	-	100	+	100	-	100
Urease	-	86.67	-	61.11	-	91.67	-	66.67
Starch hydrolysis	+	70	+	77.78	+	66.67	-	66.67
Gelatinase	+	63.33	+	50	+	83.33	-	100
Citrate	d+	43.33	d-	72.22	d+	41.67	-	100
Growth at 0% NaCl	+	76.66	+	100	+	100	+	100
Growth at 5% NaCl	-	66.67	-	55.56	-	41.67	-	100
Growth at 7% NaCl	-	63.33	-	55.56	-	50	-	100
Caesin	-	33.33	-	61.11	-	33.33	-	66.67
Resistance to 0/129	+	100	+	100	+	100	-	100
Aesculin hydrolysis	+	100	+	100	-	100	-	100

4.12 Biochemical reactions of *Aeromoas* spp. isolated from Station II

All the *Aeromonas* strains isolated from KVK were actively motile gram negative rods which could not produce brown pigment. Except for *A. sobria* which failed to hydrolyze arginine rest of the *Aeromonas sp.* hydrolysed the amino acids. 93.33% of *A. hydrophila*, 88.88% of *A. caviae* and 83.33% of *A. sobria* hydrolysed lysine. None of the *Aeromonas* strains could hydrolyse ornithine while all of them produced cytochrome oxidase.

Except for 8.33% of *A. sobria* and 5.56% of *A. caviae* none of the *Aeromonas* strains isolated from KVK produced indole. 83.33% of *A. hydrophila* and *A. sobria* produced nitrate from nitrite, while only 55.56% of *A. caviae* could produce nitrate. 100% of *A. sobria* liberated ammonia followed by 96.67% of *A. hydrophila* and 94.44% of *A. caviae*. 72.22% of *A.caviae* and 58.33% of *A.sobria* could liberate the enzyme catalase, while only 20% of *A. hydrophila* could produce the enzyme

Carbohydrate fermentation

93.33% of *A. hydrophila* could not ferment lactose while 5.56% of *A. caviae* and 8.33% of *A. sobria* could ferment lactose. 86.67% of *A. hydrophila* and 83.33% of *A. sobria* fermented maltose followed by 77.78% of *A.caviae*. 83.33% of *A. sobria* and 80% of *A. hydrophila* fermented mannitol while only 55.56% of *A. caviae* could utilize the sugar. 91.67% of *A.sobria* and 90% of *A. hydrophila* fermented sucrose while only 66.67% of *A. caviae* utilized sucrose. Percentage hydrolysis of glucose by *A. sobria* and *A. hydrophila* were silimar to that of sucrose while *A. caviae* showed variation (88.89%) (Table 55).

Temperature tolerance

90% of *A. hydrophila*, 83.33% of *A. sobria* and 72.22% of *A. caviae* gave good growth at 37^{0}C. 10% of *A. hydrophila* and 8.33% of *A. sobria* produced acetyl methyl carbinol, while only 5.56% of *A. caviae* could produce the same. None of the *A. sobria* and *A. caviae* strains isolated from KVK liberated gas from glucose while 20% of *A. hydrophila* produced gas from glucose. All the 12 strains of *A. sobria* liberated hydrogen sulphide, while only 86.67% of *A. hydrophila* produced the gas. None of *A. caviae* strains produced hydrogen sulphide from L-cystine. Except for 8.33% of *A. sobria*, 13.33% of *A. hydrophila* and 38.89% of *A. caviae* rest of the *Aeromonas* strains failed to produced urea. 70% of *A. hydrophila*, 77.78% of *A. caviae* and 66.67% of *A. sobria* hydrolysed starch. 83.33% of *A. sobria* and 63.33% of *A. hydrophila* hydrolyzed gelatin while only 50% of *A. caviae* could liquefy gelatin. 43.33% of *A. hydrophila* and 41.67% of *A. sobria* could utilize citrate while only 27.78% of *A. caviae* utilized citrate as the sole source of carbon.

Tolerance to salinity

Except for 23.34% of *A. hydrophila* all the *Aeromonas* strains isolated in the present study gave good growth in the absence of sodium chloride. 66.67% of *A. hydrophila*, 55.56% of *A. caviae* and 41.67% of *A. sobria* were unable to grow at 5% salinity. 63.33% of *A.hydrophila*, 55.56% of *A. caviae* and 50% of *A. sobria* could not grow at 7% sodium chloride (Table 55).

66.67% of *A. hydrophila* and *A. sobria* hydrolysed casein while only 38.89% of *A. caviae* could hydrolyze caesin. Except for the 12 strains of *A. sobria* isolated from KVK the rest of the *Aeromonas* strains hydrolysed aesculin. All the *Aeromonas* strains isolated in the study were resistant to O/129.

Table 56

Antibiogram of *Aeromonas* spp. isolated from Station II

Species	Resistant	Intermediate	Sensitive
A.hydrophila (n=30)	P(27) A(25) E(11) T(9) C(7) G(4) S(3) N(2)	T(6) E(3) C(2) N(1) S(1)	G(26) S(26) C(21) E(16) T(15) N(9) A(5) P(3)
A.caviae (n=18)	A(16) P(16) E(11) C(9) N(9) T(8) S(5) G(4)	C(4) T(3) S(2) E(2) N(1)	G(14) S(11) N(8) T(7) E(5) C(5) P(2) A(2)
A.sobria (n=12)	A(10) P(10) N(6) E(4) T(3) S(2)	T(3) E(2) C(2) N(2)	G(12) C(10) S(10) T(6) E(6) N(4) P(2) A(2)

Table 57

Antibiotic sensitivity pattern of *Aeromonas* spp. isolated from Station II

Antibiotics	*A.hydrophila*			*A.caviae*			*A.sobria*		
	Resi-stance (%)	Inter-mediate (%)	Sens-itive (%)	Resi-stance (%)	Inter-mediate (%)	Sens-itive (%)	Resi-stance (%)	Inter-mediate (%)	Sens-itive (%)
Ampicillin (10mcg)	83.33	0	16.67	88.89	0	11.11	83.33	0	16.67
Tetracycline (30mcg)	30	20	50	44.44	16.67	38.89	25	25	50
Chloramphenicol (30 mcg)	23.33	6.67	70	50	22.22	27.78	0	16.67	83.33
Erythromycin (15mcg)	36.67	10	53.33	61.11	11.11	27.78	33.33	16.67	50
Gentamycin (10mcg)	13.33	0	86.67	22.22	0	77.78	0	0	100
Penicillin (10units)	90	0	10	88.89	0	11.11	83.33	0	16.67
Streptomycin (10mcg)	10	3.33	86.67	27.78	11.11	61.11	16.67	0	83.33
Novobiocin (30 mcg)	66.67	3.33	30	50	5.56	44.44	50	16.67	33.33

Antibiotic Sensitivity

The pattern of drug sensitivity of *Aeromonas* species isolated from KVK is given in table 57. The water borne pathogen *A. hydrophila* exhibited the following resistotypes, P, A, . E. 90% of the strains were resistant to penicillin followed by 83.33% to ampicillin, 66.67% to novobiocin and 36.67% to erythromycin.

A.caviae the prevalent species in polluted waters exhibited the below given sensitivity pattern, G, S, N, T. *A. caviae* gave maximum sensitivity to gentamycin (77.78%), followed by streptomycin (61.11%). The resistotypes were A, P, E, C. 88.89%, of the strains were resistant to ampicillin and penicillin, while 50%

were resistant to chloramphenicol and novobiocin. 22.22% showed intermediate resistance to chloramphenicol.

The fish pathogenic strain *A. sobria* was highly sensitive towards gentamycin (100%), followed by 83.33%, to streptomycin and chloramphenicol. 50% of the strains were sensitive to erythromycin and tetracycline. 83.33% of the strains were resistant to ampicillin and pencillin followd by 50% to novobiocin. 25% of *A. sobria* exhibited intermediate resistance to tetracycline.

Table 58

ANOVA table showing the significance of the fitted multiple regression of *A.hydrophila* and the environmental parameters studied from Station I

SUMMARY OUTPUT						
Regression Statistics						
Multiple R	0.910684					
R Square	0.829345					
Adjusted R Square	0.637358					
Standard Error	1.097281					
Observations	18					
ANOVA						
	df	*SS*	*MS*	*F*	*Significance F*	
Regression	9	46.81039	5.201155	4.319804	0.025627	
Residual	8	9.632205	1.204026			
Total	17	56.4426				

The multiple regression of *A.hydrophila* on the 9 environmental parameters was significant (P<0.05). The explained variability by the fitted regression was 82.9%. In all the remaining species, the fitted regression was not significant at 5% level at both the stations.

The matrix of correlation of *A.sobria* and *P.shigelloides* showed positive correlation with the environmental parameters at Valappu while rest of the strains gave no correlation.

Table 59

Matrix of correlation between *A.sobria* and the environmental parameters from Station I

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	-0.1406	1								
Column 3	-0.31547	-0.04205	1							
Column 4	a 0.558537	0.477579	-0.55237	1						
Column 5	0.021455	0.049923	0.639921	-0.16118	1					
Column 6	0.252395	0.364579	-0.0321	0.377438	0.023215	1				
Column 7	0.129556	0.329512	0.251573	0.121023	0.209368	0.895392	1			
Column 8	-0.01866	0.211529	0.485209	-0.00265	0.221444	0.747428	0.822616	1		
Column 9	-0.2371	0.075329	0.664754	-0.29731	0.512442	0.537052	0.725038	0.797955	1	
Column 10	-0.29704	-0.37967	0.070087	-0.42273	-0.16569	-0.56791	-0.55172	-0.35041	-0.38391	1

Table 59 shows the significant correlation (P<.05) between *A.sobria* and salinity.

4.13 Bio-chemical reactions of *Plesiomonas shigelloides* isolated from both the stations

The *Plesiomonas* strains isolated from both the stations were gram negative, actively motile forms, which could not produce brown pigment. 100% of the *Plesiomonas* strains isolated in the present study hydrolyzed the amino acids

arginine, lysine and ornithine. All the strains produced cytochrome oxidase, while none of the strains could produce indole. 100% of the strains from KVK produced nitrate and ammonia while only 75% of the strains from Valappu produced the same. All the strains of *Plesiomonas* isolated from Valappu produced catalase while only 66.67% of the strains from KVK produced catalase.

Fermentation of carbohydrates

None of the *P.shigelloides* strains hydrolyzed lactose. Maltose was hydrolyzed by all the strains isolated. Mannitol and sucrose were not hydrolyzed while all the strains hydrolyzed glucose.

Temperature tolerance

None of the strains isolated from Valappu could tolerate 37^0C while 66.67% of the strains from KVK grew at 37^0C.

100% of the strains isolated throughout the study period failed to produce acetyl methyl carbinol, gas from glucose and hydrogen sulphide.

100% of the strains from Valappu (Table 52) and 66.67% of the strains isolated from KVK (Table 55) failed to utilize urea. Starch was hydrolyzed by 50% of the strains from Valappu and 33.33% of the strains from KVK. None of the strains isolated in the present study hydrolyzed gelatin nor did they utilize citrate.

Salinity Tolerance

All the strains gave good growth in the absence of sodium chloride, while none of them grew at 5% and 7% sodium chloride.

All the strains from Valappu and 66.67% of the strains from KVK failed to produce casein. None of the strains isolated in the present study hydrolysed aesculin. The 7 strains of *P. shigelloides* isolated were sensitive to O/129.

Table 60

Antibiogram of *Plesiomonas shigelloides* isolated from both the stations

Valappu	Resistant	Intermediate	Sensitive
Plesiomonas shigelloides (n=4)	S(4) A(3) N(3) G(2) E(2) P(1)	P(3)	C(4) T(4) E(2) G(2) N(1) A(1)

KVK	Resistant	Intermediate	Sensitive
Plesiomonas shigelloides (n=3)	S(3) A(2) E(2) G(2) N(2) P(1)	P(2)	T(3) C(3) A(1) E(1) G(1) N(1)

Table 61

Antibiotic sensitivity pattern of *P.shigelloides* isolated from both the Stations

Station I (Valappu) **Station II KVK**

Antibiotics	*P.shigelloides*			*P.shigelloides*		
	Resistance (%)	Intermediate (%)	Sensitive (%)	Resistance (%)	Intermediate (%)	Sensitive (%)
Ampicillin (10mcg)	66.67	0	33.33	75	0	25
Tetracycline (30mcg)	0	0	100	0	0	100
Chloramphenicol (30 mcg)	0	0	100	0	0	100
Erythromycin (15mcg)	66.67	0	33.33	50	0	50
Gentamycin (10mcg)	66.67	0	33.33	50	0	50
Penicillin (10 units)	33.33	66.67	0	25	75	0
Streptomycin (10mcg)	100	0	0	100	0	0
Novobiocin (30 mcg)	66.67	0	33.33	75	0	25

Antibiotic sensitivity

The antibiotic sensitivity of the *P. shigelloides* strains isolated from Valappu were studied. Multiple drug resistance was noticed with resistotypes being S, A, N, G, E, P (Table 60). 33.33% of the strains exhibited sensitiveness towards ampicillin, erythromycin, gentamycin and novobiocin. 66.67% of *P.shigelloides* were resistant to ampicillin, erythromycin, gentamycin and novobiocin. All the strains isolated were resistant to streptomycin. 66.67% of the strains exhibited intermediate resistance to penicillin (Table 61).

The sensitivity pattern of *P.shigelloides* strains isolated from KVK was T, C, G, A, N, E. 100% of the strains isolated from KVK were sensitive to tetracycline and chloramphenicol, while 50% were sensitive to erythromycin and gentamycin. 75% of the strains exhibited intermediate resistance to penicillin. None of the strains were sensitive to streptomycin. 75% of the strains were resistant to ampicillin and novobiocin, followed by 50% to erythromycin and gentamycin (Table 61).

Table 62

Matrix of correlation between *P.shigelloides* and the environmental parameters from Station I

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	0.029781	1								
Column 3	0.449685	-0.04205	1							
Column 4	-0.46992	0.477579	-0.55237	1						
Column 5	a 0.472696	0.049923	0.639921	-0.16118	1					
Column 6	-0.2553	0.364579	-0.0321	0.377438	0.023215	1				
Column 7	-0.03357	0.329512	0.251573	0.121023	0.209368	0.895392	1			
Column 8	0.019932	0.211529	0.485209	-0.00265	0.221444	0.747428	0.822616	1		
Column 9	0.206159	0.075329	0.664754	-0.29731	0.512442	0.537052	0.725038	0.797955	1	
Column 10	0.340293	-0.37967	0.070087	-0.42273	-0.16569	-0.56791	-0.55172	-0.35041	-0.38391	1

There was significant correlation (P<0.05) between *P.shigelloides* count and dissolved oxygen (Table 62).

Table 63

Matrix of correlation between *P.shigelloides* and environmental parameters from Station II

	Column 1	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	*Column 7*	*Column 8*	*Column 9*	*Column 10*
Column 1	1									
Column 2	-0.01447	1								
Column 3	0.128591	-0.51836	1							
Column 4	a -0.52634	0.4423	-0.3394	1						
Column 5	0.235006	-0.06401	0.37153	-0.25254	1					
Column 6	0.187559	0.329422	-0.2176	0.079505	0.235054	1				
Column 7	0.296056	0.169609	-0.14722	0.025605	0.192588	0.9085	1			
Column 8	0.272747	0.156259	-0.14899	-0.18005	0.137971	0.882879	0.814207	1		
Column 9	0.401952	-0.12986	0.023129	-0.54837	0.102599	0.524494	0.635219	0.711665	1	
Column 10	0.430389	-0.06479	-0.49157	-0.34151	-0.04326	0.252078	0.268819	0.371115	0.37174	1

There was significant negative correlation of *P.shigelloides* (P<0.05) with salinity (Table 63).

Table 64

Percentage composition of *Aeromonas* spp. isolated from the sampling sites

Species	*No. of isolates*	Percentage
A.hydrophila	63	51.22
A.caviae	35	28.46
A.sobria	25	20.33

Table 64 gives the total percentage composition of *Aeromonas sp.* isolated from both the sites of study. The three commonly occurring motile

Aeromonas spp., were isolated throughout the study period through there were variations in their percentage distribution.

A. hydrophila, the commonly occurring water borne pathogen was the most frequently isolated *Aeromonas* sp., contributing 51.22% of the total (Table 64). *A. caviae* the indicator of faecal pollution was the second predominant form (28.46%). This was followed by the fish pathogenic form *A. sobria* forming 20.33% of the total *Aeromonas* isolates.

Table 65

Percentage composition of *Aeromonas* spp. isolated from Station I

Species	No.of Isolates (n = 63)	Percentage
A.hydrophila	33	52.381
A.caviae	17	26.98
A.sobria	13	20.635

Table 66

Percentage composition of *Aeromonas* spp. isolated from Station II

Species	No.of Isolates (n = 60)	Percentage
A.hydrophila	30	50
A.caviae	18	30
A.sobria	12	20

Table 65 gives the percentage distribution of *Aeromonas spp.* isolated from Valappu. Here also *A. hydrophila* was the predominant sp. isolated (52.38%) followed by *A. caviae* (26.98%) and *A. sobria* (20.63%). Table 66 shows that at KVK too, *A. hydrophila* formed 50% of the total *Aeromonas sp.* isolated showing that at both the sites *A. hydrophila* formed 50% of the total *Aeromonas* sp. isolated. At KVK, *A. caviae* formed 30% and *A.sobria* 20%.

Table 67

Seasonal distribution of *Aeromonas* spp. isolated from Station I

	Post monsoon (n=16)	Monsoon (n=12)	Premonsoon (n=35)
A.hydrophila	8 (50%)	7 (58.33%)	18 (51.43%)
A.caviae	6 (37.5%)	3 (25%)	8 (22.86%)
A.sobria	2 (12.5%)	2 (16.67%)	9 (25.71%)

4.14 Seasonal distribution of *Aeromonas spp.* isolated from Valappu

The most frequently isolated species *A.hydrophila* was found to occur during all the seasons, contributing 50% during the post monsoon season, 58.33% during the monsoon and 51.43% during the pre monsoon season (Table 67). The second predominant flora, *A.caviae* was found to contribute 37.5% during the post monsoon season, while they formed 22.86% during the pre monsoon and 25% during the monsoon season. *A.sobria* was found predominating during the pre monsoon season (25.71%) followed by the monsoon (16.67%) and post monsoon seasons (12.5%) (Table 67).

Table 68

Seasonal distribution of *Aeromonas* spp. isolated from Station II

	Post monsoon (n=17)	Monsoon (n=11)	Premonsoon (n=32)
A.hydrophila	10 (58.82%)	5 (45.45%)	15 (46.88%)
A.caviae	3(17.65%)	5 (45.45%)	10 (31.25%)
A.sobria	4 (23.53%)	1 (9.09%)	7 (21.88%)

4.15 Seasonal distribution of *Aeromonas* spp. isolated from KVK

The predominant flora *A.hydrophila* recorded throughout the period of study was found dominating all the 3 seasons, post monsoon (58.82%), pre monsoon (46.88%) and monsoon season (45.45%). *A. caviae* was also isolated during all the 3 seasons but with maximum intensity during the monsoon (45.45%) (Table68), followed by the pre monsoon season (31.25%). Minimum strains of *A. caviae* were isolated during the post monsoon season (17.65%). *A. sobria* were frequently isolated during the post monsoon (23.53%) and pre monsoon period (21.88%) while they contributed only 9.09% during the monsoon period.

To study the seasonal variation of *Aeromonas* spp.from both the stations two-way ANOVA were carried out. There was no significant difference between seasons and between spp.

4.16 Disease Symptoms

Fishes exhibiting disease symptoms were collected from the culture ponds and the epizootological agents were isolated for pathogenicity assay.

The symptoms include turbidity of the eyes, exophthalmia (Plate9), oedema, melanization, reddening and swelling of the anus (Plate 10) and leakage of yellow viscous matter. Loss of scales, anaemia (Plate 11) and colour fading was noted in extreme chronic cases. Regardless of the surface pathological changes, the

intestine usually underwent intense inflammation. The stomach was extremely flaccid with the gall bladder exhibiting a dark reddish brown colour.

4.17 Infectivity trials Signs and Pathology

The intraperitoneal injection of *V. anguillarum* at 10^3cells/animal in *O. mossambicus* exhibited stress with melanization of the skin and slight erosion of the caudal fin, 48 hours post infection. After 96 hours total pigmentation was noted. Intraperitoneal injection of *V. anguillarum* at 10^4cells/animal in *O.mossambicus* caused pigmentation, sluggish movement and inflammation of the abdominal cavity 72 hr post injection. Intraperitoneal injection of *V. anguillarum* at 10^5cells/animal led to inflammation of the abdomen, haemorrhage at the base of the caudal fin (Plate 12), abnormal swimming behaviour and poor appetite. One of the fishes died 24 hr post injection. Intraperitoneal injection at 10^6cells/animal led to corneal opacity, swelling of the vent, extrusion of yellow fluid from the anus and loss of scales. Mortality was observed 18 hr post injection. The LD_{50} was 9.3 x 10^6cfu/fish. The dead fish exhibited enlargement of the intestine with accumulation of a yellow viscous fluid (Plate13) and enlargement of the gall bladder (Plate14). In some fishes total disintegration of internal organs was noticed (Plate15).

Injection of *V. anguillarum* into the eye socket at 10^6cells/fish caused exophthalmia within 12 hr. At 24 hr post injection, the fishes exhibited melanization with total loss of sight and degradation of the eye socket (Plate16). Slow mortality was observed.

Bath exposure of *V. anguillarum* at 10^6cells/ml in caudal cliffed fishes caused abnormal swimming behaviour, melanization and loss of appetite. Mortality was not noticed.

In all infectivity trials, control animals were injected with PBS. Control fishes and experimental fishes were periodically sampled for *V. anguillarum* isolation (Plate 17). Koch' s postulates were satisfied by the reisolation of the injected bacteria from the eyes, stomach and intestine of infected fishes.

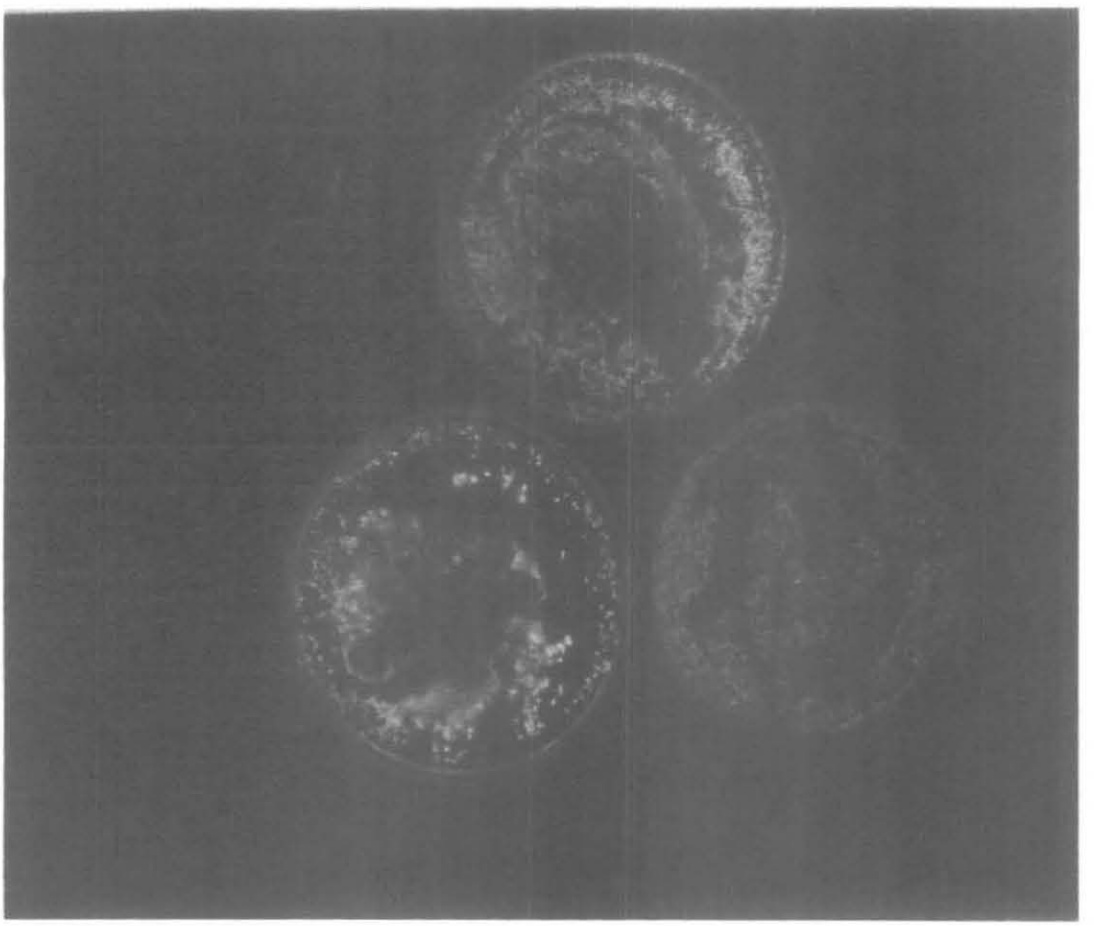

Plate 8 Bioluminescence exhibited by the pathogenic bacteria *V. harveyi* from *O. mossambicus* intestine

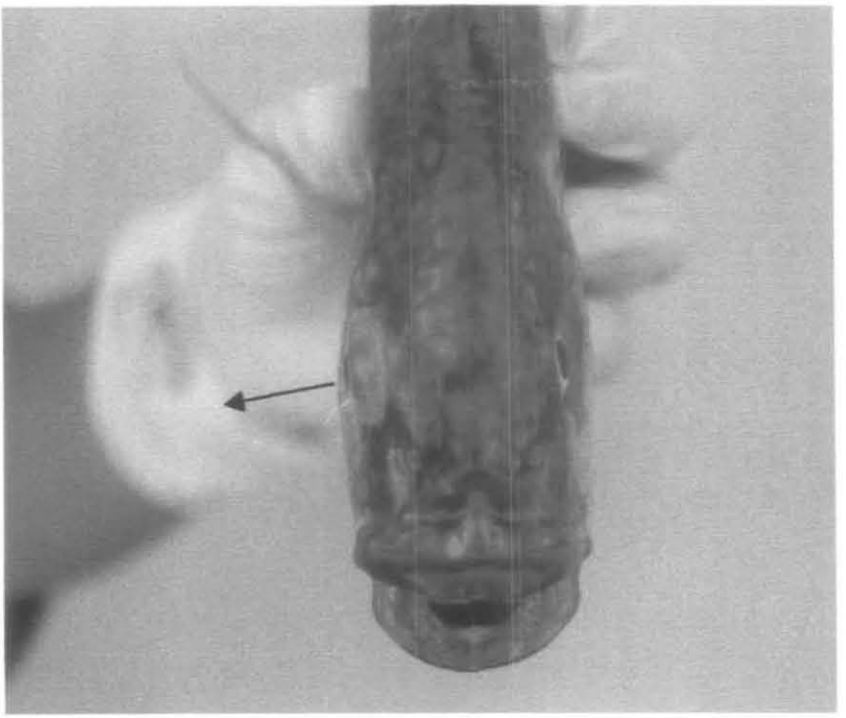

Plate 9 Exophthalmia exhibited by the cultured fish

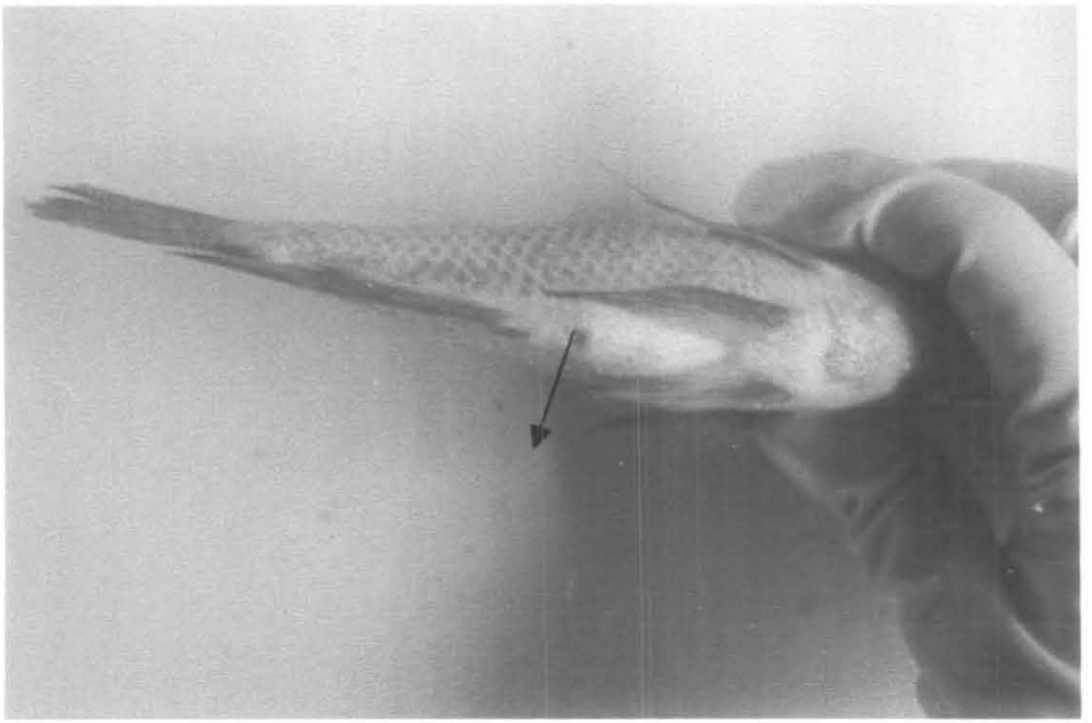

Plate 10 Swelling of the anus in culture fish collected during the study

Plate 11 Loss of scales andhaemorrhage at the base of the caudal fin in experimental fish.

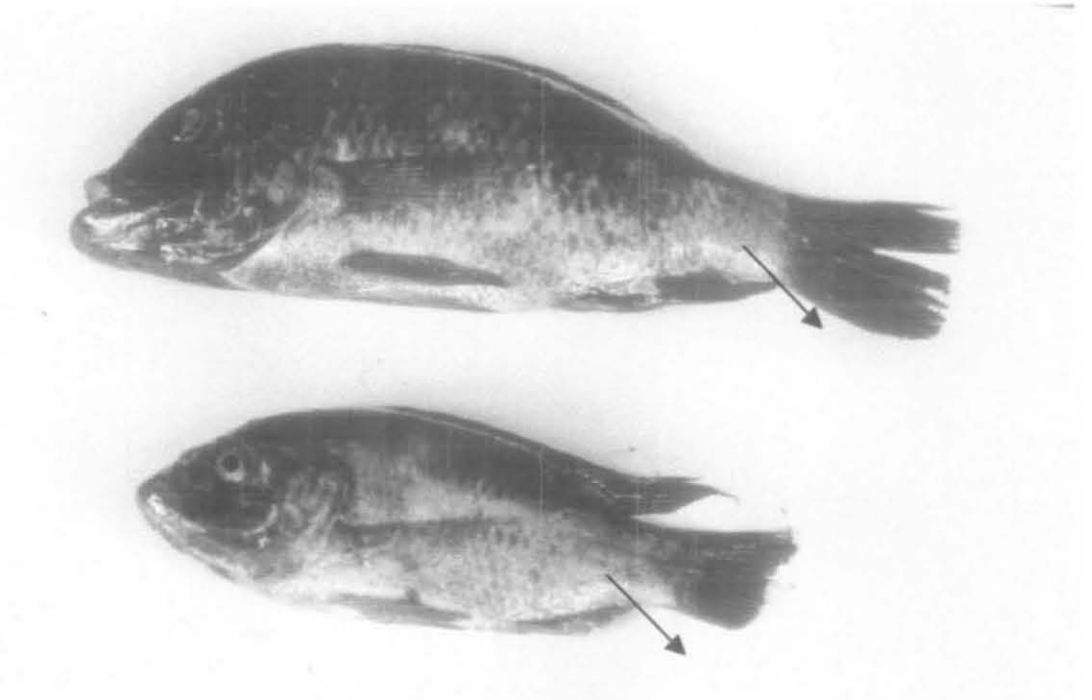

Plate 12 Melanization and haemorrhage at the base of the caudal fin in experimental fish

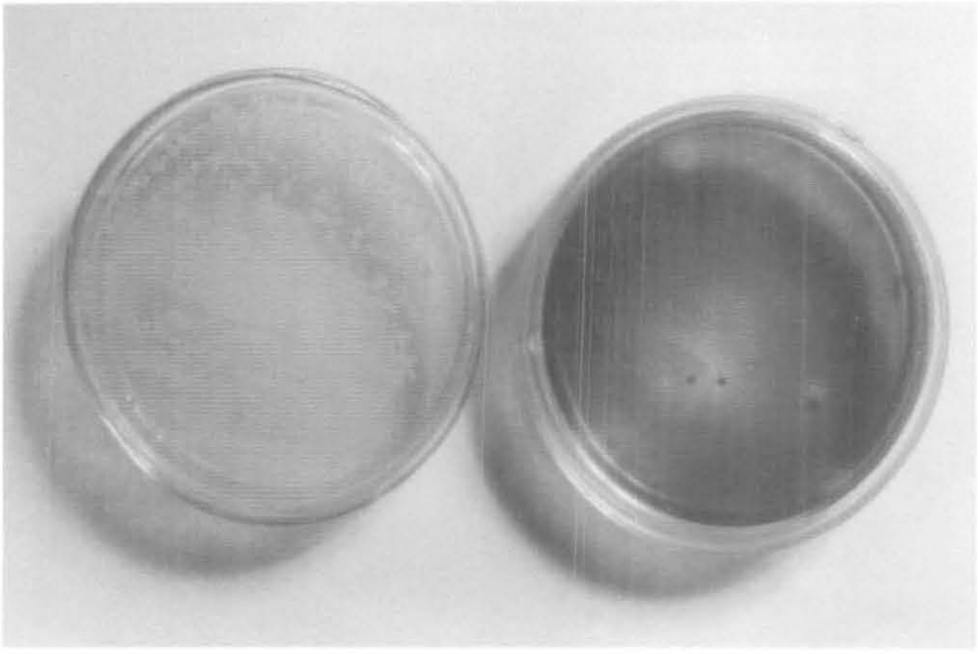

Plate 13 Accumulation of yellow viscous fluid in the intenstine

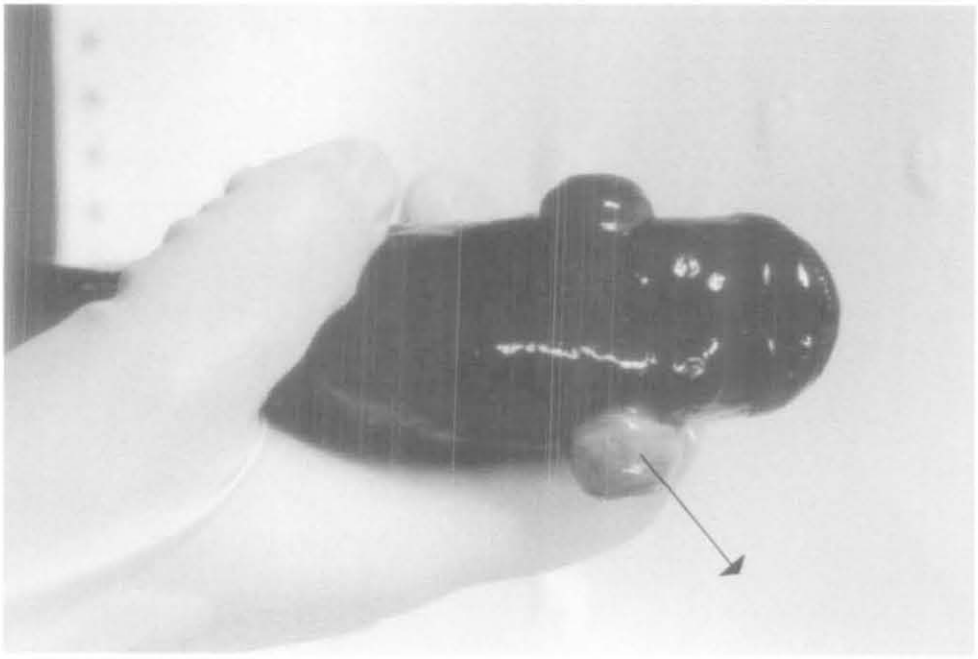

Plate 14 Enlargement of gall bladder in experimental fish

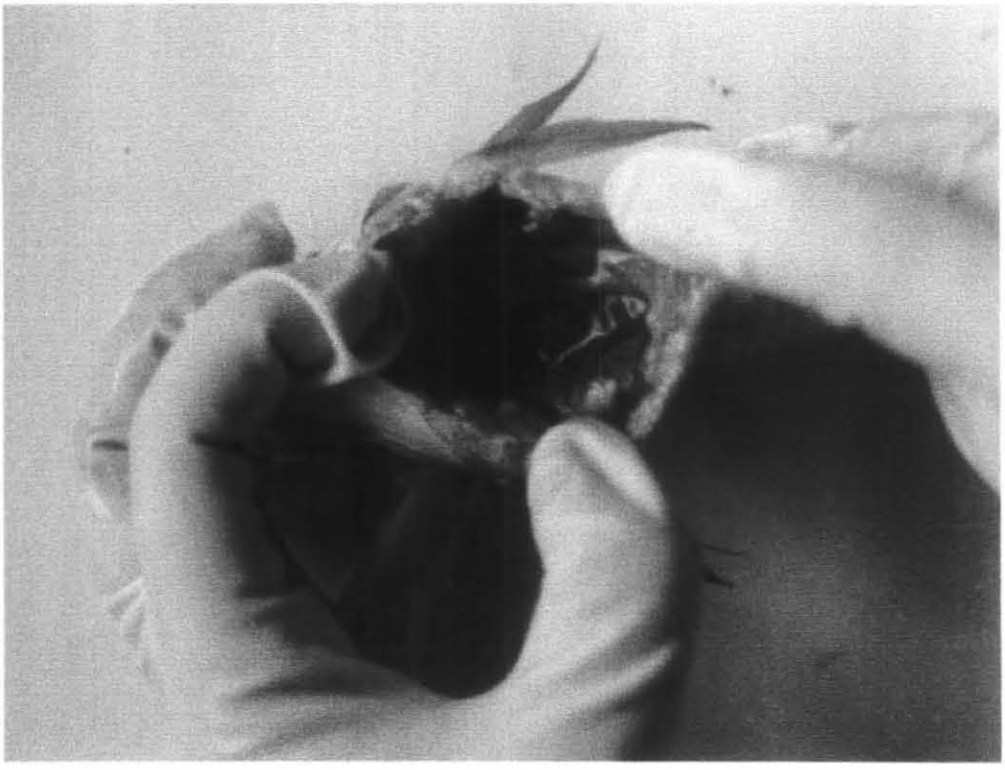

Plate 15 Total disintegration of internal organs

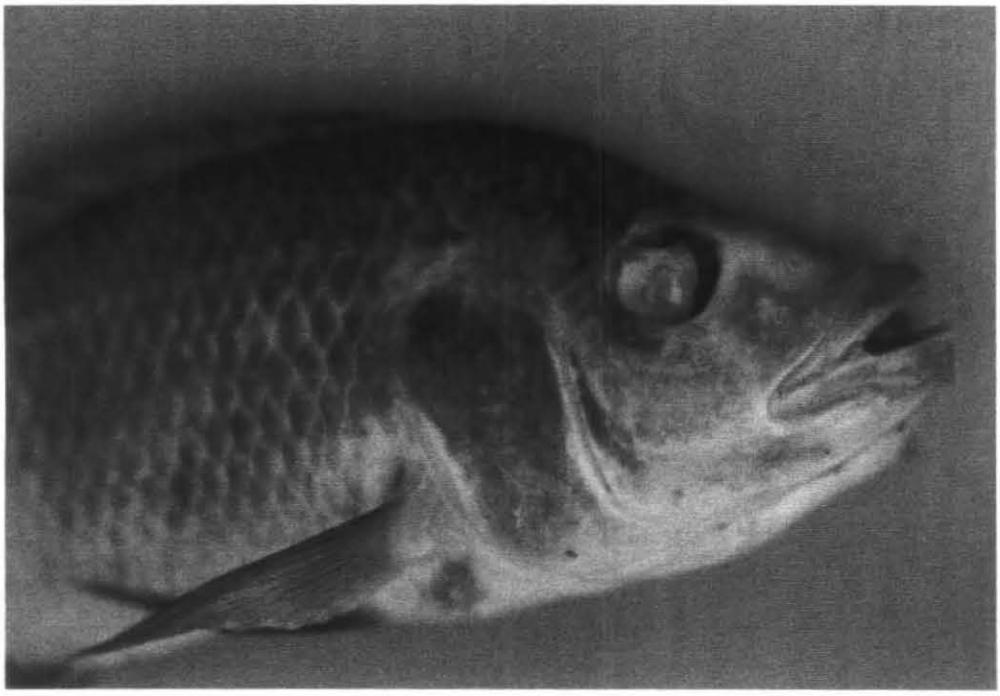

Plate 16 Disintegration of eye socket due to experimentally induced V. anguillarum infection

4.17.1 Infectivity trials with *V. fluvialis* signs and gross pathology

Intra peritoneal injection of 10^3 cells/ml of *V. fluvialis* in *O.mossambicus* showed slight stress and melanization around the injected area loss of appetite and sluggish movement after 24 hours but no mortality occurred.

Intraperitoneal injection of 10^4cells/ml of *V.fluvialis* caused stress and loss of appetite within 24 hours. Slight inflammation of the abdomen was observed which disappeared within 48 hours and the fishes started feeding normally.

10^5cells/ml of *V. fluvialis* when injected intraperitoneally caused stress with total melanization, irregular swimming movement and loss of appetite, but no mortality was recorded. Inoculation of 10^6cells/ml of *V. fluvialis* in eye socket caused melanization, exophthalmia and corneal opacity within 24 hours with no other stress symptoms.

10^6cells/ml of intra peritoneal injection in *O.mossambicus* caused melanization, fin erosion, inflamed abdomen and slight abnormal swimming movements.

Infectivity trials were carried out in caudal cliffed fishes maintained in 15ltr of water with 10^6cells/ml of thick suspension of *V. fluvialis*. The cliffed area showed slight pigmentation with the fish exhibiting irregular swimming movements for 48 hours. No mortality occurred and the fish returned to normalcy after 48 hours of experimentation.

In all infectivity trials, control animals were injected with PBS. Eyes, stomach and intestine of control as well as experimental fishes were periodically sampled for *V. fluvialis*.

4.17.2 Infectivity trials with *A. hydrophila* gross signs and pathology

Intraperitoneal injection of 10^3cells/ml of *A. hydrophila* in *O.mossambicus* caused melanization and stress in fishes, with loss of appetite and irregular movement but no mortality was recorded.

10^4cells/ml of *A. hydrophila* when injected intraperitoneally caused stress and dark pigmentation with loss of scales, melanization and loss of appetite.

10^5cells /ml of *A. hydrophila* when injected intraperitoneally caused inflammation of the abdominal cavity, corneal opacity, loss of scales, release of slimy exudates from the injected area, dark pigmentation, loss of scales and fin erosion (Plate 18).

Inoculation of 10^6 cells/ml of *A. hydrophila* in eye socket caused exophthalmia in both the eyes and total mortality occurred within 48 hours. (LD_{50} value was 8.5 x 10^6 cfu/fish) with total melanization (Plate 19).

Intraperitoneal injection of 10^6cells/ml of *A. hydrophila* caused 50% mortality within 24 hours with haemorrhage at the base of the caudal fin (Plate 20), fin erosion, total liquefaction of internal organs and melanization (LD_{50} was 8.0 x 10^6 cfu/fish).

Infectivity trials were carried out in caudal cliffed fishes maintained in 15ltr. of water. 10^6cells/ml of thick suspension of *A. hydrophila* was maintained for 7 days but no mortality occurred. The fishes exhibited irregular swimming movements for 2 days but returned to normalcy later.

Control was maintained for all experiments using PBS for the trials. Control as well as experimental fishes were observed regularly for *A.hydrophila*.

4.17.3 Infectivity trials with *A. caviae*, gross signs and pathology

A.caviae when injected intraperitoneally at 10^3, 10^4 and 10^5, 10^6cells/ml in *O.mossambicus* produced only a slight melanization in the injected area due to septicaemic conditions with no other changes. The same result was obtained when *A. caviae* was injected in the eye socket as well as when exposed to bath challenge at 10^6cells/ml in caudal cliffed *O.mossambicus* maintained in 15ltr tanks. Control was also maintained giving intraperitoneal injections with PBS.

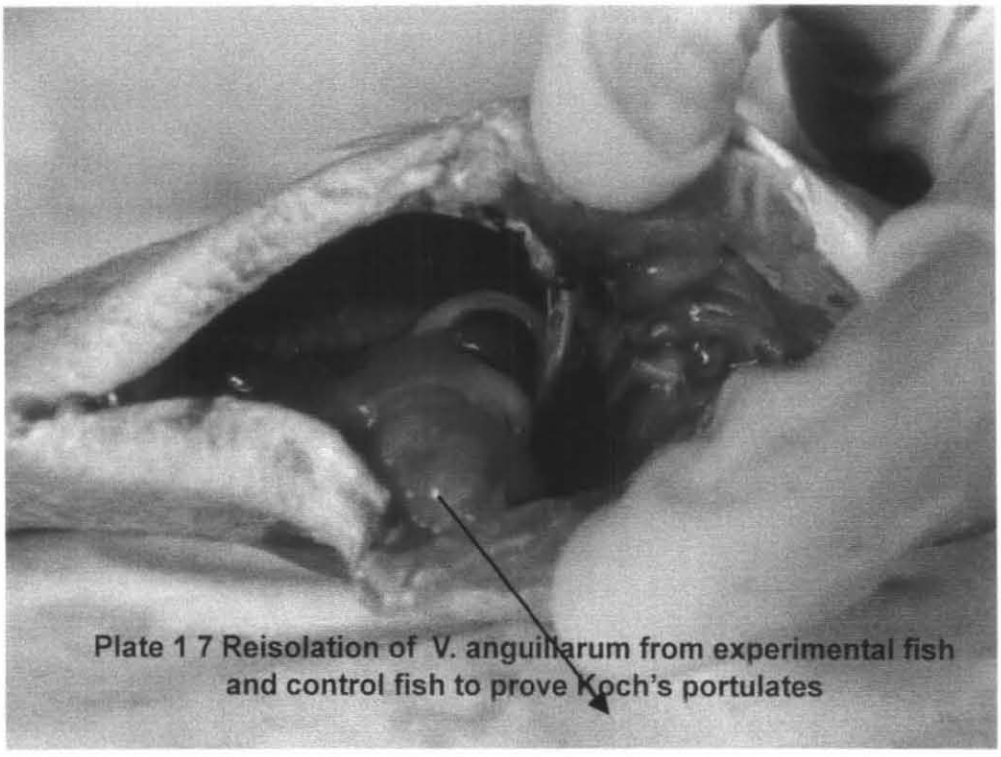

Plate 1 7 Reisolation of V. anguillarum from experimental fish and control fish to prove Koch's portulates

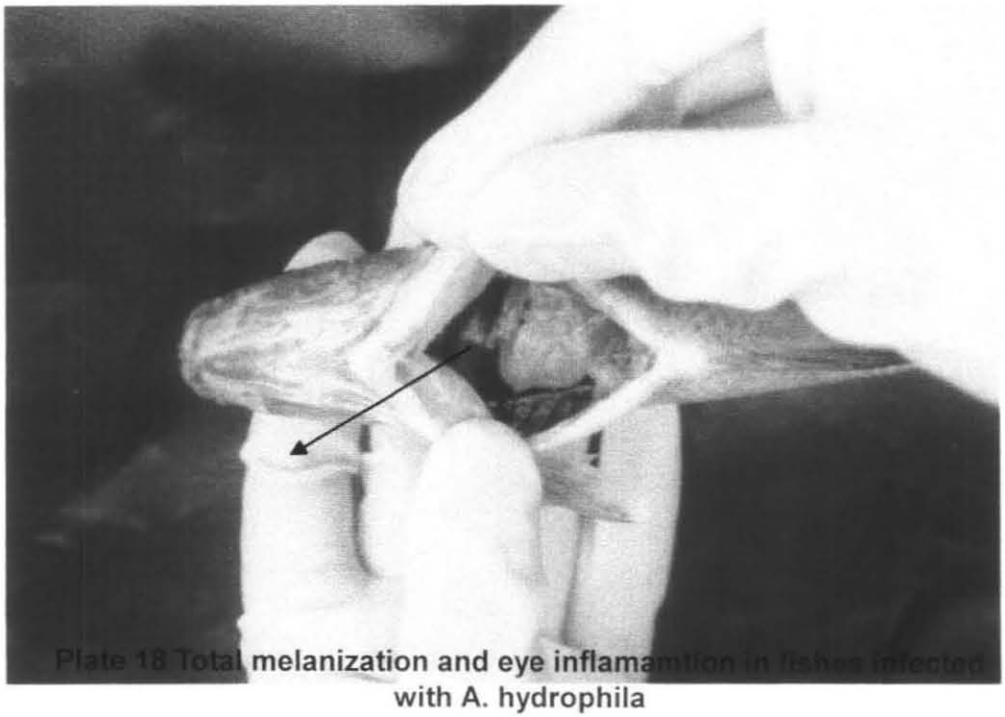

Plate 18 Total melanization and eye inflamamtion in fishes infected with A. hydrophila

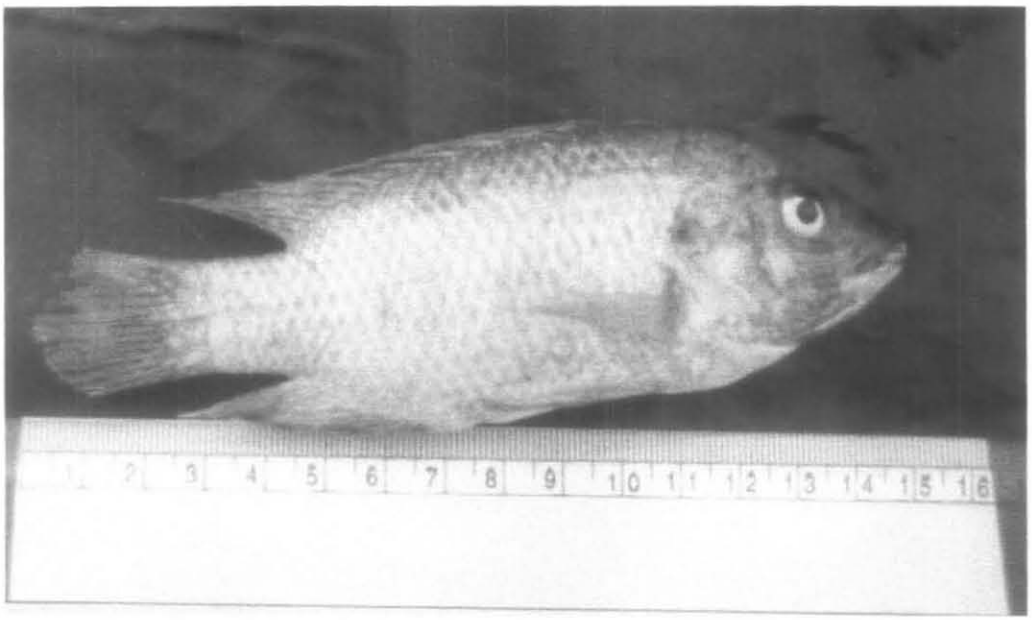

Plate 19 Loss scales and fin erosion due to *A. hydrophila* infection

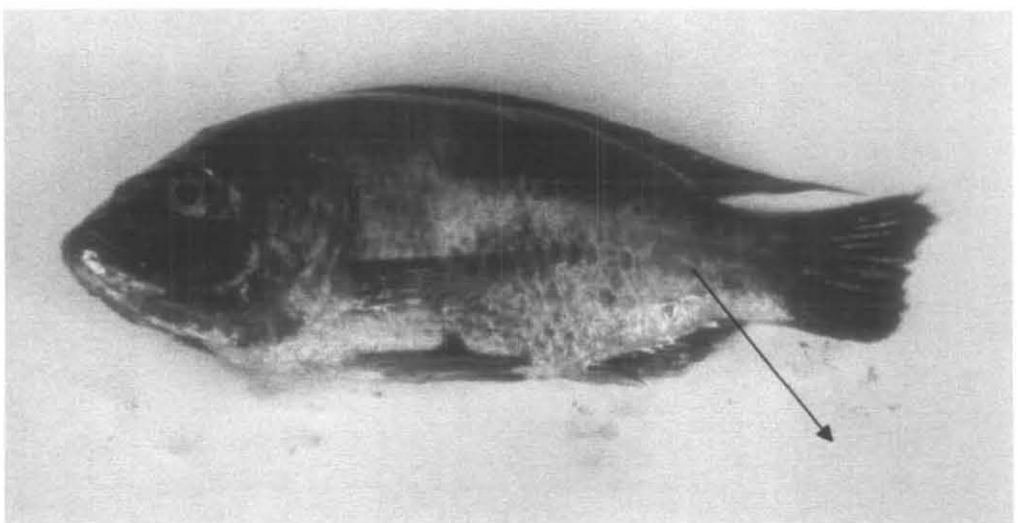

Plate 20 Haemorrhage at the base of the caudal fin

5. DISCUSSION

The principal interest of this investigation was to study the "Ecophysiology of pathogenic Vibrionaceae from cultured *Oreochromis mossambicus*" as all the problems that arise in an aquaculture system is the result of interactions of micro organisms with the environment. Vibriosis is a systemic bacterial infection primarily of marine and estuarine fishes caused by bacteria of the genus *Vibrio* (Egidius, 1987) and is a major cause of mortality in mariculture operations. The quantitative and qualitative studies on bacteria associated with cultured finfishes are confined to temperate waters. So far not much work has been done on the occurrence and distribution of Vibrionaceae in cultured finfishes in this part of the world. In this context, the knowledge of pathogenic Vibrionaceae of the culture ponds are extremely important as vibrios form 32% of the total heterotrophs (Austin, 1988). Almost all fish bacterial pathogens are capable of independent existence outside the fish and are able to persist under conditions of carbon starvation (Hoff, 1989). Obligatory pathogens are very few in numbers and even these, are capable of living for a considerable period within the tissues of their host without any deleterious effect. Usually, it is only some major changes in the ecophysiology of the host, whether internally driven as for example in the case of disease due to external stressor which allows the infection and disease to take place.

Thus it is clear that an understanding of the predominant bacterial flora of fish and their relationship with the environment is to be studied. Sneizsko (1972) was the first to enunciate this when he pointed out that communicable disease of fish occur only when the susceptible host and the virulent pathogen meet in proper environmental conditions. The work helped to understand the species diversity of Vibrionaceae in *O. mossambicus*, water and sediment in culture ponds, to develop data basis of reference for the rapid and accurate identification of unknown isolates from the environment and fish samples and to correlate the diversity within Vibrionaceae with pathogenicity to fishes.

The survey of total plate count, total *Vibrio* count and total aeromonad count were conducted in the environmental samples and in fish based on the

conclusion that bacterial diseases of fish are almost invariably stress related. The exact changes that take place in the fish which trigger the invasion or multiplication of bacteria is due to suppression of non specific defences such as reticulo-endothelial system and alterations in the integrity of mucoid surfaces. If the capacity to resist infection is reduced the micro organisms which are already within the tissues and in the gut will be able to invade and induce the disease. The capacity for such invasion is a major component of the pathogenicity of a bacterial species. Apart from surveying the TPC, TVC and TAC in the skin, gut and intestine of *O.mossambicus* and water and sediment in the culture pond, pathogenicity studies were also conducted to assess their pathogenic potential.

In the present study TPC of the skin of *O. mossambicus* was found to be maximum during the pre monsoon season 160.63 x 10^5/gm and minimum during post monsoon season 45.56 x 10^5/gm. This is in accordance with the observations of Thampuran *et.al.* (1997) where the maximum TPC and TVC were obtained during May- June from the skin of fishes. Chandrasekharan (1985) has found that the bacterial population varied from 10^6-10^7 /cm^2 on the body surface 10^6 - 10^8/gm in gill and 10^6-10^8/gm in intestinal content of *P. indicus*. The TVC of the skin of *O. mossambicus* showed maximum values (fig.4) during the post monsoon season of 1997-98 (96.685 x 10^5 /gm) while minimum value was reported during the post monsoon season of 1998-99 (29.577 x 10^5/gm). The wide fluctuations in TVC during the two post monsoon seasons indicate the variations that may occur due to the addition of organic manure to the pond. The TAC of the skin was maximum during the pre monsoon season and minimum during monsoon. This is explained by the wide fluctuations that occur in the volume of water entering the culture pond during monsoon as well as the reduction in temperature that brings down the bacterial load in the pond.

Singh (1986) has observed an increasing order of THB from body surface to gill and to alimentary canal in *P.indicus*. He has observed that the gram negative bacterial count of the alimentary canal was higher than that of the body surface.

The TPC of the stomach of *O.mossambicus* recorded high value (190.135 x 10^5/gm) during the post monsoon season. The TVC and TAC were also found to be maximum during the same season (107.69 x 10^5/gm and 69.19 x 10^5/gm respectively). Nair *et.al.* (1980) and Pradeep and Lakshmanaperumalsamy (1986) have reported peak values of TVC during the dry season. The high bacterial population in the stomach may be due to the bottom feeding nature of the animal where it feeds upon the decaying detritus and other dead matter. This along with the addition of organic manure might have resulted in a higher bacterial population in the stomach. The present observations are very much in accordance with that of Singh (1986) who reported similar results in cultured *P. indicus*.

In the present study the maximum TPC, TVC and TAC in the intestine of *O. mossambicus* were observed during the pre monsoon season (140.63 x 10^5/gm, 118.38 x 10^5/gm and 54.81 x 10^5/gm) (fig.11). Singh (1986) has reported that the THB of the stomach of *P. indicus* was much lesser than that of the intestine. Similar observation was made by Palaniappan (1982). The intestine provide a favourable environment to the tolerant strains and the active multiplication of the bacterial genera takes place in this region.

The microenvironment of alimentary canal in general is highly suitable for *Vibrio* where it undergoes a few cycles of division. The vibrios are versatile groups capable of elaborating various hydrolytic enzymes which may enhance the digestive process in the alimentary canal. At the same time when the environmental conditions become adverse mounting stress on the animal, these bacteria may invade the tissue from the alimentary tract and if the skin factor persists for a long duration, septicaemia due to vibrios may result.

The maximum TPC and TVC observed during the pre monsoon season during the present study can be substantiated by the standard pearson correlation coefficient analysis performed by Oliver *et.al.* (1983) between the bacterial population and the environmental parameters which indicated that several significant correlations existed with TPC and TVC. The results suggest that the aeromonad density in *O.mossambicus* samples were greater than that of the surrounding water

and sediment suggesting that the motile aeromonads are normal inhabitants of the fish intestine and that they become pathogenic only under conditions of stress.

Water is the reservoir of infection for pathogenic Vibrionaceae as they can persist for long periods in water of high hardness and organic matter content. The maximum values of TPC, TVC and TAC of water from the culture ponds were observed in the post monsoon season (191 x 10^4/ml, 66.5 x 10^4/ml and 37.5 x 10^4/ml) and the minimum values were observed during the monsoon season whereas Maugeri *et.al.* (2000) while carrying out quantitative analysis of total *Vibrio* sp. from mussel farms found the total vibrios to be predominant during May (500x10^3, 190 x 10^4). Austin (1988) has reported that bacterial count in sea water usually ranged between 10^3-10^6/ml. Gunn *et.al.*(1982) reported the reduction of aerobic heterotrophic bacteria from 10^4/l at an Atlantic coastal site of USA to 2 x 10^3/l at an oceanic site. However these counts seem to be low when compared to other workers. During the study on shrimp culture system, Anand *et.al.* (1996) reported maximum THB in water as 322 x 10^5 cfu/ml. The observation suggest that aquaculture ponds always have a higher load of bacterial population than the coastal off shore waters. The total bacterial counts were found to vary between culture ponds. Surendran *et.al.* (2000) have reported that the total bacterial counts of the water, mud and the cultured prawn from fresh water farms were lower then those from the brackish water farms. The apparent fluctuation in bacterial counts may reflect complex nutritional and physico chemical variations within the ecological niches. Thampuran *et.al.* (1997) have found that the total TPC in the coastal waters of Cochin upto 12 nautical miles where most of the fishing activity is concentrated was in the range of 10^5/ml or above. The aeromonad count was found to vary between 4x10^2/ml of the nearshore waters to 2.1 x 10^3/ml of the farthest point. Kaper *et.al.* (1981) while studying the aeromonad count of estuarine waters found a range of 10.3 – 5 x 10^3/ml and also noted a positive correlation with TPC. Alonso and Garay (1989) have reported that motile aeromond counts were high when using mADA (0/129) and mSA (0/129) (> 10^4/100ml). They have recorded aeromonad counts ranging from 40x10^4 to 650 x 10^5 in mADA (0/129) to 33 x 10^4 to 63 x 10^5 in mSA (0/129). This is in conformity with the present study where a selective medium was used for the isolation of motile aeromonads.

In the present study the TPC and TVC of the aquaculture sediments was found to be maximum during the premonsoon season (71.97 x 10^4/gm and 32.87 x 10^4/gm). Maximum TAC was noted during the post monsoon season. Anand *et.al.* (1996) have reported that in culture systems the maximum THB in sediment was 3100x10^5cfu/gm. Both pond and estuarine sediments recorded high number of heterotrophic bacteria than water.

Usually sediments harbour more bacteria because of its static nature and containing more nutrient concentration levels. The heterotrophic bacteria of sandy beaches showed maximum count of 138.46 x 10^3/g and minimum of 0.47 x 10^3 /g, was obtained in July and October (Nair and Lokabharathi, 1980). Venkateswaran *et.al.* (1989) have reported <2 to 80 cfu/gm in the sediments of the seto inland sea. Manavalan *et.al.* (1977) while studying the occurrence of *V. parahaemolyticus* in Porto – Novo isolated VLO 2.3 x 10^2, 240 x 10^2/gm dry weight of sediment and VPLO – 1.3 x 10^2/gm dry weight sediment. Maximum counts of both were encountered during the post monsoon. The present study showed variation from the observation period of the other workers, in that maximum counts were observed during the pre-monsoon period.

Thampuran *et.al.* (1997) employed a direct plating procedure using TCBS agar, considering the high densities of vibrios expected from fish samples. The TPC and TVC were in the same range of 10^5/ml. This is in accordance with the present observation where the TPC, TVC and TAC of skin, gut and intestine were in the same range (10^5/g).

Factors such as short generation time of *Vibrio* (Ulitzur, 1974; Thomas, 1982), ability of marine bacteria for the uptake of substrate at low concentrations (Wright, 1973), confinement of water for a longer time resulting in the loss of interaction of bacteria and the environment (Brock, 1967) must have resulted in the increase of *Vibrio* in the system. Moreover ability of microbes to attach to and colonise particulate material affords them with a micro environment higher in nutrient concentration than the surrounding water (Stevenson, 1978). This may be the reason for the sharp increase of *Vibrio* seen in the cultured animal than in water.

Recent review of microorganism in aquaculture ponds emphasized the need for investigation of the factors controlling bacteria (Moriarty, 1997). However the first step is to characterize, the spatial and temporal changes in bacterial numbers with ecological parameters and nutrient concentration so that effective sampling strategies can be developed. Water column and the sediment are highly heterogenous and the greatest difference occurs in the pond centre where the flocculated sludge accumulates.

At Station I (Valappu aquaculture pond) the maximum temperature recorded was 35.7^{0}C,while at KVK it was 35.1^{0}C which occurred during the pre monsoon season (April 1998) at both the sites. This explains the increase in temperature which normally coincides with the summer season (fig.12, 18). The temperature range within which individual micro organisms can live differs a great deal, which implies that their molecular constituents are very stable and this is particularly true of their enzymes. Marine microbial enzymes allow for high temperature biotransformations with more efficient enzymatic process due to greater speed. Increasing temperature promotes microbiological reactions, because all metabolic activities are favoured at summer temperatures (Rheinheimer, 1985). This explains the increase in bacterial density which coincides with the pre monsoon and post monsoon seasons when temperature is comparatively high in the present study.

The minimum temperature recorded at Station I was 28^{0}C which occurred in July 1998 (Fig.12) and at Station II (Fig.18) it was 27^{0}C which was reported both during July 1998 as well as January 1999. The fall in temperature may be due to the south west and north east monsoon rains. At low temperature all metabolic reactions are slowed down so that the survival of the bacteria is prolonged. In any case the seasonal temperature fluctuations cause a change of heterotrophs (Rheinheimer,1985).

The maximum salinity recorded at Station I was 20.778 ppt (fig.13) which occurred during March 1998, and at Station !I maximum salinity was observed during March 1999 (24ppt) (fig.19). The degree of salinity determines to a particularly large extent the living communities in water. Majority of marine bacteria are halophilic .It is the Na^+ which are the vital necessity for most marine creatures and for some, in

additional Cl^- ions (MacLeod, 1965, 1968). Salinity which deviates to some degree from the optimum, prolongs the generation time in all bacteria (Rheinheimer, 1985). Some metabolic activities like oxidation of organic acids and sugars or production of indole, are encouraged by certain salt concentrations (Mac Leod, 1965). A small group of bacteria can grow over a very wide salinity range (15-25%) by acclimatizing themselves. According to Meyer-Rail (1972), adaptation is largely restricted to within certain salinity ranges characteristic for the particular ecological groups ie. the bacteria with the greatest salt tolerance are as a rule, also the most adaptable.In the present observation, minimum salinity at station I was recorded during September 1998 (1.66ppt) (Fig.13) and at Station II during October 1998(3.601) (fig.19). This is due to the monsoon and post monsoon rains, which in turn brought down the salinity to low ranges and thereby the bacterial counts.

Maximum dissolved oxygen was encountered during June 1998 (9.754 ml/l) (Fig.14) at Station I. At Station II maximum value was reported during April 1999 (5.67ml/l) (Fig.20). The increase in oxygen levels in June may be due to the monsoon rains and that during April 1999 due to the pre monsoon rains. Four groups of micro organisms are distinguished with regard to their oxygen requirements; obligate aerobes, micro aerophilic organisms, facultative aerobes or facultative anaerobes and obligate anaerobes (Rheinheimer, 1985). The majority of aquatic micro organisms particularly of the marine environment are facultative anaerobes. The vibrios aeromonads and *Plesiomonas* isolated during the study period can be classified into anaerobic forms when they are in the gut and intestine, microaerophilic in sediments, aerobic in water, skin and gill of fish which showed the stable enzymatic potential of Vibrionaceae in different oxygenic and anoxygenic environment.

The low dissolved oxygen at Station I during March 1999 (3.57ml/l) (fig.14) and at Station II, (1.08ml/l) during March 1998 (fig 20) may be due to the increased density of micro organisms and less oxygen evolution from phytoplankton, which in turn brought down the oxygen level.

At Valappu aquaculture pond, maximum pH of 9.3 was recored during June 98 (Fig.15) and at Station II the maximum pH recorded was 7.87, during May

1999 (Fig.21). Singh (1986) has observed similar results during his study where he reported that 35.7% of the isolates from cultured *P. indicus* gave good growth at pH 9. The growth and reproduction of Vibrionaceae was found to be affected by the hydrogen ion concentration. Vibrios prefer high alkaline pH eventhough the optimum for most aquatic bacteria is between 6.5 and 8.5. The high pH range of 9.3 observed at Station I may be due to the vigorous phytoplankton bloom. Nellen (1967) has reported that the Schlei Fjord (Baltic) at a time of vigorous plankton bloom had a pH of 9.5. The large fluctuations of pH affects the composition of the bacterial populations. Maeda and Taga (1980) have reported that alkali tolerant bacteria shows good growth at pH 7.3 –10.6 in the pacific. In the present observation, the high pH coincided with increased bacterial growth especially Vibrionaceae. At Valappu and KVK minimum pH of 7.49 and 6.62 were observed during January 1998 (Fig.15) and May 1998 (Fig. 21) respectively which was found optimum for the growth of both Vibrionaceae and aerobic heterotrophs.

Maximum nitrite was observed at Valappu aquaculture pond during May 1998 (1.4µg at/l) and at Station II, KVK perennial pond during March 1998 (1.2µg at/l). Minimum nitrite was recorded during November 1998 (0.2µg at/l) at Valappu and at KVK during February 1998 (.15µg at/l). In the present study, high nutrient levels were noticed during the pre monsoon season and low values during the monsoon season coinciding with the influx of rainwater.

At Valappu, maximum nitrate values were recorded during May 1998 (6µg at/l) (fig.16) and minimum during November 1998 (0.82µg at/l) while at KVK maximum value was recorded during June1998 (5.5µg at/l) and minimum during December 1998 (0.53µg at/l)(Fig.22). At Valappu maximum ammonia value was noted during May 1998 (12.1µg at/l) and minimum during February 1998, (2.6µg at/l). At KVK, maximum values were noted during June 1998 (8.5µg at/l) and minimum during January 1999 (2.1µg at/l). Ammonia and nitrite play an important role in the supply of energy for nitrifying bacteria, and the oxygen bound in nitrate can be used by the numerous bacteria capable of denitrification under anaerobic conditions for the oxidation of organic material. Optimum concentrations of nitrate and ammonia were found to enhance bacterial activity whereas subacute or chronic exposure to as little

as 1ppm unionized ammonia or 10 ppm nitrite may result in gill damage, black gills, and low level mortalities (Chen *et.al.*, 1990). At Valappu maximum phosphate values were noted during May 1998, (7.5μg at/l) and minimum during January 1998, January 1999 and March 1999 (1.1μg at/l).). At KVK maximum phosphate values were noted during June 1998 (8.4μg at/l) and minimum during February 1999 (.15 μg at/l). The activity of heterotrophic micro organisms causes enrichment of nitrate and phosphate, consequently, regions where water from the deeper parts which are rich in nutrients wells upto the surface show high productivity and a more abundant growth (Rheinheimer, 1985).

At Valappu maximum organic carbon was observed during November 1998 (1.82 mg/g) and minimum during April 1999 (.707 mg/g) (Fig.17). At KVK maximum values were observed during May 1998 (1.257 mg/g) and minimum during April 1999 (0.092mg/g) Fig (23). The increase in the concentration of organic carbon during the post monsoon season (1999) is due to the addition of organic manure to the pond. Organic substances, dissolved or suspended in water are particularly important as food for heterotrophic micro organisms (Seki, 1982). The size and composition of the bacterial populations of water depend to a large degree on the concentration and composition of organic substances. There is a positive correlation between the numbers of micro organisms and the concentration of organic substances and wherever organic material accumulates, high total counts will be obtained. Rheinheimer (1985) is of the view that it is not the quantity of organic substance but the fraction of organic compounds which are easily assimilated like proteins, starch,sugar, fats etc., which decides the fertility of the water. The works of Kusnezow (1959) supported the view that the large total quantity of organic matter in dystrophic waters has no effect on the microbial numbers, but only the assimilable part of it can influence the same.

The microbial populations in aquatic systems are known to be influenced by a variety of physico chemical and biological factors. Total bacterial count isolated from skin of *O.mossambicus* was found to be significantly positively correlated with salinity and nitrite at 1% level and with nitrate at 5% level which showed that all the strains were of marine origin (Table 2). Watkins and Cabelli (1985) have reported similar findings stating that slight correlations of bacteria with

the nitrogen, salinity, phosphorus and pH do exist. No correlation was found between environmental parameters and total bacterial count of stomach, which may be due to the diverse range of enzymatic activity attributed to the total aerobic heterotrophs in the gut. There have also been results, which contradict the correlation of high densities of *Vibrio* sp. with the presence of organically enriched water (Simidu and Taga, 1974; Thompson *et.al.*, 1976). Intestinal heterotrophs showed significant negative correlation with pH; which showed that there was a consortium of heterotrophs, other than vibrios which was very predominant during the observation (Table 3) as vibrios prefer high alkaline pH . Table 4 explained the fitted regression of TPC of water on the environmental parameters. It explained 82% of the variability, which was significant at 5% level. Chandrasekharan *et.al.* (1991) have reported that under extreme conditions each individual environmental factor has an independent influence on the growth of bacteria. Significant negative correlation was obtained between total bacterial count in water and pH, (Table 5) which indicated the prevalent distribution of fermentative type of heterotrophs other than *Vibrio*. This is similar to the correlation between intestinal TPC of *O.mossambicus* and the environmental parameters studied from Station I (Table 3). TPC of water was also negatively correlated with nitrite, phosphate and ammonia indicating the predominance of nitrifiers and phosphate solubilisers in active degradation of ammonia. Nitrogen enters the nitrogen cycle as ammonia, resulting from the break down of proteins by a process called ammonification. Burford *et.al.* (1998) reported that organic carbon and nitrogen are limiting factors to bacterial growth whereas the studies of Janakiram *et.al.* (2000) on the bacterial abundance in brackish water culture ponds show that in pond water, the bacterial loads fluctuated erratically and no relationship could be found between water quality parameters and the bacterial load in the pond water. A high correlation between nitrogen concentration and bacterial number have also been recorded in fish pond sediments by Jana and De(1990).

Total count of sediment was found to be positively correlated with temperature (Table 6). The findings of Chandrika (1983) showed significant negative correlation with temperature at 1% level in surface water, indicating that negative correlation between TPC and temperature may be due to poor nutrient concentration of sea water. The significant positive correlation of TPC with temperature at 5% level

shows that the water contains high nutrient concentration influencing the increased productivity level.

Skin flora (Table 7) showed positive correlation with nitrite, which indicated that high concentration of nitrite was there in the pond due to the influx from land water run off and rainwater, moreover activity of heterotrophic micro organisms causes enrichment of nitrate. The multiple regression of TPC of intestine with the environmental parameters was significant at 5% level explaining 80% of the variability in the data.

The distribution of intestinal flora was positively correlated with temperature at 5% level and with salinity at 1% level (Table 9) which showed that the bacterial multiplication rate increased with temperatue and salinity. As all metabolic activities are favoured at high temperatures microbial reactions are promoted which in turn increases the bacterial density. The positive correlation with salinity implies the predominance of halophilic forms. In Table 10, the fitted regression of water with parameters explains 86% of the variability in the data which was significant at 5% level. Significant negative correlation of TPC was obtained at the perennial pond of KVK with nitrate, nitrite and ammonia at 1% level (Table 11). This is similar to the findings of Benny and Kurup (1991) who reported the variations in microbial flora of estuarine sediments and molluscs, depending on the phosphate, nitrate and organic carbon content. They found that the microbial flora decreased with increase of phosphate and nitrate, whereas Burford *et.al.* (1998) have reported that bacterial numbers are highly correlated with organic carbon and nitrogen in the sediment suggesting that they are limiting factors to bacterial growth. At Valappu, significant positive correlation was obtained between total *Vibrio* and salinity at 1% level and with nitrate at 5% level, which indicated the halophilic nature of the total vibrios (Table 12). Depaola *et.al.* (2000) have reported a slight but significant ($P<0.05$) negative correlation (-0.25) between *V. parahaemolyticus* density and salinity. The factors of organic enrichment, temperature and salinity present a complex inter relationship, each of which has been shown to aid in the development and survival of vibrios in the marine environment (Singleton *et.al.* 1982; Bockemuhl *et.al.* 1986 and Powell and Loutit, 1994). The relationship between total vibrios and other biotic and abiotic factors in the different regions of the seto Inland sea were studied by

Venkateswaran *et.al.* (1989). The inverse relationship with salinity showed that this factor is a crucial one governing the distribution as salinity was found to be statistically significant in most water sampling regions in the present study. Intestinal flora of enteric vibrios was significantly positively correlated with salinity, which showed their halophilic nature. Ramesh *et.al.* (1987) and Sivasankar and Jayabalan (1994) during their studies on the influence of hydrological parameters on the seasonal distribution of *V. harveyi* has also found that salinity has a great influence on bacteria. Table. 14 shows the significant negative correlation of TVC of water with pH(P<0.01), phosphate at 5% level and positive correlation with salinity. The negative correlation of water vibrios with phosphate indicated the high uptake of phosphate in these environments and the observation was similar to that of Benny and Kurup (1991) who found that microbial flora decrease with increase in phosphate and nitrate. The positive correlation indicates the predominance of halophilic forms mainly vibrios.

The analysis of data carried out by Groberg *et.al.* (1978) by using standard correlation coefficient with the parameters against the bacteriological counts revealed the number of vibrios in the water column to be positively correlated with turbidity and total bacterial levels ($P<0.05$) and inversely correlated with pH ($P<0.05$) and nitrate levels of water. Total *Vibrio* count in the skin was found to be positively correlated with nitrate (Table 16) which indicated the role of nitrate in the adsorption and colonization of vibrios in skin. Shinoda *et.al.* (1985) studied the distribution of *V.parahaemolyticus* and *V. fluvialis* in the estuarine regions and found that *V. parahaemolyticus* preferred brackish water than seawater due to the optimum concentrations of salt contents such as phosphates and nitrates. In the absence of nitrogen, nitrate present is used in place of oxygen for respiration reducing nitrite, ammonia or nitrogen gas. The regression of entric vibrios in the intestine with 9 environmental parameters explained 82.5% of the variability, which was highly significant ($P<0.05$) (Table 17). The intestinal TVC was highly influenced by salinity and nitrate (Table 18) as vibrios are halophiles, whereas the studies of Kelly and Stroh (1988) showed that highest counts of *V. parahaemolyticus* were detected when the average salinity remained low suggesting that salinity was important in regulating the occurrence of *V. parahaemolyticus* in the environment. Table (19) explained 92%

of the variability of TVC of water with the 9 environmental parameters which was highly significant.

Shinoda *et.al.* (1985) has stated that *V.parahaemolyticus* prefers water with optimum phosphates and nitrates.

Vibrios isolated from water at KVK showed positive correlation with temperature at 5% level (Table 20) as growth rate was found to be highly influenced by temperature. Barbieri *et.al.* (1999) have reported the positive correlation between *Vibrio* and temperature. The findings of Kelly (1982) indicated that the occurrence of vibrios is influenced by temperature and salinity. The high positive correlation at 5% level indicated the marine origin and halophilic nature of the vibrios. The significant negative correlation with ammonia indicated that the sudden increase in concentration of nitrate in the water which are derived from bio-fertilizers, and the agricultural run-off that might have entered the pond was not broken down as *Vibrio* concentration was low. Thereby the load of ammonia increased, signifying the role of vibrios in the breakdown of ammonia. Dumitrescu and Voicu (1984) studied the influence of environmental parameters on the occurrence of vibriosis in rainbow trouts reared in sea water and the occurrence of vibriosis was directly related to high ammonia and organic matter. The total vibrios in KVK water showed negative correlation with ammonia, indicating that the resident phytoplankton utilize, excessive ammonium ions as the source of nitrogen and perhaps it may be due to the slow rate of ammonification process. The negative correlation of phosphate with TVC showed that these elements were taken up by autotrophs and phototrophs as mineral salts (Table 20).

The TAC of water at Valappu was found to be negatively correlated with nitrate and ammonia at 5% level and with phosphate at 1% level which indicated the dissimilation of nitrate to gaseous nitrogen, via nitric oxide and nitrous oxide which involves at least 3 separate enzymes. This explains the low incidence of *Aeromonas* at high concentration of nitrates (Table. 24), whereas the studies of Monfort and Baleux(1990) have shown that the concentration of *Aeromonas* spp. in estuaries is strongly correlated with temperature as well as organic nutrients such as phosphate, nitrogen and carbon concentrations.

The growth of aeromonads in skin has been shown to be related to temperature and assimilable carbon (Table 25). A significant correlation between organic matter content and total numbers of mesophilic aeromonads in water has also been reported by Araujo *et.al.* (1989). Goberg *et.al.* (1978) has found that high densities of *A. hydrophila* is significantly correlated with temperature and pH whereas no correlation was noted with organic carbon. Hazen (1979) has found that pH was significantly correlated with densities of *A.hydrophila* in the water column whereas they were not related to dissolved oxygen and organic carbon. Species of the genus *Aeromonas* are considered to be autochthonous inhabitants of aquatic environments depending on temperature (Vanderkooj *et.al.* 1980). Alonso *et.al.* (1994) has reported that *Aeromonas* did not exhibit seasonal fluctuations in the majority of zones analyzed and was independent of environmental temperatures. The observations of Hazen *et.al.* (1978), Chowdhury *et.al.* (1990) also support the report of Alonso *et.al.* (1994). Kaper *et.al.* (1981), Monfort and Baleux (1990) have reported an abundance of *Aeromonas* sp. in estuarine waters that has been found to be seasonally distributed, with a maximum occurring during the summer. This is in accordance with the present observation, where higher aeromonad count was observed during the pre monsoon and post monsoon seasons. The multiple regression of TAC of intestine on the 9 environmental parameters was highly significant explaining 80% of the variability in the data (Table 26). Table 27 explains the negative correlation of aeromonads with organic carbon at 1% level showing that the fishes were actively feeding on the available organic matter. This may be the reason for the negative correlation of organic matter with TAC of stomach. The complex molecules in the organic matter were not degraded by micro organisms like *Aeromonas*. Eventhough high proportion of bateria were there in marine sediments the saccharolytic activity may not be well marked at certain times which will lead to the accumulation of organic carbon eventhough mineralization is a dynamic phenomenon. Goberg *et.al.* (1978) and Hazen (1979) have reported that no correlation was observed between the density of *A. hydrophila* and organic carbon. Total intestinal aeromonads were significantly positively correlated at 1% level with salinity which implies that motile aeromonads have got a very stable enzyme potential to acclimatize themselves with the changing saline conditions, as they have often been reported from off shore waters also (Thampuran and Surendran, 1998).

The multiple regression of TAC of water with the 9 environmental parameters explains 81.4% of the variability in the data which is a good fit (Table 28) suggesting that environmental parameters had a significant influcence over the density of aeromonads. The influence of environmental parameters such as oxygen, temperature, nitrogen and ammonia on the abundance of *A. hydrophila* was studied by Hazen (1983). The multiple regression and correlation of the data produced a best fit regression which explained 38% of the variation observed in *A. hydrophila* density.

Table 29 shows the matrix of correlation between the TAC of water and the 9 environmental parameters isolated from Station II. Total aeromonad count was significantly negatively correlated with nitrite, phosphate and ammonia at 5% level and with nitrate at 1% level, which showed active denitrification of water column microbes at KVK in microaerophilic conditions. Phosphate showed negative correlation with total aeromonads, which indicated the active utilization of phosphate for the active denitrification process. Plumb (1981) has reported the occurrence of infectious diseases in cultured channel cat fish caused by *A. hydrophila* under the influence of high levels of ammonia and nitrite. Matrix of correlation of TAC in sediment showed negative correlation with phosphate and organic carbon, which may be due to active recycling of organic matter in the sediment (Table 30). Organic carbon and phosphate also showed negative correlation with aeromonads as the source of organic carbon was depleted by the microbes in the anaerobic cycle of organic matter in the sediment. The reports of Huq and Colwell (1994) have shown that concentration and availability of nutrients directly affect bacterial survival and multiplication. The association of aeromonads with high levels of nutrients such as phosphates, nitrates and organic matter were studied by Boira (1996). The results show that correlation between different environmental factors and bacteria associated with *O. mossambicus* and corresponding water and sediment samples were not similar in all the samples throughout the collection.

Taxonomy of vibrios is still in a state of uncertainities (West and Colwell, 1984). Several taxonomic studies of vibrios and related species have been conducted by various workers. Baumann and Schubert (1984) have listed 20 spp., Alsina and Blanch (1994) have isolated 34 *Vibrio* sp. and 4 unidentified phenons, from environmental samples. More sp. will be probably added to this list in the future.

Further problems that arise in making comparisons are that different set of characters may have been used and the same test may not give the same results in different laboratories explaining the variations that occur in certain tests for the classification of bacterial isolates (Bryant *et.al.*, 1986). The present study helped to establish whether data collected from different strains isolated from different locations at different times by employing standard techniques were reliable and could be combined to provide a useful taxonomic result. The conventional procedure for the identification of fish pathogens exemplified by Baumann and Schubert (1984), Kreig and Holt (1984) and Alsina and Blanch (1994) were referred.

The comprehensive and practical description of conventional test media and methods set out in Alsina and Blanch (1994) are appropriate and widely used for the characterization of most of the fish pathogenic vibrios. The fundamental characters of the organism isolated were determined to assign it to the family Vibrionaceae and genus *Vibrio, Aeromonas* and *Plesiomonas*. With the schemes of Alsina and Blanch (1994) & Popoff & Veron (1976) and other selected tests the identification of Vibrionaceae were done.

In the conventional test system, human pathogenic vibrios are capable of metabolic activity at 37^{0}C. The time and temperature of incubation was also modified appropriately for aquatic marine vibrios (RT) (Colwell- personal communication). Discrepancies between the reactions obtained in the conventional methods for citrate utilization, gelatin liquefaction and Voges proskauer test were borne in mind when determining the identification of the isolate with strain variation. Vibrionaceae are nutritionally non fastidious and the majority of species may be isolated in general media like Zobell. Even though sodium is not the absolute requirement of *Vibrio* the growth of a few spp. of vibrios are however stimulated by sodium chloride, so the medium supplemented with 2% NaCl is advantageous for the primary isolation of these organisms (Hjeltness and Roberts, 1993). Aged sea water based media provided an excellent alternative.

All Vibrionaceae, except *V. cholerae* and *V. metschnikovii* have an absolute requirement for Na^+, whereas *Aeromonas* and *Plesiomonas* are non-halophilic. Vibrios are therefore, principally found in marine and estuarine habitats

and are predominantly pathogens of fish. *Aeromonas* are essentially of fresh water origin and consequently potential pathogen of fresh water fish. This distinction is not absolute, however aeromonad infection of marine fish and vibriosis of fresh water fish both do occur. The disease progresses whether it is in fresh water or sea water and are similar for both group of organisms and extend over a spectrum of conditions from acute septicaemia to chronic ulceration.

Stained smears of *Vibrio*, *Aeromonas* and *Plesiomonas* were gram negative, short to medium rods. Vibrionaceae are facultative anaerobes and all species were highly motile with darting movement. Hjeltness and Roberts (1993) reported that *Vibrio* consists of gram negative straight or slightly curved rods, which are non-spore forming and motile by monotrichous or multitrichous sheathed polar flagella. This is similar to the present observation where all the strains isolated were gram negative actively motile rods. *Vibrio* and *Plesiomonas* were sensitive to (150µg of O/129) and this test was used to distinguish the genera from the O/129 resistant *Aeromonas.*

Distribution of *Vibrio* spp. in different finfishes have been reported by Dhevendaran and George kutty (1998) and Santha *et.al.*(1985). In the present study *V. anguillarum* (31.86%), *V. parahaemolyticus* (14.71%), *V. fischeri* (15.19%), *V. fluvialis* (9.31%), *V. furnissii* (9.80%), *V.harveyi* (4.41%), *V.campbellii* (3.92%), *V.metschnikovii* (5.88%), *V.cincinnatiensis* (3.43%) and *V. vulnificus* (1.47%) were isolated from the culture ponds, based on the scheme of Alsina and Blanch (1994). Mathew(1996) has reported the predominance of *V. anguillarum* in culture systems (24.40%) followed by *V. fischeri* like bacteria (19.05%), *V. parahaemolyticus* (17.26%), *V. nereis* (11.90%), *V.ordalli* (9.52%), *V. fluvialis* biovar II (8.33%), *V. cholarae* non-O1 (5.36%) and *V. proteolyticus* (4.17%). Singh (1986) has reported that from 902 isolates taken from both larvae of *P. indicus* and water vibrios were found to be dominant (41.9%)

Chandrika and Nair (1992) have reported that *Vibrio* sp. form only 4% of the total heterotrophs in Trivandrum coastal waters, which were asperogenous gram negative short rods with darting motility. The difference in the percentage composition may be due to different methods followed in the isolation procedures.

The *V. anguillarum* strains isolated in the present study exhibited arginine positive, lysine negative and ornithine negative, reaction whereas Muroga (1976 a and 1976 b) reported that the isolates from *Anguilla japonica* did not decompose arginine and were positive to lysine decarboxylation. The properties mentioned in these reports generally agree but there were differences in bio-chemical reactions, including the decarboxylation of amino acids. *V.anguillarum* is positive to arginine and negative to lysine and ornithine but among vibrios with piscine pathogenicity the converse is true (Egusa, 1991). Moreover there are also cases in which not one of the 3 amino acids are not decomposed Onishi and Muroga (1976). Hendrie *et.al.* (1971) Alsina and Blanch (1994), Baumann *et.al.* (1984) have reported *V.anguillarum* strains which were positive to arginine and negative to lysine and ornithine.

Egusa (1991) reported voges-proskauer reaction of *V.anguillarum* to be positive but negative types were also reported among piscine pathogenic vibrios. Harrel *et.al.* (1976) have isolated vibrios with positive VP test in strains isolated from *Oncorhynchus kisutch* fingerlings. In the biochemical characteristics of *Vibrio* isolates from *Salmo gairdenerii irideus* differences were noticed. Hoshina (1957) has reported a type with negative VP, sucrose and indole reactions (Partially +), while Smith (1962) reported results of ++–. The variations are believed to be due to the variation in test methods and in the evaluation methods. In the present investigation, *V. anguillarum* at Valappu showed 12.5% VP + reaction and 2.44% at KVK which indicated very low production of acetyl methyl carbinol which in turn indicated high incidence of pathogenic *Vibrio anguillarum* strains in these environments.

Egusa (1991) has stated that among those identified as *V. anguillarum* there are many changes involving H_2S production. In the present study 79.17% from Valappu and 90.24% from KVK gave positive reaction to H_2S production. Hoshina (1957) reported that *V.anguillarum* like strains produced H_2S and indole in trace amounts. In the present study all *V. anguillarum* strains were indole negative. L-cystiene was not utilized by any strains as sole source of carbon (West *et.al.* 1983).

Egusa (1991) also observed changes involving utilization of citrate, MR test, indole production etc. Similarly in the present study, citrate was utilized by only

(33.33% of the strains isolated from Valappu and 41.46% from KVK). Indole was not at all produced from the aminoacid tryptophan at both the stations which showed that they lacked tryptophanase enzymes as they were environmental isolates. As the substrate was not available in the environment, the environmental isolates might have lacked this enzyme potential which showed that all microbial enzymes are adaptive enzymes. Moreover differences were also reported in the decomposition of carbohydrates such as sucrose, mannitol, sorbitol, arabinose, cellobiose and glycerin (Egusa 1991). In the present observation, variations, in carbohydrate utilization by different strains of the same sp. was observed. Maltose was utilized by all the *V.anguillarum* strains whereas mannitol was utilized by only 95.83% at Valappu and 87.80% at KVK. Sucrose was utilized by only 95.83% of *V.anguillarum* from Valappu, whereas 100% of the strains from KVK utilized sucrose. All the vibrios from Valappu utilized glucose while only 97.56% of the strains from KV K utilized glucose. Nybelin (1935) differentiated *V.anguillarum* into A and B types based on indole production and decomposition of sucrose and mannitol. Subsequently Smith (1962) added a C type.

Type	Indole production	Sucrose decomposition	Mannitol decomposition
A	+	+	+
B	-	-	-
C	-	+	+

Mc Carthy *et.al.* (1974) have also reported similar findings. This classification was convenient but Onishi and Muroga (1976) reported the existence of types isolated from diseased *Salmo gairdnerii irideus* with + sucrose, - mannitol and – indole which are neither A, B, nor C.

Based on Nybelin's classification (1935) the vibrios isolated in the present study from aquaculture ponds are found to be of type C (-++)

Hendrie *et.al.* (1971) reported the temperature range of growth of *V. anguillarum* to be 5 to 30^{0}C with no growth occurring at 37^{0}C, but however, Bergmann (1909), Schaperclaus (1927), Muroga and Egusa

(1967) and Ross *et.al.* (1968) reported that growth is possible at 37^{0}C. 95.83% of the strains from Valappu and 85.37% from KVK restrained from growing at 4^{0}C while 58.33% from Valappu and 95.12% from KVK failed to grow at 40^{0}C, showing that some of the strains had the stable enzyme potential to grow at widely fluctuating temperatures which is an outstanding characteristic of the isolates of marine origin.

According to Evelyn (1971) all the strains grew at temperatures ranging from 1 or 2^{0}C (light growth in 14 days) to 35^{0}C (detectable growth in 20 hour). They failed to grow at 37^{0}C at which temperature they were killed within a few days. The bacteria isolated by Muroga *et.al.* (1976 b) has specific properties of good growth at 18-39^{0}C. This explains the variation in the temperature tolerance of bacteria between temperate and tropical waters. The relation between growth and temperature seems to be a relation with the temperature of the environment in which the bacterial strain is present. The temperature range of *V.anguillarum* isolated from the eels in summer was 18-39^{0}C in peptone water culture with 3% salt, while the optimum temperature is 30-35^{0}C. The relation between temperature and growth is well known to differ depending on the concentration of salt in the culture (Muroga *et.al.*, 1976 b). Apart from a few psychrophilic sp., vibrios are mesophiles (Baumann and Schubert, 1984). Temperature is a major factor in bacterial growth kinetics. Most marine organisms are slight halophiles and grow best in media containing 1.2 – 2.9 % salt (Kushner, 1978). According to Larsen and Willeberg (1984), though vibriosis is primarily a salt-water disease, the density of the *Vibrio* was highly influenced by temperature and organic pollution, whereas salinity was of minor importance.

The relation between salt content and growth was studied which indicated that 58.33% of the *V.anguillarum* strains from Valappu and 65.85% of the strains from KVK could not grow well in the absence of NaCl, whereas 79.17% of the Valappu strains and 85.37% from KVK gave good growth at 6% salinity. Only 62.5% of *V.anguillarum* from Valappu and 51.22% of the KVK strains gave good growth at 8% salinity. Schaperclaus (1934), Nybelin (1935), Muroga and Egusa (1967), Ross *et.al.* (1968), Muroga and Egusa (1970), Evelyn (1971) and Onishi and Muroga (1977) have reported very good growth of *V.anguillarum* in the presence of NaCl ranging from 0.07 to 8.0. This may be the upper limit of possible growth in the culture medium, which has got very wide range 4.5% to 8% (Egusa, 1991). When medium

free of salt is used in bacterial isolation, strains which are incapable of growth in media free of salt, will be eliminated during isolation (Egusa, 1991).

Faublee *et.al.* (1995) states that growth temperature range as well as the salt requirement and tolerance are very useful taxonomic tools. They state that with the exception of *V. cholerae*, *Vibrio spp.* are slightly halophilic organisms, they have a specific and absolute requirement for Na ions. *V.anguillarum* has a minimum NaCl requirement of 0.15% and an optimum around 2% depending on the stain, it may tolerate 6% and 10% NaCl for growth. Salinity which deviates to some degree from the optimum prolongs the generation time in all bacteria. The rise in salinity interferes with the normal reproductive mechanism, the cells can grow but are unable to divide.

Grisez *et.al.* (1991) during their study on *V. anguillarum* identified 5 scandinavian strains which were negative for indole. Another strain also showed variation in arginine dihydrolase. Isolates identified as *V. anguillarum* showed many changes involving catalase (12.19% at KVK and 25% at Valappu), nitrate and ammonia (95.12 at KVK and 100% at Valappu) and they can be designated as variants (Table 31 & 32).

V.anguillarum strains isolated were positive for arginine dihydrolase reaction, acetoin production and amylase production. In the present observation all the strains from Valappu and 85.37% from KVK hydrolyzed starch.

None of the strains of *V. parahaemolyticus* isolated from Valappu in the present study grew at 4^0C, while 82.34% grew at 40^0C. At KVK 53.85% of the stains gave no growth at 4^0C while 92.31% gave good growth at 40^0C. Sudha *et.al.* (1998) found that maximum growth of *V.parahaemolyticus* was observed at 37^0C while at 15 and 42^0C growth was very slow. Kelly (1982) reported that there was no growth of *V. parahaemolyticus* at 4^0C. The studies of Sudha *et.al.* (1998) showed that *V. alginolyticus* and *V. parahaemolyticus* were able to tolerate higher temperature and were biochemically active at RT and 37^0C. Kelly and Stroh (1989) while studying the biochemical characteristics of clinical and environmental isolates of *V. parahaemolyticus* found that all of the isolates required salt for growth. West and Colwell (1984) found that almost 94% of the *V. parahaemolyticus* strains were able to

grow in the presence of 3 to 7% NaCl and 80% were unable to grow in the presence of 10% NaCl. Colwell (1970) reported that *V. parahaemolyticus* is capable of growth in the presence of 10% NaCl.

V. parahaemolyticus is considered to be restricted to saline environment and it requires Na^+ for survival and growth, but studies suggest that it also occurs in fresh water (Bockemuhl *et.al.*, 1986).From the studies of Sarkar *et.al.* (1985) on the distribution of *V. parahaemolyticus* in fresh water environments, they found that the Na^+ requirement of *V. parahaemolyticus* vary with the substrate serving as the carbon and energy source in the medium. This would imply that under certain specific nutrient conditions, the Na^+ requirement for *V. parahaemolyticus* is not mandatory and that the halophile can survive well in conditions where the salt concentration may be equal or even lower than physiological concentrations. 53.85% of *V. parahaemolyticus* strains isolated from KVK gave good growth in media without NaCl while 92.31% and 53.85% gave good growth at 6% and 8% NaCl respectively. At Valappu only 29.41% of the strains could grow at 0% NaCl while 82.34% and 58.82% of the strains reported good growth at 6% and 8% NaCl respectively, which indicated the wide salt tolerance of *Vibrio parahaemolyticus* and the metabolic potential of these enzymes at various NaCl concentration. Chan *et.al.* (1989) found that some of the strains were able to grow in 10% NaCl supporting the view that this characteristic is not enough to reject an identification of *V. parahaemolyticus* (Schandevyl *et.al.* 1984).

In the present study 52.95% of the strains isolated from Valappu and 84.62% from KVK, lead to the production of H_2S from lead acetate. *V.parahaemolyticus* strains isolated from sea fishes in Senegal produced H_2S and grew in 10% NaCl peptone water (Schandevyl *et.al.* 1984)•Both characteristics were only observed when strains were grown at their optimal temperature and for H_2S production in a medium containing 3% NaCl. This is in agreement with other authors, who concluded that NaCl tolerance and H_2S production depend on various parameters such as temperature, duration of incubation and nutrient composition of the medium (Thomson *et.al.* 1976, Van der Brock *et.al.* 1979). West and Colwell (1984) found that 59% of *V. parahaemolyticus* strains produced H_2S in TSI agar. Production of H_2S by *V. parahaemolyticus* has been reported to be dependent upon

the method and medium used (Kaper *et.al.*,1983). Sulphur reduction has been reported by Schandevyl *et.al.*(1984) for almost 50% of tropical *V. parahaemolyticus* isolates after 2 days when NaCl supplemented Klinger – Iron Agar was used. Production of H_2S has been reported by Colwell (1970) for 100% of 32 *V. parahaemolyticus* strains studied, however this trait is one that is frequently overlooked possibly by insufficient incubation times.

West and Colwell (1984) while studying the *V. parahaemolyticus* strains found that most of the strains hydrolysed aesculin. In the present observation very few strains hydrolyzed aesculin (5.88% from Valappu and 7.69% from KVK) and produced ammonia.

Kelly and Stroh (1989) while studying the biochemical characteristics of clinical and environmental isolates of *V. parahaemolyticus* found that almost all of the isolates were sucrose negative while Bose and Chandrasekharan (1976) observed opposite results with some strains of *V. parahaemolyticus*. Bose and Chandrasekharan (1976) found that some of the haemolytic vibrios differed in one or two of the tests for sucrose fermentation and salt tolerance. The Japanese investigators differentiated *V. parahaemolyticus* and its biotypes by sucrose fermentation reaction but the American workers recorded that 7 out of 40 Japanese type strains of *V. parahaemolyticus* utilized sucrose.

Baross and Liston (1970) concluded that all haemolytic mesophilic vibrios meeting the general classification of *V.parahaemolyticus* without regard to sucrose fermentation are to be described as *V. parahaemolyticus*. West and Colwell (1984) during their studies on *V. parahaemolyticus* strains found that only <6% were unable to produce acid from sucrose. At Valappu 64.71% and at KVK 84.62% of the isolates fermented sucrose. Colwell (1970) has reported that *V. parahaemolyticus* strains produce acid from sucrose. The observation of Chan *et.al.* (1986) indicate that sucrose positive vibrios predominated over sucrose negative vibrios in all sea food samples reflecting the relative abundance of these sp. in sea water.

Sucrose negative strains of *V. parahaemolyticus* indicated the origin and source of the bacterium which was reported from Porto novo waters, which in

turn indicated the ecology of the *Vibrio*, whether from intestine of warm blooded animals, as they are mostly sucrose (+) or from the natural environment (-) for sucrose assimilation as saccharolytic enzymes are adaptive enzymes.

82.35% from Valappu and 92.31% from KVK fermented maltose. 70.59% of the strains from Valappu and 92.31% from KVK fermented mannitol (Table 31 and 32). All the strains from Valappu and 92.31% from KVK fermented glucose without production of gas, which showed the saccharolytic potential of these isolates. Bacteria have also been attributed with the ability to utilize dissolved carbohydrate. This can be interpreted as indicating active bacterial metabolism.

West and Colwell (1984) during their studies on *V. parahaemolyticus* strains found that <8% could not produce acetoin, and they have been found to be influenced by both incubation time and temperature. None of the strains of *V. parahaemolyticus* isolated during the present study could produce acetoin.

Kelly and Stroh (1989) suggested that urease positive strains are the predominant form of *V. parahaemolyticus* associated with gastroenteritis in the pacific north-west. Urease positive *V. parahaemolyticus* strains are found frequently in estuarine water samples and 50% of the urease positive strains did not have plasmids. Clinical and environmental isolates of *V. parahaemolyticus* from the pacific north west had similar biochemical characteristics whereas West and Colwell (1984) during their studies on the biochemical characteristics of *V. parahaemolyticus* found that less than 10% hydrolyzed urea. Similarly only 5.88% and 7.69% of *V. parahaemolyticus* hydrolyzed urea at Valappu and at KVK respectively.

Kelly and Stroh (1989) while studying the biochemical characteristics of clinical and environmental isolates of *V. parahaemolyticus* found that almost all the isolates were lysine positive and arginine negative. The environmental and clinical isolates were less often ornithine decarboxylase positive than predicted. West and Colwell (1984) during their studies on *V. parahaemolyticus* strains found that 69% of the isolates produced ornithine decarboxylase. This is similar to the present study where none of the strains of *V. parahaemolyticus* isolated lead to arginine hydrolysis, whereas all the strains hydrolysed both lysine and ornithine which indicates their animal origin.

Brenner *et.al.* (1984) showed that *V. fluvialis* strains did not produce gas from glucose. None of the *V. fluvialis* strains isolated throughout the period of study could produce gas from glucose indicating their fermentative potential without producing gas.

Lee *et.al.* (1981) described 2 biogroups within *V. fluvialis.* Group I represented by the strains that are able to hydrolyze aesculin (72%) and Group II that did not hydrolyze aesculin (0%). Brenner *et.al.* (1983) have found that all strains of *V. furnissii* and 75% of *V. fluvialis* strains were aesculin negative. 75% of *V. fluvialis* isolated from Valappu and 54.55% of the strains isolated from KVK lead to the hydrolysis of aesculin.

Benediktsdottir *et.al.* (1998) stated that *V. fluvialis* isolated were able to grow in media from 0% to 8% NaCl. Furniss *et.al.* (1977) found that *V. fluvialis* like other vibrios can grow in the presence of high concentrations of NaCl. They noticed the variations in the biochemical characteristics of the strains isolated. Brenner *et.al.* (1983) reported that *V. fluvialis* was absent in the absence of NaCl whereas Lee *et.al.* (1981) reported positive or negative requirement. Several differences were seen in biochemical results obtained in the study of Brenner *et.al.* (1983). 100% of *V.fluvialis* isolated from KVK gave good growth in the absence of sodium chloride whereas only 87.5% of the strains from Valappu could grow well in the absence of sodium chloride which indicated that all these isolates are highly halophilic in nature. While the API 20E strains (*V. fluvialis* and *V. furnissii*) were usually positive for indole production and VP, they gave only 4% (+) for *V. fluvialis* and 14% (+) for *V. furnissii* for indole. Both species were negative for VP. The difference in the biochemical studies emphasize the variability in results that can occur when different media and methods are used (Brenner *et.al.*,1983).

Brenner *et.al.* (1983) found that 4% of *V. fluvialis* were found to be indole positive. 12.5% of the *V. fluvialis* strains isolated from Valappu gave positive result for indole test while none of the strains from KVK gave positive results. Biochemical diversity of *V. fluvialis* is vast as they vary in indole reaction, arabinose and sodium chloride growth pattern. All the *V. fluvialis* strains isolated gave good growth at 5% NaCl, and all the strains isolated from KVK gave good growth at 8%

NaCl while only 87.5% of the strains from Valappu reported good growth at the same salinity.

Furniss *et.al.* (1977) found that *V. fluvialis* is not as sensitive to 0/129 as other *Vibrio* spp. Sensitivity to 0/129 varied among strains of *Vibrio* spp. including *V. fluvialis*. Benediktsdottir *et.al.* (1998) during their studies on the *Vibrio* sp. isolated from diseased salmonids found that all strains were sensitive to 150µg of 0/129. One of the main clusters of strains within phenon 1 isolated from diseased fish was resistant to 10µg 0/129. All the strains of *V. fluvialis* were sensitive to O/129 at 150µg in the present study.

The studies of Brenner *et.al.* (1984) showed that *V. furnissii* produced acid and gas in glucose medium. 75% of the strains isolated from KVK, produced acid and gas from glucose, while only 62.65% from Valappu could liberate acid and gas.

Brenner *et.al.* (1983) have used peptone water supplemented with 0.5% NaCl to enrich *Vibrio* in environmental samples. 100% of *V. furnissii* isolated from Valappu grew well in the absence of NaCl while only 83.33% of the strains from KVK, grew at 0% NaCl. 100% of the strains from KVK and 75% of the strains from Valappu reported good growth at 6% NaCl. 75% of the strains isolated from KVK and 87.5% from Valappu reported good growth at 8% NaCl indicating that they harbour a stable enzyme potential to tolerate vast salinity range which is characteristic of typical marine bacteria.

Brenner *et.al.* (1983) during their studies on *V. furnissii* found that only 14% of the strains were found to be indole positive. Esteve *et.al.* (1995) found that atypical positive responses to indole production were observed in many *V. furnissii* strains. Except 12.5% of *V. fluvialis* and 20% of *V. metschnikovii* from Valappu rest of the strains isolated in the present study could not produce indole indicating their environmental origin.

Esteve *et.al.* (1995) found that false negative results were obtained for the citrate test in most of the *V.furnissii* isolates, whereas false positive responses were observed for VP test in the strains. Moreover atypical positive responses to

aesculin hydrolysis were observed in many *V. furnissii* strains. 62.5% of *V. furnissii* isolated from Valappu and 41.67% from KVK utilized citrate as a sole source of carbon. None of the strains isolated throughout the period of study gave positive result for VP test. 100% of the strains from Valappu and 91.66% from KVK could not hydrolyse aesculin.

The *Vibrio* sp., that are human pathogens include non-01 *V. cholerae*, *V. parahaemolyticus, V. alginolyticus, V. hollisae, V. damsela, V. mimicus, V. fluvialis, V.metschnikovii* and *V. vulnificus.* They produce a variety of intestinal and extra intestinal infections (Tacket *et.al.*, 1984).

The *V. vulnificus* strains exist in brackish waters as well as in coastal waters and should be considered as possible pathogens in wounds exposed to any body of brackish water. The recovery of autochthonous halophilic vibrios from inland waters in New Mexico and Oklahoma suggests that other potentially pathogenic halophilic *Vibrio* sp. can also occur in brackish inland waters under certain conditions (Tacket *et.al.* 1984).

V. vulnificus is widespread in the environment and environmental isolates as they produce virulence factors identical to those of clinical isolates. The studies of Arias *et.al.* (1999) suggests that *V. vulnificus* may be present in low numbers in water during the whole year but cannot compete successfully with other predominant *Vibrio* sp. either in the cold or in the warm season. This may be the reason for the low count of *V. vulnificus* in the present study. Some of the *V. vulnificus* strains which fermented lactose were sensitive to penicillin and differed in their growth on citrate (Oliver *et.al.*, 1982.). All the strains of *V. vulnificus* isolated during the study period fermented lactose. Citrate utilization by *V. vulnificus* isolated from the two sites showed considerable variation. None of the strains isolated from Valappu could utilize citrate whereas all the strains from KVK could utilize citrate as the sole source of carbon. As mentioned by Esteve *et.al.* (1995) for *V. furnissii* in this case also false negative results could have occurred contributing to the wide fluctuations.

V. vulnificus is capable of causing disease in cultured eels (Nishibuchi *et.al.*1979, 1980) and the eel isolates have distinct phenotypic, cultural and

serological properties. Survival of this bacterium in the environment and the spread of the disease depend on the temperature and salinity of the water. The environmental studies demonstrated that this bacterium is widespread along the coasts (Oliver *et.al.*, 1983).

Oliver (1981) have reported that *V. vulnificus* showed substantial decrease in growth with complete elimination of viable cells within 24 hours at 4^0C probably due to cold shock injury. Sudha *et.al.* (1998) have found that *V. vulnificus* was metabolically inactive at 4 and 42^0C. Tamplin *et.al.* (1982) has reported that *V. vulnificus* was isolated only in water with a temperature greater than 30^0C. The *V. vulnificus* strains isolated from Valappu as well as from KVK could not grow at 4^0C, while all the strains isolated in the present study gave good growth at 40^0C.

Oliver *et.al.* (1982) found that some strains of *V. vulnificus* differed primarily in the production of H_2S (by 97 and 100% of the members). None of the strains of *V. vulnificus* isolated from Valappu produced H_2S while 50% of the strains from KVK produced H_2S.

Baumann *et.al.* (1981) have stated that wild type strains of *V.vulnificus* are unable to utilize lactose. Reichelt and Baumann (1973) have reported that lactose fermentation is detected only after 1-3 days of incubation because of spontaneously arising mutants which are capable of fermenting this sugar. A possible reason for the commonly encountered negative result observed for lactose fermentation by marine vibrios concerns the rate of the reaction. In the present study, *V. vulnificus* showed positive lactose fermentation whereas all other vibrios gave negative results which showed that this particular sp. had the potential to synthesize the enzyme needed to ferment lactose by adapting itself by mutation. All the *V. vulnificus* strains fermented lactose.

Tison *et.al.* (1982) while studying the *V. vulnificus* sp. found that there were phenotypic differences between the eel isolates and typical *V. vulnificus* strains, the major one being that strains isolated from diseased eels were constantly indole negative, whereas clinical and environmental strains of *V. vulnificus* as described in the original sp. description (Reichelt *et.al.*, 1976) were indole positive. In the present

study the *V. vulnificus* strains were found to be lacking tryptophanase and hence indole was not produced, indicating the pathogenic potential.

V. vulnificus sp. isolated from diseased eels were ornithine decarboxylase negative (Tison *et.al.*, 1982) whereas clinical and environmental strains (Reichelt *et.al.*, 1976) were generally ornithine decarboxylase positive. All the *V. vulnificus* strains isolated during the study decarboxylated the amino acid ornithine, giving 100% positive result.

Tamplin *et.al.* (1982) have stated that *V. vulnificus* was found only in waters with salinity greater than 17%. *V. vulnificus* has been recovered in salinities upto 34% (Kaysner *et.al.*, 1987) and in competition with other related *Vibrio* sp. better adapted to this specific environment. The ability to tolerate salinity fluctuations were found to depend upon the bacterial species and the environment. In the present study the *V. vulnificus* strains isolated from both KVK and Valappu could not grow in the absence of NaCl. Whereas all of them grew well in 5% NaCl. None of the strains isolated from Valappu could tolerate 8% NaCl, whereas 50% of the strains isolated from KVK gave good growth at the same salinity.

Intrinsic resistance of Vibrionacae is usually associated with the presence of plasmids and the ability of plasmids for transconjugation. In the study of Li *et.al.*(1999) a large number of strains were devoid of plasmids but were resistant to ampicillin, kanamycin and trimethoprim. The presence of plasmids in these isolates seemed to increase their antibiotic resistance. The different types of antibiotics used for control of *Vibrio* suggest that few drugs were sensitive whereas others were found to be resistant due to indiscriminate use of such antibiotics leading to the production of resistant strains.

Published reports suggests that vibrios are more sensitive to chloramphenicol and resistant to erythromycin, kanamycin and streptomycin (Lio-po, 1984). This is in accordance with the present observations where 37.5% *V.anguillarum* strains from Valappu and 26.83% from KVK were resistant to streptomycin. 54.17% from Valappu and 51.22% from KVK were resistant to erythromycin. Evelyn (1971) has reported that *V.anguillarum* were sensitive to 5µg of novobiocin. In the present study 20.83% of *V. anguillarum* from Valappu and 24.39%

from KVK were sensitive to novobiocin (30mcg). Pybus *et.al.* (1994) states that the production of antibiotic substances by strains of *V.anguillarum* could give them a competitive advantage over closely related organisms in the same habitat and could also explain the apparent ecological success of *V. anguillarum* as a pathogen, as a commensal of marine fish and as a colonist of the marine environment.

The different strains of *Vibrio* spp. exhibited a common trend in their sensitivity pattern as well as resistance pattern, though variations among isolates from the same strains were quiet noticeable. This observation is substantiated by the reports of Rosily *et.al.* (1987) who have stated that *Vibrio* sp. isolated from different stages of prawn larvae varied in their resistance pattern as well as frequency of multiple drug resistance. Vibrios exhibited sensitivity towards streptomycin, chloramphenicol, aureomycin, terramycin, tetracycline, erythromycin, neomycin, novobiocin and 0/129. No sensitivity was exhibited to penicillin (Egusa, 1969). This is in accordance with the present observation. In the present study *Vibrio* strains exhibited greater sensitivity than resistance to chloramphenicol (*V.fischeri*- 72.73% (Valappu) and 65%(KVK), *V. fluvialis* 75% and 72.73%, *V.furnissii* 62.5% and 50%, *V.campbellii* 60% and 100% intermediate resistance, *V.vulnificus* 100% and 50% intermediate resistance at KVK). Variations were observed in the case of strains like *V.metschnikovii* where resistance to chloramphenicol was exhibited by a large number of strains 60% at (Valappu) and 57.14% at (KVK), *V. harveyi* (60% at Valappu) and 50% at KVK) and *V. cincinnatiensis* (50% at Valappu and 100% intermediate resistance at KVK). *V.parahaemolyticus* strains isolated exhibited greater sensitivity to chloramphenicol (47.06% Valappu and 69.23 KVK). But 29.41% at Valappu and 7.69% of the strains from KVK exhibited resistance to the drug tested. Sanjeev (1999) in his study has observed that all the *V.parahaemolyticus* strains were found sensitive towards chloramphenicol. This variation is substantiated by the observations of Li *et.al.* (1996), which imply that different strains have different sensitivities and that the mechanism of resistance is related to gene mutation. *V.parahaemolyticus* strains exhibited greater resistance to erythromycin (23.53% were resistant and 58.82% exhibited intermediate resistance at Valappu; and at KVK, 46.15% were resistant and 30.77% exhibited intermediate resistance). 88.24% of the strains from Valappu and 61.54% from KVK were sensitive to gentamycin. All the

strains isolated from Valappu and 84.62% isolated from KVK were found to be sensitive to streptomycin.

Multiple drug resistance (MAR) was more prevalent then resistance to one drug alone by most of the isolates. Gibotti *et.al.* (2000) while studying the virulence properties of *V. cholerae* non-01, *Aeromonas* spp. and *Plesiomonas shigelloides* isolated from Cambe stream found that all strains exhibited multiple drug resistance. Sanjeev (1999) has observed that 68.4% of *V. parahaemolyticus* studied were sensitive to gentamycin, 18% to tetracycline and 16.8% to streptomycin. None of the strains were sensitive to penicillin. The isolates from the culture pond were more resistant than the isolates from fish/shell fish towards gentamycin, neomycin and ampicillin. 100% of the *V. parahaemolyticus* strains from Valappu and 92.31% from KVK were found to be resistant to ampicillin (10mcg). Antibiotics are used extensively in brackish water culture system to cure amphibians, fish and shellfish diseases. Higher incidence of antibiotic resistant bacteria in brackish water culture system might be due to antibiotic thrust. 41.18% of the strains from Valappu and 53.85% from KVK were sensitive to tetracycline. Molitoris *et.al.* (1985) have shown that most *V. parahaemolyticus* strains have an intermediate reaction to tetracycline, an antibiotic important in the treatment of gastroenteritis. The wide variety of resistance patterns found among *V.parahaemolyticus* isolates in their study and their intermediate resistance reactions to several antibiotics create a high potential for refractory infections caused by *V.parahaemolyticus.* Grisez *et.al.* (1991) have found that *V.parahaemolyticus* was resistant to novobiocin. 64.71% of the strains from Valappu and 30.77% from KVK were found to exhibit intermediate resistance to novobiocin.

V.parahaemolyticus has been reported to be resistant to ampicillin and penicillin, and very few strains were susceptible to tetracycline, chloramphenicol, gentamycin and streptomycin (Colwell, 1970; Joseph, *et.al.*, 1982, Kaper, *et.al.*, 1983). In the present study 94.12% from Valappu and 92.31% of the strains from KVK were resistant to penicillin.

V.harveyi strains isolated from both the sampling sites exhibited 100% resistance to ampicillin and 80% and 100% resistance to penicillin at Valappu and

KVK respectively whereas in the present study high resistance was exhibited towards tetracycline (80% and 100%) while they were highly sensitive towards streptomycin (80% and 100%). 60% of the strains from Valappu and 50% from KVK were resistant to chloramphenicol. The strains exhibited higher sensitivity towards gentamycin (80% and 100%)(Table 34&36). The antibiotic sensitivity study carried out by Abraham *et.al.* (1997) revealed that *V. harveyi* developed resistance to many of the antibacterial agents tested. All strains were sensitive to chloramphenicol and most to tetracycline. Multiple antibiotic resistance (MAR) was more common in these strains. The *V. harveyi* strains showed resistance to at least 5 antibacterials. Occurrence of antibiotic resistant bacteria are more common in aquaculture system because of the intense use of antibiotics (Toranzo *et.al.*, 1992). The strains that exhibited resistance to 18 antibacterials was more virulent than the strain that exhibited resistance to 9 antibacterials (Abraham *et. al.*, 1997).

V. furnissii strains isolated from both the stations exhibited resistance to ampicillin (75% and 100%) and penicillin (100%).Brenner *et.al.* (1984) have shown that *V. furnissii* strains exhibited intermediate resistance to ampicillin. High resistance was recorded against erythromycin (87.5% and 66.67%) while chloramphenicol (62.5% and 50%) and gentamycin (50% and 75%) and streptomycin (100% and 91.67%) were sensitive. Novobiocin exhibited higher resistance (75%). Sundaram and Murthy (1983) have reported that *V.furnissii* isolates were resistant to tetracycline. In the present study 37.5% of the strains from Valappu and 25% from KVK were resistant to tetracycline.

V. campbellii strains isolated from both the sampling sites were found to be resistant to ampicillin (100%), penicillin (60% at Valappu and 100% KVK) erythromycin (60% at Valappu and 100% KVK) and novobiocin (80% at Valappu and 100% intermediate resistance at KVK) (Table 34 & 36). Maximum sensitivity was exhibited towards gentamycin and streptomycin (100%) and tetracycline (60% Valappu and 100% KVK). Balebone *et.al.* (1998) have reported that more than 80% of resistance was recorded for streptomycin (98.6%) and erythromycin (83.0%) by *Vibrio* spp. The combined resistance to antimicrobials was very frequent among the *Vibrio* strains tested. 21 different resistotypes were detected, the most frequently observed being Sm. Km, An, Tm, Gm, Ap, E (12.4%), Sm,Ap, E (14.4%) and Sm, Ap,

E (14.4%) and Sm-E (13.4%). In the present study a clear homogeneity in the antimicrobial resistance pattern has been detected in the strains of several *Vibrio* sp, thus all the strains of *V.campbellii* showed the following resistotypes, A, N, P, E, C (Table 33).

V. vulnificus strains isolated from both the sampling stations showed high resistance to ampicillin (100% and 50%), erythromycin (100%) penicillin (100% and 50%) and novobiocin (100% and 50%) (Table 34 &36). Whereas variation has been observed in the case of tetracycline and chloramphenicol (100% sensitiveness at Valappu whereas the 2 strains isolated from KVK, were not sensitive). All the strains were sensitive to streptomycin (100%) whereas variations were noticed in the case of novobiocin(100% at Valappu and 50% at KVK). According to Dalsgaard *et.al.* (1996) *V. vulnificus* were found to be susceptible to ampicillin, gentamycin, chloramphenicol and tetracycline. 2 strains were found to be resistant to ampicillin. They have also found that the presence or absence of plasmids did not appear to correlate with antibiotic resistance pattern.

V.fluvialis strains isolated from both the sampling sites were ampicillin resistant (100%). Tacket *et.al.* (1982) observed that *V.fluvialis* isolated from human specimen were found to be resistant to ampicillin. Furniss *et.al.* (1977) found that *V. fluvialis* was not as sensitive to novobiocin as other *Vibrio* spp. Nishibuchi *et.al.* (1983) found the strains of *V. fluvialis* to be resistant to novobiocin. Toranzo *et.al.* (1993) obtained low resistance percentage to these antimicrobial agents among the *Vibrio* strains isolated from diseased cultured sea bass, however between 50 and 100% of the strains of *Vibrio* isolated from diseased turbots were streptomycin resistant. In the present study 25% from Valappu and 9.09% from KVK were resistant to streptomycin. On the contrary low percentage resistance to chloramphenicol, oxytetracycline and tetracycline were recorded among the *Vibrio* strains tested. These findings may be interpreted as a result of a limited use of these antimicrobial agents in the aquaculture practice in Spain. In the present study the strains were sensitive to tetracycline (50%, 63.64%), chloramphenicol (75%, 72.73%) gentamycin (87.5% and 81.82%) and novobiocin (75% and 63.64%). However resistance mediated by plasmids to these antimicrobials especially to tetracycline has been

described in *Vibrio* strains isolated from fish cultured in other geographical zones (Levy, 1988; Speer *et.al.*, 1992; Zhao *et.al.*, 1992).

Grave *et.al.* (1990), Zhao *et.al.* (1992)and Gyles *et.al.* (1978)have observed a close relationship between the use of antimicrobials in aquaculture and the selection of resistant strains, with the potential risk of acquisition of resistance by means of microbial genetic transfer in the aquatic environment.

Most of the *V.fischeri* strains isolated were found to be resistant to ampicillin (100% and 90%) and penicillin (100% and 85%), whereas most of the strains were found to be sensitive to tetracycline (63.64% and 55%), chloramphenicol (72.73% and 65%) erythromycin (72.73% and 25%) gentamycin (81.82% and 70%) streptomycin (100% and 85%) and novobiocin (54.55% and 60%) (Table 34 & 36). Li *et.al.* (1999) studied the antibiotic susceptibility of *Vibrios* towards 16 antibiotics. Almost all strains were sensitive to chloramphenicol (98%) and 30 strains were highly resistant to ampicillin. This is in concordance with the present observation. Different *Vibrio* strains have different antibiotic resistance profiles. Most of the strains were susceptible to chloramphenicol and tetracycline and resistant to ampicillin (French *et.al.* 1989). The results of Li *et.al.* (1999) are similar to that of French *et.al.* (1989) who reported similar antibiotic susceptibility profiles of *Vibrio* sp. in a clinical and environmental setting.

V. cincinnatiensis were totally resistant to ampicillin and penicillin(100%). Much variation was observed in their sensitivity towards tetracycline (75% were resistant at Valappu whereas none were resistant at KVK). Baumann and Schubert (1984) have reported that very few strains were susceptible to tetracycline, chloramphenicol, gentamycin and streptomycin. Most of the strains were resistant to erythromycin (75% and 100%). In the present study all strains were sensitive to gentamycin and most to streptomycin (75% and 100%). Baumann and Schubert (1984) and Joseph *et.al.*(1982) found that most of the *Vibrio* strains were resistant to ampicillin, penicillin. The strains isolated from the 2 sites showed variations, in their sensitivity to novobiocin; 100% of the strains were resistant (Valappu) to novobiocin while they exhibited intermediate resistance at KVK (Table 34&36). Studies conducted by Clarridge and Zighelboin Daum (1985) proved that vibrios were

susceptible to ampicillin, chloramphenicol, gentamycin and tetracycline, whereas the studies of Rivonker *et.al.* (1999) showed that gentamycin at higher dosages appeared to be more effective as compared to erythromycin, kanamycin, streptomycin and tetracycline. No resistance to chloramphenicol and potentiated sulfonomides was observed in the vibrios but high levels of resistance to ampicillin and streptomycin were detected in vibrios isolated from turbot farms (Toranzo *et.al.*, 1993).

The increasing number of drug resistant bacteria in the environment may pose health hazards, so the unrestricted and often unnecessary use of antibiotics in the culture system has to be curtailed.

Antibiotics and other chemotherapeutic agents are used as feed additives or added directly to the water to prevent and treat vibriosis. In Japan, ampicillin, chloramphenicol, nalidixic acid derivaties, nitrofuran derivatives, sulphonamides and trimethoprim have been routinely used to treat vibriosis (Aoki *et.al.*,1984). However, the use of these compounds has resulted in drug resistant strains (Aoki *et.al.*, 1980; Hayashi *et.al.*, 1982; Toranzo *et.al.*, 1984). Tetracycline resistant isolates were recovered from cultured ayu (*Plecoglossus altivelis*), until 1977, when the use of this antibiotic was discontinued. Analysis of the resistant profile of *V.anguillarum* recovered since 1978 has shown that only one isolate was resistant to tetracycline (Aoki *et.al.*,1984), demonstrating a correlation between the use of tetracycline and the appearance of resistance. Tetracycline is used frequently as "cure all" for all diseases in aquaculture.

The correlation studies between *V. fischeri* and the environmental parameters (Table 42) indicated the positive influence of temperature with density of the species. West (1989) has reported that a significant positive relationship at 1% level was observed between *Vibrio* and water temperature confirming the findings that pathogenic vibrios are more frequently isolated in an aquatic environment with temperature varying between 10^0 and 30^0C.

The correlation between *V. furnissii* and the environmental parameters (Table 43) showed that ammonia and phosphates have a positive influence on the density of the species. Dumitrescu and Voicu (1984) have proved that the

propogation of vibrios was more pronounced during summer and that it was directly related to high ammonia. The high phosphate content correlating with the density of *V. furnissii* is contradictory to the observations of Benny and Kurup (1991) who noted that the microbial population decreased with increase of phosphates. At Station II *V.furnissii* was found to be influenced by the dissolved oxygen content (Table 45) whereas Dumitrescu and Voicu (1984) and Thune *et.al.* (1991) have reported that low dissolved oxygen accelerated systemic vibriosis.

The regression analysis of the total count of *V.campbellii* on the environmental parameters explains a significant part of the variability in the data. The fitted regression explains 85.5% of the variability (Table 40). The correlation studies showed that the density of the species was influenced by salinity (Table 44). Studies conducted by Kaper *et.al.* (1979) have stated that changes in population of *Vibrio species* could be correlated to salinity. It has been demonstrated by "invitro" experiments that the vibrios persisted in seawater but perished within 3-5 hours in fresh water specifying the significance of salinity on the incidence of vibrios.

The matrix of correlation between *V. metschnikovii* and the environmental parameters (Table 46) showed that the species was significantly positively correlated with salinity. Baumann and Schubert (1984) reported that most strains of *V. metschnikovii* are not able to grow in 1% tryptone broth with no added sodium chloride medium used in the salt tolerance test for identification. It is in accordance with the present observation showing that they are obligatory halophilic forms. Marchand (1986) while studying 203 strains of *V. metschnikovii* found that low temperature promoted the growth of the bacterium and that they were very tolerant to salinity.

The correlation of *V. campbellii* on the 9 environmental parameters at Station II (Table 47) showed significant positive correlation with pH at 1% level and with organic carbon at 5% level respectively preferring, alkaline pH for all their metabolic activities. They are having a direct relationship with hydrogen ion concentration accentuating the fact that they are highly alkaline forms. As the organic load increases, the nutrient level also increases, thereby providing the best conditions for the multiplication of the bacteria.

The factors of organic enrichment, temperature and salinity present a complex interrelationship, each of which has been shown to aid in the development or survival of vibrios in the marine environment (Singleton *et.al.*, 1982). Pollitzer (1959) have shown that the survival of *V. cholerae* in sea water was found to be related to temperature, pH and salt concentration. Watkins and Cabelli (1985) have reported poor to slight correlation of *Vibrio* densities with pH. Singh (1986) has shown that in culture environment, the bacterial population in general are alkalophilic. The alkaline pH of the culture pond makes it congenial for the different genera to exist together.

Seasonal distribution in the present study showed maximum *Vibrio* isolates during the pre-monsoon (n=53 at Valappu and n=44 at KVK) (Table 48 &50). The strains isolated include *V. anguillarum* , *V. parahaemolyticus, V. fischeri, V. fluvialis, V. furnissii, V. campbellii, V. metschnikovii, V. harveyi, V. cincinnatiensis* and *V. vulnificus*. The high bacterial load recorded during the pre monsoon season is in agreement with the observations of Davis and Seizemore (1982) who found that during summer the average *Vibrio* sp. levels (70%) increased and were significantly higher at 5% level than during the other 3 seasons. Significantly greater numbers of *V. vulnificus* and *V. parahaemolyticus* were isolated during the summer (50%) and fall (35%). Intesive sampling of oysters for *V. parahaemolyticus* gave very high levels during the summer (10,000 g^{-1}), (Depaola *et.al.*, 2000).

Kaneko and Colwell (1973) have reported high levels of *V. parahaemolyticus* in sea water during the warm months and low levels in cold months, appearing mostly in sediments. Watkins and Cabelli (1985) and Sarkar *et.al.* (1985) have found that the density of *V. parahaemolyticus* increased during summer. There appeared to be higher numbers and more frequent isolation of *V. vulnificus* from samples taken during the summer and fall months (Tamplin *et.al.*, 1982). Gjerde and Boe (1981) reported that the incidence of *V.alginolyticus* increased in mussel, fish, water and sediment samples from the Norwegian coast during the summer months when water temperature was warmest (10 to 16^0C). In the results reported by Molitoris *et.al.*(1985) seasonal fluctuation in the occurrence of *V. alginolyticus* did not appear to be dependent upon warm water temperatures during the dry season in tropical areas. The incidence of *V. parahaemolyticus* increased during the dry season

(Molitoris *et.al.*, 1985) as has been reported in other studies from tropical areas (Nair *et.al.*,1980). Pradeep and Lakshmanaperumalsamy (1986) observed peak values of total viable count in fish during April and maximum *Vibrio*-like organisms was noticed in July, May and April at different stations. A high incidence of *V. vulnificus* infections has been reported from northern Europe during the unusually warm summer of 1994. Bockemuhl *et.al.* (1986) found that highest incidence of vibrios was observed during the summer months. Water temperature appeared to be of primary importance for the incidence of vibrios. A temperature rise over 10^0 to 20^0C was followed by a distinct increase of *Vibrio* numbers, the highest counts were obtained at about 20^0C water temperature. Seasonal increases of *Vibrio* numbers have been shown in relation to zooplankton on which the organisms adsorb and from which they are released during the mineralization process (Kaneko and Colwell 1973). The periodical production and decomposition of biomass might be the nutritional basis for a distinct increase of zooplankton organisms during the summer months and thereby the increase in bacterial number. Furthermore, the constant alkaline pH around 7.5 might favour an association of *V.parahaemolyticus* with zooplankton and the requirement of these halophilic organisms for Na^+ might be spared in the presence of sufficiently high organic nutrient concentration as has been demonstrated for *V.cholerae* 01 (Singleton *et. al.*, 1982; Huq *et. al.*, 1984). Molitoris *et. al.* (1985) found that the occurrence of *V. parahaemolyticus* was present in sea water every month, with the highest occurrence in May 1972 (83.3%).

Quantitative occurrence of *Vibrio* spp. in the present study was very low during the monsoon (Table 48 & 50). Sarkar *et.al.* (1985) during their study in fishes of Calcutta reported that *V. parahaemolyticus* was recovered from plankton, water and sediments mainly during the summer months and the incidence progressively declined with the onset of monsoons. Sanjeev (1999) noted that *V. parahaemolyticus* count of brackish water pond was maximum during February to April and it was absent during July to November, when the salinity was minimum. In sediment the load was maximum in March and it was absent during June, July, October and November. Tanaka *et.al.* (1993) isolated drug resistant *V. anguillarum* frequently from April to July whereas Thampuran *et.al.* (1997) noted that the maximum diversity of *Vibrio* sp. occurred during the monsoon period July – September.

During the post monsoon season, the strains isolated from KVK include *V. anguillarum* followed by *V.parahaemolyticus*, *V. fischeri*, *V. fluvialis*, *V. harveyi* and *V. metschnikovii*. *V. campbellii* and *V. cincinnatiensis* were not isolated.

Kelly (1982) obtained a marked seasonal variation in the occurrence of *V. vulnificus* in seawater, with a peak in the late summer and a disappearance in the winter.

V. anguillarum was the most frequently isolated *Vibrio* sp. contributing 31. 86% of the total *Vibrio* population (Table 37). Of the total vibrios isolated from Valappu, 27.27% was contributed by *V.anguillarum* (Table 38) while at KVK, it formed 35.34% of the total *Vibrio* sp.isolated (Table 39). Anand *et.al.* (1996) has found *V. anguillarum* to be a potential penaeid pathogen which was more in the culture pond in comparison with estuary. Prasad and Rao (1994) while studying the distribution of pathogenic vibrios in prawn and finfishes found that prawns harboured a variety of sp. like *V. anguillarum*, *V. cholerae and V. parahaemolyticus*. George Kutty (1989) made a survey on the distribution of *Vibrio* spp. in water and infected parts of diseased fish in Trivandrum coast. The sp. encountered were *V.parahaemolyticus*, *V.anguillarum*, *V. alginolyticus*, *V. cholerae*, *V.costicola*, *V. vulnificus* and *V. fischeri*. *V. anguillarum* was the most common sp. isolated from diseased fish constituting 31.42% of the total *Vibrio* spp. This is in accordance with the present study (Table 37). *V. anguillarum* and *V. anguillarum* like has been isolated from diseased *Sparus aurata* (Balebona *et. al.*, 1995).

The third most important *Vibrio* isolated (Table 37) was *V.parahaemolyticus* which constituted 14.71% of the total vibrios isolated. They were more pronounced at Valappu (19.32%) than at KVK (11.21%). Sanjeev and Stephen (1993) Matte *et.al.* (1994) have stated that the incidence of *V. parahaemolyticus* in marine fresh fish and shell fish varied from 67 to 92%. Sanjeev and Stephen (1993) isolated 38.5% of *V. parahaemolyticus* from *O. mossambicus* from markets. They also isolated 51.26% of *V. parahaemolyticus* from finfish samples.

V. fischeri was the 2nd predominant bacterial flora (15.19%) among the vibrios isolated in the present study followed by *V. fluvialis* (9.31%) and *V. furnissii* (9.80%). *V. fluvialis* and *V. furnissii* are widely distributed throughout the aquatic environment but more particularly in brackish and estuarine water (Lee *et.al.*,1981). Toranzo *et. al.* (1993) isolated 68.5% of *Vibrio* sp. from turbot farms. The percentage vibrios from different sites of South eastern United States averaged 34.6% of the total bacterial counts. Schandevyl *et. al.* (1984) while studying the distribution of *Vibrio* sp. from sea fish in senegal isolated 42.9% of *V. fluvialis*.

V. furnissii first described by Lee *et. al.* (1981) as the gas producing biovar II of *Vibrio fluvialis*, is a rare species that occurred in brackish and estuarine waters (Lee *et. al.*, 1981; West *et. al.*, 1986). The first report of the isolation of *V. furnissii* strains from an European eel culture system was made by Esteve *et. al.* (1995). *V. fischeri* and *V. harveyi* were detected in apparently healthy turbot and in diseased fish by Nova *et. al.* (1992). Li *et. al.* (1999) isolated *V. fluvialis* from moribund sea bream.

4.41% of the luminescent bacteria *V. harveyi* was isolated in the present study. At Valappu they formed 5.68% whereas at KVK it formed 3.45%. Sanjeev *et.al.* (personal communication) while studying the vibrios isolated from iced, frozen fish and fish products found that *V. harveyi* was the major sp., among non pathogenic halophilic vibrios (12.30%). Halophilic vibrios were isolated from 50.52% of the samples and pathogenic halophilic vibrios were isolated from 44.76% of the samples. V.*harveyi* (17.3%) was the predominant sp. of the genus isolated throughout the sampling period from the Seto inland area (Venkateswaran *et. al.*, 1989). Arias *et.al.* (1999) has reported *V. harveyi* (19%).

Abraham *et.al.* (1999) isolated 5 different sp. of LB from Tuticorin coastal and shrimp farm viz *V. fischeri*, *V. harveyi*, *V. orientalis*, *V. splendidus* I and *P. leiognathi*. *V. harveyi* was the dominant sp. comprising 88.66 – 90.93% of the total luminous bacterial population. Toranzo *et.al.* (1993) isolated *V. splendidus*, *V. pelagicus*, *V. fischeri* and *V. harveyi* from the turbot farms.

V. metschnikovii formed 5.88% of the total *Vibrio* isolates. Valappu recorded 5·68% and KVK 6·03% respectively *V. vulnificus* formed 1.47% of the total

vibrios isolated. The incidence of *V. vulnificus* in water samples and bivalves has been reported as about 19% (O'Neil *et. al.*, 1990), whereas Matte *et.al.* (1994) has reported only 8 to 17%. Sakata and Hattori (1988) have reported the occurrence of V. vulnificus from diseased tilapia. Li *et. al.* (1999) have isolated *V. vulnificus, V. parahaemolyticus* and *V. fluvialis* from moribund silver sea bream. *V. vulnificus* and *V. parahaemolyticus* were confirmed to be virulent to sea bream by experimental challenge. Anand *et.al.* (1996) in the course of their studies found *V. parahaemolyticus, V. anguillarum, V. vulnificus* and *V. cholerae* to be the predominant forms. Karunasagar *et.al.* (1990) reported that *V. parahaemolyticus* was the most commonly encountered halophilic pathogenic *Vibrio* followed by *V. vulnificus* in samples collected from the markets of Karnataka state. Prasad and Rao (1994) studied the distribution of pathogenic vibrios in fresh, iced and frozen prawns and fish of Karnataka coast and reported the incidence of halophilic vibrios viz *V. parahaemolyticus, V. vulnificus, V. metschnikovii, V. anguillarum* and group F vibrios.

Thampuran *et.al.* (1997) have reported *V. alginolyticus* as the dominant sp. throughout the year followed by *V. parahaemolyticus* which appeared in all periods except the last quarter in Cochin waters. Thampuran and Surendran (1998) found that the majority of sucrose negative colonies were *V. campbellii* followed by *V. parahaemolyticus. V. campbellii* and *V. cincinnatiensis* occurred in very low percentage (3.92%, 3.43%). Thampuran *et. al. (*1997) isolated *V. algniolyticus, V. parahaemolyticus, V. vulnificus, V. cincinnatiensis, V. damsela* and *V. metschnikovii* from coastal waters and fishes of Cochin .From the Bisan seto region, *V. vulnificus, V. parahaemolyticus* and *V. metschnikovii* from the sediment samples were isolated by (Venkateswaran *et. al.*, 1989).

Schandevyl *et. al.* (1984) while studying the distribution of *Vibrio* sp. from sea fish in senegal isolated 26.6% typical for *V. parahaemolyticus* and *V. vulnificus*, 42.9% typical for *V. alginolyticus, V. fluvialis* and *V. metschnikovii.* The most frequently isolated pathogenic strains from farmed gilt- head sea bream was *Vibrio* genus 67.8% (Balebona *et. al.*, 1998).

A. hydrophila are found in all waters and are generally halophilic. Although they occur in relatively unpolluted waters, they are much more abundant in

waters with high organic load (Hazen *et.al.*, 1978, Kaper *et.al.*, 1981). *A.hydrophila* is cosmopolitan in distribution, it is widely distributed in water and sediments of ponds and can be transmitted by discharge from the intestinal tract and the external lesions on the skin (Aoki, 1974). Motile aeromonads are characterized by active motility, achieved by means of a single polar flagellum and production of gas as well as acids from carbohydrates. Biochemical profile showed aerobic, and facultatively anaerobic forms fermenting carbohydrates with the formation of acids. They produce 3-butanediol from glucose. They are highly cytochrome oxidase positive, reduced nitrates and are resistant to pteridine vibriostat, 0/129 compound, (Popoff, 1984).

In the present study 12.12% of *A. hydrophila* from Valappu, and 20% from KVK produced gas from glucose, while 15.38% of *A. sobria* from Valappu and none of the stains from KVK produced acid and gas. Eddy (1960) and Kou (1972) reported that non-virulent or weakly pathogenic strains did not produce gas and acetone from glucose.

Catalase was produced by 45.45% of *A. hydrophila* isolated from Valappu and 20% from KVK, which indicated that all aeromonads were micro aerophilic and facultative anaerobic forms as high catalase production is a characteristic of aerobic bacteria. Saccharolytic activity of *A. sobria* was found to be poor in the strains isolated as mannitol was found to be fermented by only 38.46% of the isolates from Valappu.

From the studies of Turnbull *et.al.* (1984) it was found that 95% of strains identified as *A. hydrophila biovar hydrophila* and 94% of those identified as *A. sobria* were enterotoxigenic. In contrast only 11% of *A. hydrophila* biovar anaerogenes (*A. caviae*) strains and none of the saccharolytic group were enterotoxigenic, implying that *A. caviae* are non pathogenic. They found that there was a significant positive relationship between enterotoxigenicity and lysine decarboxylation, VP reaction and production of gas from glucose. They state that there was a highly significant relationship between the ability to elaborate enterotoxin and both taxonomic grouping and positive results in 5 biochemical tests (lysine decarboxylase, VP, production of gas from glucose, gluconate oxidation, xanthine hydrolysis) and haemolysis of human erythrocytes. This indicates that a laboratory

not equipped to do enterotoxin testing may still assess with a high degree of confidence whether an isolate is likely to be enterotoxin positive.

Of the 100 characteristics tested by Altwegg *et.al.*(1990) 6 were invariably positive and 18 were invariably negative. Of the remaining 76, only 19 exhibited major differences among the 3 main phenons. These were production of gas and acetoin (VP) from D-glucose, decarboxylation of lysine, hydrolysis of esculin and fermentation of mannose. In the present study lysine was carboxylated by 84.85% of *A. hydrophila* from valappu and 93.33% from KVK. 76.92% of *A. sobria* from Valappu and 83.33% from KVK decarboxylate lysine. Aesculin was hydrolyzed by all the strains of *A. hydrophila* and *A. caviae* whereas *A. sobria* failed to do so (Table 52 & 55).

Esteve *et.al.* (1995) while studying a new species *A. encheleia* from eels found that 4 strains produced acid and gas from glucose and 3 of 4 strains were VP negative. Alonso & Garay(1989) while studying the *Aeromonas* strains found that *A. caviae* did not produce acid and gas from glucose. This is in accordance with the present observation where none of the *A. caviae* strains produced acid and gas from glucose.

Antibiotic sensitivity studies of motile aeromonads showed maximum resistance against penicillin and ampicillin. The different strains of the same species isolated during the period of study also showed variation in their antibiotic sensitivity pattern. Many workers have observed that almost all strains of *A. hydrophila* were resistant to ampicillin. But Rahim *et.al.* (1984) found that eight strains were susceptible to ampicillin containing 10µg of the antibiotic. The MIC for the strains were 12.5 µg/ml by the plate dilution technique. The observation suggest that many strains of *A. hydrophila* may go undetected if a selected medium containing 30µg of ampicillin /ml is used with the aim of eliminating other enterobacteria. 16.67% of *A. hydrophila* isolated from KVK and 12.12% of the strains isolated from Valappu showed sensitivity to ampicillin (10µg). Ishimura *et.al.* (1988) during their observations on the antibiotic sensitivity of motile aeromonads isolated from aquatic environmental samples found the majority of isolates to be resistant to ampicillin. Although most of the strains have intrinsic resistance to ampicillin, (Akashi and Aoki, 1986) drug

sensitive strains are also observed. Son *et.al.* (1997) while studying the antibiotic resistance of *A. hydrophila* isolated from *Tilapia mossambica* found all the strains to be resistant to ampicillin which indicated R+ plasmid occurrence in them.

In the present study 90% of the strains from KVK and 87.88% from Valappu were found to be resistant to penicillin. Chen and Chien (1978) while studying the antibiotic sensitivity of *A. hydrophila* isolated from cultured black porgy infected with tail rot found the *A. hydrophila* strains isolated from them to be resistant to pencillin.

Lipton (1991) while studying the sensitivity of *A. hydrophila* from diseased carps found the strains to be sensitive to tetracycline, streptomycin and penicillin. 86.67% from KVK and 66.67% from Valappu were sensitive to streptomycin. 50% of *A. hydrophila* from KVK and 66.67% from Valappu gave sensitivity to tetracycline. 87.88% of the strains from Valappu & 86.67% from KVK were sensitive to gentamycin. Hua *et.al.* (2000) while studying the *A. hydrophila* strains isolated from the frenetic disease in *Anguilla anguilla* found the strain to be sensitive to gentamycin. The multiple drug resistance showed the occurrence of R^+ factor harbouring plasmids with transferable drug resistance.

70% of *A. hydrophila* isolated from KVK and 57.58% from Valappu were found to be sensitive to chloramphenicol, 53.33% (KVK) and 15.16% (Valappu) were sensitive to erythromycin. Reungpraeh and Kasornchan (1983) found similar observation where *A. hydrophila* were found to be highly sensitive to chloramphenicol, erythromyin and tetracycline, while Chen and Chien (1978) found the strains to be resistant to streptomycin, tetracycline and chloramphenicol. The observations of Ishimura *et.al.* (1988) are similar to that of Reungpraeh and Kasornchan (1983) as they also found *A. hydrophila* strains to be sensitive towards tetracycline, chloramphenicol and gentamycin. Son *et.al.* (1997) found most of the strains to be resistant towards streptomycin (57%) tetracycline (48%) and erythromycin (43%). Multiple drug resistant strains carrying transferable R plasmids are now distributed widely in cultured amago ayu, carp and eel in Japan (Aoki *et.al.*, 1971, Aoki and Watanabe, 1973). Drug resistant strains carrying transferable R plasmids which encodes resistance to tetracycline, chloramphenicol and streptomycin are present

(Akashi and Aoki 1986). The results of Rhodes *et.al.* (2000) on the resistance plasmids of aeromonads in hospital and aquaculture environments evidence that related tetracycline resistance encoding plasmids have disseminated between different *Aeromonas* sp. and *E.coli* and between the human and aquaculture environments in distinct geographical locations. The findings provide evidence to support the hypothesis that the aquaculture and human compartments of the environment behave as a single interactive compartment. In the present study, 66.67% of the strains from KVK and 69.70% from Valappu were resistant to novobiocin. Plasmid encoded resistance to novobiocin has been observed by Hanes and Chandler (1993).

In the present study, antibiotic resistance of *A. sobria* was found to be very high towards ampicillin (84.62% at Valappu and 83.33% at KVK), tetracycline (38.46% from Valappu and 25% from KVK), erythromycin (30.77% from Valappu and 33.33% from KVK) penicillin (92.31% from Valappu and 83.33% from KVK) and novobiocin (76.92% from Valappu and 50% from KVK). The present study is substantiated by the findings of Dixon *et.al.* (1990) who studied the antibacterial resistance exhibited by *Aeromonas sp.* towards 11 antibacterials isolated from tropical fishes. *A. sobria* was found to be the most resistant, often showing susceptibility to only 3 of the 11 drugs tested.

The antimicrobial susceptibility of *Plesiomonas shigelloides* towards eight antibiotics was under taken in the present study. Multiple antibiotic resistance (MAR) was prevalent with the resistotypes S, A, E, G, N, P (Table 60). The increase of the antibiotic resistant proportions observed among the culturable microflora was more pronounced and statistically significant among the motile aeromonads (Schmidt *et.al.,* 2000). High levels of individual and multiple antimicrobial resistance were demonstrated within the collected aeromonads, thus indicating a substantial impact of fish farming on several groups of bacteria associated with aquacultural environments.

Claesson *et.al.* (1984) found that in addition to ampicillin and chloramphenicol the strains were sensitive to gentamycin and intermediately susceptible to pencillin. Gonzalez *et.al.* (1999) while studying the antibiotic sensitivity

of *P. shigelloides* isolated from the intestinal content of pike found the strains to be resistant to ampicillin and streptomycin. Penn *et.al.* (1982) found the *P. shigelloides* strains to be sensitive to tetracycline whereas resistant forms were also isolated.

P. shigelloides strains isolated from integrated fish farms (Twiddy and Reilly, 1995) were resistant to chloramphenicol, oxytetracycline, tetracycline and neomycin. This explains the multiple drug resistance (MDR) and the variations observed in the various strains of the same species throughout the period of study.

Sugita *et.al.* (1995) isolated *A.caviae, A. hydrophila, A. jandaei, A. sobria* and *A. veronii* from fish intestines water and sediments from the Hikijii river. *A. caviae* appears to be the most frequent mesophilic aeromonad isolated (Alonso *et.al.*, 1994). Nakano *et.al.* (1990) also detected *A. caviae* as predominant in polluted marine waters. However, other authors (Seidler *et.al.*, 1980) found *A. hydrophila* or *A. sobria* (Monfort and Baleux, 1990) to be the most numerous.

Alonso and Garay (1989) while studying the enumeration of motile aeromonads in sea water found that *A. caviae* was the most abundant sp. followed by *A. hydrophila* and *A. sobria*. The number of *A. hydrophila* isolates increased in less polluted station (Alonso *et.al.*, 1991). Arribas *et.al.* (1987) detected *A. caviae* as predominant in polluted marine waters. In the present observation the most frequent spp. identified were *A. hydrophila* > *A. caviae* > *A. sobria* indicating that the pond water was comparatively less polluted.

Araujo *et.al.* (1991) reported that out of 883 cultures isolated from salt waters, *A. caviae* formed 55%, *A. hydrophila* 34% and *A. sobria* 6%, whereas in the present study, *A. hydrophila* formed 51.22%, *A.caviae* 28.46% and *A. sobria* 20.33% of the total aeromonads isolated (Table 64). According to Schubert (1975) and Araujo *et.al.* (1991) *A. caviae* predominated in sewage and water with high degree of pollution while in less polluted waters of fresh or marine origin *A. caviae* and *A. hydrophila* are almost equally distributed. The study shows that aeromonads like *Vibrio* sp. is an inhabitant of the coastal waters and the skin surface or intestine of the fishes are good reservoirs of this bacteria (Thampuran and Surendran, 1998). Biotyping at the sp. level identification of 96 strains showed 46% as *A. caviae*, 22% as *A. sobria* and 16% as *A. hydrophila*. *A hydrophila* was always associated with

cleaner water (Fiorentini *et.al.*, 1998). Janda *et.al.* (1996) have stated that, regardless of the origins of the strains tested, *A. hydrophila* was the single most common genomospecies detected, accounting for between 30 and 40% of all strains identified. This is similar to the present observation where the most frequently isolated *Aeromonas* sp., *A. hydrophila* formed 52.38% at Valappu (Table 65) and 50% at KVK (Table 66). Araujo *et.al.* (1991) have reported that though *A.caviae* was the most predominant sp. isolated, the proportion of *A. hydrophila* was also high, especially in less polluted waters. Thampuran and Surendran (1998) reported the dominance of *A. caviae* in sea water and fresh fish. *A. sobria* was isolated from fresh fish muscle in very low numbers.

Okpokwasili and Obah (1991) while studying the correlation between the bacterial load and hydrological parameters during the different seasons found that the bacterial counts of water and sediment samples increased during dry season when the hydrological parameters like temperature was high.

Pathak *et.al.* (1993) while studying the density of *A. hydrophila* in the different organs of infected fishes found that infections were found to be maximum during summer (26%) followed by monsoon (12%) and winter (5%). In the present study at Valappu, maximum % of *A. hydrophila* were isolated during the monsoon season (Table 67) while at KVK, maximum % of *A. hydrophila* were isolated during the post monsoon season, followed by the premonsoon season (Table 68). Alavandi *et.al.* (1989) while studying the aerobic heterotrophic bacterial flora in the coastal waters of Cochin found that the counts were highest during summer and that aeromonads formed 13% of the total count. Thampuran and Surendran (1998) reported that in the Cochin coastal water, the concentration of *A. caviae* was very high followed by *A. hydrophila.*

At KVK, *A. caviae* was found predominating the monsoon season (45.45%) followed by the pre monsoon season (31.25%) (Table.68). Fiorentini *et.al.* (1998) have found *A. caviae* to be the most prevalent species in estuarine water with a high degree of pollution. No correlation was established between temperature and number of aeromonads in either estuary. The high counts of *A. caviae* observed during the monsoon season indicates contamination which could have occurred due

to the influx of contaminated water from the land water run off into the estuaries. At Valappu, *A.hydrophila* was predominant during the monsoon season (58.33%) followed by the pre monsoon season (51.43%) and post monsoon season (50%). At Valappu maximum % of *A.caviae* were isolated during the post monsoon season (37.5%). At both the ponds, *A.sobria* was the least isolated sp. supporting the view of Thampuran and Surendran (1998). At KVK, maximum % of *A.sobria* were isolated during the post monsoon season (23.53%) whereas at Valappu, their maximum % were observed during the pre-monsoon season (25.71%). From the studies of Fiorentini *et.al.* (1998) it can be assumed that identical types of aeromonads can occur in estuaries at different times of the year indicating that certain *Aeromonas* strains can survive in more widely varying physico-chemical conditions. Sugita *et.al.* (1995) observed that *Aeromonas* spp. occurs at high densities with high incidences regardless of season suggesting that *Aeromonas* are indigenous in fish intestines, water and sediments and have the potential to be predominant in aquatic environments. Pathak *et.al.* (1988) has reported that the highest isolation rate of *A.hydrophila* occurred in water during the late winter followed by a progressive decline in density during the summer and monsoon seasons. *A.hydrophila* was recovered from fish throughout the period from which it was concluded that they form a reservoir which is unrelated to their density in water. Boussaid *et.al.*(1991) concluded that highest numbers of aeromonads in water occurred during the cold month and lowest during the warm months whereas Alonso *et.al.* (1991) have observed that motile aeromonads did not exhibit seasonal fluctuations and was independent of environmental temperatures.

Chandrika and Nair (1992) during their studies on the bacterial flora of the coastal waters of Trivandrum found that *Aeromonas* sp. contributed 20.9% of the total 43 bacterial strains isolated. Twiddy and Reilly (1995) found that in integrated fish farms, the contribution of *A. hydrophila* towards the level of pathogenic bacteria was 67%. In the present study, of the 123 *Aeromonas* strains isolated *A. hydrophila* formed 51.22%, *A. caviae* 28.46% and *A. sobria* 20.33% (Table 64).

A hydrophila contributed 52.38% at Valappu and 50% at KVK respectively. At KVK *A. caviae* contributed 30% whereas at Valappu it formed 26.98%. The percentage distribution of *A. sobria* was almost the same at both the

sits (20% at KVK and 20.63% at Valappu). The high percentage distribution of *A. caviae* may be due to the inflow of contaminated water which finds entry through the inlet. Sugita *et.al.* (1995) found the percentage distribution of *Aeromonas* sp. isolated from water, sediment and intestinal tract of fishes as *A. veronii* (22%), *A. caviae* (18%) *A. hydrophila* (13%) and *A. sobria* (8%). Noterdaeme *et.al.* (1996) reported that of the percentage distribution of fish associated motile aeromonads, *A. hydrophila* formed 43.8%, *A sobira* (26.9%) and *A. caviae* (16.3%). Garcia *et.al.* (1999) while studying the *Aeromonas* sp. obtained from different cultured fin fishes found that *A. hydrophila* formed 50.7% followed by *A. sobria* and *A. schubertii.* All these studies are in corroboration with the present study on motile aeromonads.

In the study of Burke *et.al.* (1984) *A. hydrophila* formed 14% in water and *A. sobria* formed 41%. Of the 138 strains analyzed phenotypically (Altwegg *et.al.* 1990) 56.5% clustered in the phenon corresponding to *A. caviae* group. 20.3% clustered in the phenon corresponding to *A. hydrophila* and 18.1% clustered in the phenon corresponding to the *A. sobria* group. In the study of Janda *et.al.* (1996) *A. hydrophila* was the single most common genomo species detected, accounting for between 30 and 40% of all strains identified. *A. hydrophila, A. caviae* and *A. veronii* species accounted for 56% of all animal isolates and 69% of all environmental isolates. Kirov *et.al.* (1986) while studying the virulence characteristics of *Aeromonas* sp. found that the isolates when divided into sp. according to the criteria of Popoff (1984) *A. hydrophila* was the predominant isolate comprising 79% of the environmental isolates and *A sobria* formed 16%. The studies of Thampuran and Surendran (1998) indicated that motile aeromonads formed 0.29 to 2.5% of the total bacterial population of seawater.

In recent years great emphasis has been put upon understanding the pathogenicity of pathogens of fishes to elucidate the mode and mechanism of infection and nature of virulence. There has been considerable progress on the pathogecinity of some of the representatives of *Vibrio* particularly *V.anguillarum* (Egidius,1987). However most of these studies relate to fishes in temperate waters and there is only limited information available in Indian waters.

In the present study intra peritoneal injection method, bath challenge method as well as injection to the eye socket were used to test the pathogenicity of bacterial strains from diseased *O.mossambicus.* Austin and Austin (1994) found that 10^5-10^6cells/ml of *V.anguillarum, V.salmonicida, P.damsela, V.esturanianus, V.carchariae and V. costicola* resulted in infection while, rest of the 25 species tested showed no signs of pathogenicity. Intraperitoneal injection of 10^3 cells/animal of *V. anguillarum* caused stress with melanization of the skin with caudal erosion. Jaikumari *et.al.*(1995) found that *V. anguillarum* ATCC 19264 when injected caused 50% mortality at 10^3 dilution. Nordmo *et.al.* (1997) found that intra peritoneal injection to be the most reliable one.

Esteve *et.al.* (1995) found the LD_{50} of *V. furnissii* strains injected intra peritonially in elvers to be 8 x 10^6 and 4.4 x 10^6 cfu/fish. In the present study intraperitoneal injection of *V. anguillarum* at 10^6cells/animal caused mortality within 18hr of injection. The LD_{50} was 9.3 x 10^6cfu/fish. The clinical strains of *V.furnissii* ATCC 350/6^{T}was avirulent for fish (LD_{50}>10^8 cfu/fish) under the same assay conditions. Hamid *et.al. (*1978) have found LD_{50} of *V. campbellii* like in *P. indicus* as 2.18x10^7 cfu/animal.

The mortality data in the pathogenicity experiments showed that the pathogenicity of the present isolates depends on the dosage, period of exposure and the fish species. Similar observations have been recorded by Lightner and Lewis (1975) and Hameed (1995). In the immersion method, fishes exhibited loss of appetite, melanization and abnormal swimming behaviour, but no mortality occurred. Injection of 10^6 cells into the eye socket of *O.mossambicus* caused exophthalmia and slow mortality.

The results suggests that *V. anguillarum* is pathogenic to adult *O.mossambicus* (10^6cells/animal). Intra peritoneal injection of 10^6cells/animal caused mortality within 18 hr. When 10^6cells/ml was injected into the eye socket, exophthalmia occurred with slow mortality post injection. Immersion experiments failed to produce infection.

Yambot and Inglis (1994) have reported the phenomenal eye disease affecting Nile Tilapia. 70% mortality was reported due to *A. hydrophila* infection where the animals exhibited whitening, exophthalmia and bursting of the eye. Ogara *et.al.* (1998) have reported that farmed turbouts infected with *A. hydrophila* and *A. caviae* exhibited varying degrees of eye pathology. Although gross external signs of disease were not observed in moribund fish, they all presented haemorrhages in the intestinal tract. Bacteria used for challenging were reisolated as pure cultures from internal organs. The symptoms of the experimentally infected fish were similar to those of natural infection. When large numbers of *V. anguillarum* were injected intra peritoneally the organisms rapidly entered and reached all the organs examined.

Esteve *et.al.* 1993 has noted that *A. sobria* and *A. caviae* are non pathogenic for eels. In the present study with tilapia also *A. caviae* proved non-pathogenic. Shakila *et.al.* (1999) during their studies with *A. hydrophila* in *Cirrhinus mrigala* found that all the fish survived through experimental inoculation. No mortality was recorded throughout the period of experimentation. Of the different routes of administration of the pathogen into the fish, intra peritoneal route appeared to cause more symptoms than the other routes. This was strongly supported by the studies of Omprakasham and Manohar (1991), Ramaiah and Manohar (1987). Arujo *et.al.* (1991) & Majeed *et.al.* (1989) have reported, *A. hydrophila* and *A. sobriae* as enterotoxigenic by animal assay while *A. caviae* was found to be non –toxigenic. The infectivity trials carried in the present study showed that *A.caviae* injected intraperitoneally produced a slight melanization in the injected area but no mortality occurred showing that the strain injected was not toxigenic. It was found that *A. caviae* was relatively non pathogenic but the frozen fishes harboured greater number of *A. hydrophila* posing a risk to human health. Symptoms like exophthalmia, oedema, haemorrhage, accumulation of bloody purulent fluid in the peritoneal cavity, internal pathology with translucent intestine, pale liver and reddish gall bladder observed in the present study were in line with those of Omprakasham and Manohar (1991). LD_{50} values obtained for *V. anguillarum* in gilt head sea bream are higher than the values determined for other fish sp, such as turbot, eel and salmonids (Santos *et.al.*, 1991). In an investigation by Buras *et.al.* (1985) uptake of microorganisms by tilapia, *O. aureus* and mirror carp, *Cyprinus carpio* were studied.

The concentrations of micro organisms in internal organs were higher when bacteria were orally inoculated as compared to a bath challenge using the same microbial concentration.

80% of the disease occurrence in culture ponds are due to environmental stress and are therefore unavoidable. In the present study, except for *A. sobria* isolated from Valappu rest of the *Aeromonas* sp. were not influenced by any of the 9 environmental parameters. Multiple regression and correlation analysis to relate the count of *A.hydrophila, A. caviae, A. sobria* and *P.shigelloides* at the two sampling sites to the 9 environmental parameters were carried out. The regression equation accounted for 82.9% of the variation in the count of *A.hydrophila* at Valappu influenced by the environmental parameters (Table 58). The fitted regression was found to be significant at 5% level.

Environmental factors that predispose fish to infection by *A. hydrophila* include crowding and handling (Snieszko and Bullock, 1968) high temperature and low dissolved oxygen (Rock and Nelson, 1965), high nitrite, high ammonia and combinations of low dissolved oxygen, high ammonia and high carbondioxide levels (Walters and Plumb, 1980). The density of *Aeromonas* spp. isolated from both the sampling sites were in the order *A. hydrophila* > *A. caviae*> *A. sobria*.

Under culture conditions, *A. sobria* may adopt to higher salt concentrations and originally halophilic strains after appropriate adaptation may grow in fresh water and fresh water strains in sea water (Baars, 1930). This explains the significant correlation at 5% level between *A. sobria* and salinity observed at station I. *Aeromonas* sp., which are not usually highly halophilic have acclimatized themselves to the halophilic condition and has become significantly correlated to salinity at 5% level (Table 59).

Only 7 *Plesiomonas shigelloides* strains were isolated throughout the study period. Significant positive correlation of *P. shigelloides* with dissolved oxygen was noted at Valappu (Table 62) which indicated that *Plesiomonas* are highly aerobic and micro aerophilic in nature. Moreover oxygen content of water is governed by bacterial activity and respiration is often predominantly due to microbial processes and water quality factors such as oxygen.

The 2 way ANOVA of vibrios with seasons at Valappu showed that there is significant difference between seasons as far as *Vibrio* sp. is concerned at 1% level (Table 49). The sp. composition of the *Vibrio* is partly determined by nutrients and other environmental factors. In the present study, pre monsoon season showed significantly higher count and monsoon showed significantly lower count. The lower count obtained during monsoon may be due to low temperature and low salinity which are not highly favourable for vibrios.

At KVK vibrios showed significant difference between the species count but there was no significant difference between seasons (Table 51). The nitrate and oxidase negative forms of *V. metschnikovii* obtained in the present study was found to be in significantly higher numbers followed by *V. parahaemolyticus*. In natural ecosystem, sp. composition is controlled by physiological factors and the bacteria has a selective advantage. Predominance of any one species is enhanced by oxygen, pH, and nutrients like phosphate. In the present study, the count of bacterial sp. which harbour wide range of exoenzymes, efficient at breaking down polymers were predominant. This explains the significant difference between sp. though the substrate availability and physiological factors were the same.

The study adds information on the prevalence of Vibrionaceae in cultured fin fishes and their environment. It highlights the fact that though Vibrionaceae are autochthonous inhabitats of fishes under stress conditions they may turn pathogenic. It also signifies the multiple antibiotic resistance that develops in vibrionaceae due to the indiscriminate use of drugs in culture operations. Considerable attention has to be given towards the management, disease diagnosis and its control in cultured fishes.

6. SUMMARY

The study "Ecophysiology of pathogenic Vibrionaceae from cultured *Oreochromis mossambicus*" was conducted during December 1997 to May 1999 in order to understand the ecophysiology and biodiversity of Vibrionaceae and their potential to cause disease in cultured *Oreochromis mossambicus*. Motile aeromonads were also studied as *Aeromonas hydrophila* was found to cause haemorrhagic septicaemia at low salinity.

Data on physico-chemical parameters were collected to determine their influence on Vibrionaceae.

A proper understanding of bacteria and environmental parameters and their effects on bacterial productivity is a pre requisite for the effective management of any acquaculture system. As Vibrionaceae forms 32% of the total bacterial flora, it can be broadly taken as sensitive indicators for estimating pathogenic potential.

The data collected will be helpful in assessing the present status of Vibrionaceae in this vital ecosystem and can be utilized for ecological monitoring and future evaluations.

The present study observation, suggests that aquaculture ponds always have a higher load of bacterial flora and the apparent fluctuation in *Vibrio* and aeromonad counts reflected complex nutritional and physico chemical variations within the ecological niches.

Adaptation was largely restricted to within certain salinity ranges (1.66ppt - 24ppt) characteristic for the particular ecological groups.

High nutrient levels were noted during the pre monsoon season and low values during the monsoon season coinciding with the influx of rain water.

Ammonia and nitrite play an important role in the supply of energy for nitrifying bacteria, and the oxygen bound in nitrate can be used by the numerous bacteria capable of denitrification under anaerobic conditions for the oxidation of organic material.

An increase in the concentration of organic carbon during the post monsoon season (1999) was due to the addition of organic manure and bio-fertilizers to the pond. Organic substances, dissolved or suspended in water were metabolized as food by heterotrophs.

The bacterial parameters were influenced by physico chemical and biological factors. Total bacterial count of skin of *O.mossambicus* was significantly positively influenced by salinity and nitrite at 1% level and with nitrate at 5% level which showed that all the strains were of marine origin. No correlation was found between environmental parameters and total bacterial count of stomach, which may be due to the diverse range of enzymatic activity attributed to the total aerobic heterotrophs in the gut. Intestinal heterotrophs showed significant negative correlation with pH; which showed that there was a consortium of heterotrophs, other than vibrios, which was very predominant during the observation as vibrios prefer high alkaline pH. The fitted regression of TPC of water on the environmental parameters also explained 82% of the variability, which was significant at 5% level.

TVC of water was positively correlated with salinity implying the predominance of halophilic forms.

Total count of sediment and intestinal flora were positively correlated with temperature, at 5% level which showed that eventhough many other environmental factors play an important role in the occurrence, distribution, physiology and pathogenicityof bacteria, probably none are more important than temperature.

Skin flora showed positive correlation with nitrite, which indicated the high concentration of nitrite in the pond due to influx from land water run off and rainwater, moreover higher metabolic activity of heterotrophic microorganisms might have caused enrichment of nitrite.

At Valappu vibrios showed significant positive correlation with salinity at 1% level and with nitrate at 5% level, indicating the activity of halophilic nitrifiers.

Total *Vibrio* count in the skin was found to be positively correlated with nitrate, which indicated the role of nitrate in the adsorption and colonization of vibrios in skin of tilapia.

The total intestinal *Vibrio* count was highly influenced by salinity and nitrate as vibrios are halophiles.

The growth of aeromonads in skin has been shown to be related to temperature and assimilable carbon. Higher aeromonad count were observed during the pre monsoon and post monsoon seasons which indicated their halotolerance.

The results of physicochemical and bacterial parameters highlighted the synergestic effect of all these 9 environmental parameters. The results also indicated the impact of tidal effect, agricultural run off, sewage outflow, with its heterogenous nature, rain water input and fertilization of the pond.

In the present study percentage composition of the 10 *Vibrio* spp. isolated were as follows: *V. anguillarum* (31.86%), *V. parahaemolyticus* (14.71%), *V. fischeri* (15.19%), *V. fluvialis* (9.31%), *V. furnissii* (9.80%), *V.harveyi* (4.41%), *V.campbellii* (3.92%), *V.metschnikovii* (5.88%), *V.cincinnatiensis* (3.43%) and *V. vulnificus* (1.47%).

Based on Nybelin's classification (1935) the *V. anguillarum* strains isolated in the present study from aquaculture ponds were found to be of type C (-++)

95.83% of the *V. anguillarum* strains from Valappu and 85.37% from KVK refrained from growing at 4^0C while 58.33% from Valappu and 95.12% from

KVK failed to grow at 40^{0}C, showing that some of the strains had the stable enzyme potential to grow at widely fluctuating temperatures which is an outstanding characteristic of the isolates of marine origin.

Bacterial genera varied from season to season but their amylolytic, proteolytic and lipolytic enzyme potential remained almost the same.

Multiple antibiotic resistance (MAR) was more prevalent then resistance to one drug alone. Higher incidence of antibiotic resistant bacteria in brackish water culture system might be due to indiscriminate use of antibiotics and its thrust.

The increasing number of drug resistant bacteria in the environment may pose health hazards, so the unrestricted and often indiscriminate use of antibiotics in the culture system has to be curtailed.

Seasonal distribution showed maximum *Vibrio* isolates during the pre-monsoon (n=53 at Valappu and n=44 at KVK).

V. anguillarum was the most frequently isolated *Vibrio* species contributing 31. 86% of the total vibrios encountered. The second most predominant *Vibrio sp.* isolated was *Vibrio fischeri* which constituted 15.19% of the total vibrios.

The most predominant aeromonads were *A. hydrophila* > *A. caviae* > *A. sobria* indicating that the pond water was comparatively less polluted. Out of the 123 aeromonad strains isolated *A. hydrophila* formed 51.22%, *A. caviae* 28.46% and *A. sobria* 20.33%.

The study showed that aeromonads like *Vibrio* sp. is an autochthonous inhabitant of the coastal waters and it has the ability to colonize the skin surface and intestinal lumen of the fishes .

Maximum % of *A. hydrophila* were isolated during the monsoon season at Valappu while at KVK, *A. caviae* was found predominating the monsoon (45.45%) followed by the pre monsoon season (31.25%).

Intraperitoneal injection of 10^3 cells/fish of *V. anguillarum* caused stress with melanization of the skin and caudal erosion. Intraperitoneal injection of *V. anguillarum* at 10^6cells/animal caused mortality within 18hr of injection. The LD_{50} was 9.3×10^6cfu/fish.

Intraperitoneal injection of 10^3 and 10^5cells/fish of *A. hydrophila* caused inflammation of the abdominal cavity, melanization, corneal opacity and loss of scales. No mortality occurred. Intraperitoneal injection of 10^6cells/ml caused 50% mortality within 24 hours post injection. (LD_{50} was 8.0×10^6cfu/fish).

The experiments showed that the pathogenicity depends on the dosage, period of exposure and the fish species and that the gastro intestinal tract is the main portal of entry. In the immersion method, fishes exhibited loss of appetite, melanization and abnormal swimming behaviour, but no mortality occurred. Injection of 10^6 cells into the eye socket of *O.mossambicus* caused exophthalmia and slow mortality.

The results suggests that *V. anguillarum* and *A hydrophila* are pathogenic to adult *O.mossambicus* (10^6cells/animal).

Immersion experiments failed to produce any infection in adult tilapia.

V. fluvialis and *A. caviae* were not found to have any mortal effect on *O. mossambicus*. The high counts of *Vibrio* spp. at high salinity and *Aeromonas* spp. at low salinity indicated that density of these spp. does not have any influence on their pathogenicity. The pathogenicty depended upon the species of fish cultured and the influence of the environmental factors on them.

Intra peritoneal injection appeared to be the most reliable method for pathogenicity studies. The infectivity trials carried out in the present study showed that *A.caviae* injected intraperitoneally produced a slight melanization in the injected area but no mortality occurred showing that the strain injected was not toxigenic and possessed only limited invasive qualities.

The analysis of variance of the fitted multiple regression between the total count of *V. campbellii* and the environmental parameters was found significant

at 5% level indicating that the fitted regression explains significant part (85.5%) of the variability in the data.

The correlation studies of *V. anguillarum* and *V. fischeri* with the 9 environmental parameters showed that the spp. were significantly positively correlated with the temperature.

The correlation matrix of *V. furnissii* on the environmental parameters showed significant positive correlation with ammonia and phosphate at 5% levels while *V. campbellii* was found to be significantly positively correlated with salinity at 5% level. *V. furnissii* and *V. metschnikovii* were found to be significantly positively correlated with dissolved oxygen and salinity at 5% levels respectively.

The matrix of correlation of *V. campbellii* on the environmental parameters was found to be significantly positively correlated with pH and organic carbon.

The vertical gradient in temperature and salinity was very less, this was due to the shallow nature of the pond .The general drop of salinity nearly to fresh water conditions, during monsoon caused drastic decline in the count of *Vibrionaceae*. Wide variation in pH can be attributed to the intermittent addition of lime as fertilizer to the pond. The fairly steady pH values in some months was governed by the influence of sea water intrusion.

The two way ANOVA of *Vibrio* spp. with seasons at Valappu showed significant difference between seasons, whereas at KVK there was significant difference between *Vibrio* spp. but no significant difference between seasons.

All the *Aeromonas* strains isolated exhibited resistance to the Vibriostat 0/129 whereas *Plesiomonas* and *Vibrio* strains were found to be highly sensitive.

A clear homogeneity in the anti microbial resistance pattern has been detected with a high percentage of *Vibrio* and *Aeromonas* spp. exhibiting resistance towards ampicillin and pencillin. Maximum sensitivity was exhibited towards gentamycin.

The multiple regression of *A. hydrophila* on the environmental parameters was significant at 5% level, which explained 82.9% of the variability in the data.

At KVK there was significant negative correlation of *P.shigelloides* with salinity.

Control of vibriosis can be accomplished using chemotherapeutants, but this method is not satisfactory because drug resistant forms can occur.

These studies considerably enhance our knowledge of occurrence and distribution of Vibrionaceae and their pathogenicity to *O. mossambicus*.

Future research should attempt to improve our understanding of how various stressors interact to produce disease in fishes and efforts must focus on preventive measures to ensure the health of such animals. Stress associated with environmental pollution should be the top priority for research. Environmental pollutants are suspected but not proven, to contribute to infectious disease by weakening immune systems, suppressing reproductive function and as a predisposing factor to neoplasia. Periodic study of fish toxicological pathology is necessary for protection and survival of fishes.

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